LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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TECHNOLOGICAL FUNCTIONALISATION OF MICROENCAPSULATED GENISTEIN AND DAIDZEIN DELIVERY SYSTEMS SOLUBLE IN THE STOMACH AND INTESTINES

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LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

Jurga Andrėja Kazlauskaitė

SKRANDYJE IR ŽARNYNE TIRPIŲ MIKROKAPSULIUOTŲ GENISTEINO IR DAIDZEINO PERNAŠOS SISTEMŲ TECHNOLOGINIS FUNKCIONALIZAVIMAS

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LIST OF ABBREVIATIONS

ABTS	–	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
API	–	active pharmaceutical ingredient
ATCC	–	American type culture collection
CD	–	cyclodextrin
CI	–	centrifugation index
CPE	–	cytopathic effects
DPPH	–	2,2-diphenyl-1-picrylhydrazyl
dw	–	dry weight
ER	–	estrogen receptors
FE(II)	–	ferrous sulfate
FRAP	–	ferric reducing antioxidant power
GC	–	gas chromatography
HPLC	–	high-performance liquid chromatography (liet. ESC)
IBV	–	infectious bronchitis virus
LOD	–	limit of detection
LOQ	–	limit of quantification
MS	–	mass spectrometry
PDA	–	photodiode array
PVP/VAC	–	polyvinylpyrrolidone-vinyl acetate copolymer
RU	–	rutin
SSG	–	sodium starch glycolate
$TCID_{50}$	–	median tissue culture infectious dose
TE	–	trolox

INTRODUCTION

Medicinal plants have long played a crucial role in traditional health care globally, serving as the primary source of therapeutic agents for the majority of the world's population, particularly in developing countries [1]. As scientific methodologies have evolved, the pharmacological evaluation of medicinal plants has moved from empirical observations to rigorous scientific investigation, facilitating the integration of plant-derived compounds into evidence-based medicine [2, 3].

Plant-derived compounds' multifunctionality and high structural diversity offer clear advantages over their synthetic counterparts. The synergy among these compounds within plants provides additional benefits, a rarely applied feature by synthetic molecules [4, 5]. As a result, phytochemicals play a significant role in drug discovery and development.

Red clover (*Trifolium pratense* L.) has long been recognised for its medicinal properties, playing a significant role in traditional and modern herbal medicine [6, 7]. It was selected for this research due to its wide application and high biological activity. Red clover is a rich source of bioactive compounds, including isoflavones, coumarins, flavonoids, and phenolic acids [8]. These compounds contribute to red clover's wide-ranging health benefits, making it a valuable ingredient in nutraceutical preparations [9].

Isoflavones, including genistein and daidzein, stand out as the most significant phytochemicals found in red clover and are called phytoestrogens [9, 10]. Phytoestrogens structurally resemble estrogens (such as 17β-estradiol) and compete with it for binding ER. These compounds have a stronger affinity for binding ER_β than ER_α. By interacting with ERs, isoflavones can exert estrogenic effects in humans [10, 11]. However, depending on the dose or the duration of exposure, isoflavones can also act as antiestrogenic agents [12]. This makes red clover a popular choice for alleviating menopausal symptoms, such as hot flashes and osteoporosis, by providing a natural alternative to hormone replacement therapy [13]. Beyond menopausal relief, red clover's isoflavones exhibit antioxidant, anticancer, and cardioprotective properties, offering potential protection against various chronic diseases [14–17]. Red clover's antioxidant activity is another key factor in its nutraceutical applications, and these properties, coupled with anti-inflammatory effects, further broaden red clover's appeal in formulations that promote overall health and well-being, but nonetheless, much more clinical trials are needed [18, 19].

In research on legumes from the *Fabaceae* family, particular attention is given to isoflavones like genistein, daidzein, glycitein, formononetin and biochanin A [12, 20]. These compounds are usually encountered as conjuga-

tes and are hydrolysed to aglycones (biologically active form) in the human gut. The isoflavone aglycones are absorbed faster and in greater amounts than their glucosides. This underscores the importance of sugar component separation in making isoflavones readily available to the body [21, 22]. Additional substances, such as excipients, can be applied to aid in the extraction of isoflavone aglycones using water or another solvent, thereby improving the yield from the extract. Moreover, for enhancing the solubility of isoflavones substances used in the manufacture of oral pharmaceutical forms – such as α-, β-, γ-CDs, PVP/VAC, croscarmellose sodium, SSG, or magnesium aluminometasilicate, which are integral for increasing the active pharmaceutical ingredients' stability and bioavailability – can be effectively utilised [23–28]. The study of excipients in plant extraction processes is an underexplored area that offers new opportunities to improve the efficiency and safety of these methods. While there has been considerable exploration into the efficacy of deep eutectic solvents as safer alternatives to conventional toxic organic solvents for extracting specific bioactive compounds, limited attention has been paid to excipients [29, 30]. Notably, one study investigated the increase of phenols from peach pomace using β-CD as an excipient, and it was successful [27]. Additionally, various studies have explored distillation from different plant materials by incorporating excipients such as seawater, polysorbate 20, and magnesium aluminometasilicate, all aimed at enhancing essential oil yields [31–33]. Therefore, this study aims to fill this research gap by assessing novel excipients, including α-, β-, and γ-CDs, and polymers like PVP/VAC, croscarmellose sodium and SSG. These excipients increase isoflavone yield and can increase the extract's antioxidant potential.

Simply obtaining isoflavone-enriched extracts is insufficient. Due to their sensitivity to environmental factors such as heat and light, as well as inherent challenges like low water solubility, rapid metabolism, and quick elimination from the body, it is essential to incorporate isoflavones and phenols into emulsions when formulating pharmaceutical products. This method enhances their stability and bioavailability, ensuring more effective therapeutic outcomes [34–36]. As biphasic systems, emulsions offer a robust solution for effectively encapsulating these hydrophobic compounds, thereby facilitating their administration and absorption within the body [37]. This approach enhances the efficacy of the active ingredients and reduces potential side effects by precisely controlling the release rate of encapsulated compounds. Moreover, it boosts these compounds' stability and bioavailability, thereby optimising their therapeutic potential [34]. Microencapsulation of the emulsions further increases the stability and efficacy of bioactive compounds. This technology involves encasing tiny particles or droplets of the active

compounds within a protective coating, offering irreplaceable advantages [38, 39].

Microencapsulation enables the controlled release of active ingredients, targeting specific sites for improved therapeutic outcomes and reduced side effects while ensuring sustained effects over time [40, 41]. Furthermore, the efficacy of microencapsulation in achieving controlled release of active ingredients is influenced by various factors such as the selection of excipients used in the production process, the specific techniques employed and other parameters [39, 42].

The preparation of microcapsules through various methods, such as freeze-drying, spray-drying, and extrusion, offers distinct advantages, catering to the unique requirements of different pharmaceutical applications [43]. Freeze-drying, for instance, is renowned for its ability to preserve the structural integrity and bioactivity of sensitive compounds, including phenols, by removing water under low temperature and pressure, thus minimising thermal degradation [44, 45]. Spray-drying, on the other hand, offers a rapid and cost-effective means of encapsulating active ingredients, with the added benefit of producing particles with a wide range of sizes suitable for oral and inhalable therapies [45–47]. Extrusion, characterised by its versatility and scalability, enables the continuous production of microcapsules with a uniform size distribution, which is essential for controlled release formulations [48, 49]. The strategic use of polymers that are soluble in either the stomach or the gut for microcapsule shells is particularly advantageous in optimising the organoleptic parameters, bioavailability and therapeutic efficacy of isoflavones combined with the microencapsulation methods [50, 51]. These polymers, like chitosan and alginate, can be engineered to dissolve at specific locations depending on pH, ensuring that the encapsulated isoflavones are released precisely where their absorption is most efficient [51].

The best extraction methods and parameters were carefully selected to yield isoflavone-rich extracts from red clover, preparing diverse formulations of isoflavone-containing emulsions tailored for various microencapsulation techniques and polymer matrices. This strategic approach aimed to comprehensively harness bioactive compounds' unique properties and actions. The study identified the most effective formulations from a broad spectrum of samples, establishing the important role of technological factors in influencing the efficacy and functionality of the final products.

The aim of the dissertation is to assess the influence of technological factors on the quality of microcapsules containing genistein and daidzein produced by different methods, and on the release of active compounds *in vitro*.

Objectives of the dissertation:

1. To investigate the effect of various extraction methods and excipients on the yield of genistein and daidzein (isoflavones) extracted from red clover (*Trifolium pratense* L.) flowers and analyse the antioxidant and antimicrobial properties of the resultant extracts.
2. To assess the impact of isoflavones, selected extracts, and emulsifiers forming direct-type simple emulsions on the quality of emulsion matrices and enhance their properties.
3. To evaluate the influence of technological factors and isoflavones on the formation and quality of microcapsules produced by different methods.
4. Investigate how technological parameters influence the development, quality characteristics, and active compounds release *in vitro* of products containing microcapsules.

SCIENTIFIC NOVELTY

Given the growing interest in isoflavones, this study aimed to address the gap in comprehensive data regarding the total phenolic content and antioxidant activities of red clover flower extracts. This research developed new techniques for extracting natural compounds and enhancing the extraction of bioactive phenols and isoflavones from *Trifolium pratense* L. The approach was characterised by using excipients, including magnesium aluminometasilicate, croscarmellose sodium, SSG, PVP/VAC, and α-, β-, γ-CDs. SSG, PVP/VAC, and CDs led to increased yields of bioactive compounds (phenols, flavonoids and isoflavones) in both aqueous and ethanolic extracts. The study also highlighted an improvement in the antioxidant potential of these extracts, an aspect critical to their pharmaceutical application. It was noted that the use of excipients did not affect the antimicrobial properties overall, although there was a notable reduction in the growth of Gram-positive bacteria and an observed extract ability against the IBV virus strain.

In exploring emulsion formulations, the study observed red clover extract alone or red clover extract combined with liquorice root extract and nutmeg essential oil, using methods such as spray-drying, lyophilisation, and extrusion to produce stable microcapsules. Mustard extract, a natural emulsifier, was among the several emulsifiers tested, highlighting the impact of different excipients and their concentrations on microcapsule formation. Materials such as alginate sodium and chitosan were utilised to enhance solubility in the stomach and gastric media, facilitating the formation of microcapsule shells.

The outcome of this research included the development of extruded microcapsules encapsulated in capsules containing chitosan and alginate-based microcapsules, offering an advantageous medium for incorporating oil-soluble vitamins. Additionally, the research introduced chewable tablet technology, employing pectin as a gelling agent without the need for added sugars, utilising only the natural sweetness of the fruits used, and incorporating freeze-dried microcapsules with not only red clover extract but also the addition of liquorice root extract and nutmeg essential oil.

This study provides valuable insights into the field, promotes the development of more effective and environmentally friendly pharmaceutical products derived from natural sources, and illustrates the potential of new extraction and formulation methods to increase the utility of plant compounds.

PRACTICAL VALUE

The use of SSG, PVP/VAC and CD excipients increases the extraction yield of bioactive phenols and isoflavones using 50% ethanol and water as solvents. This method not only enhances the antioxidant activity of the extracts but also increases their therapeutic potential, paving the way for the development of stronger, natural remedies. These advances address the long-standing problem of poor solubility of isoflavones while ensuring that the extraction method remains cost-effective, environmentally friendly and compliant with green chemistry principles.

In addition, natural emulsifiers such as mustard extract were effective compared to traditional synthetic emulsifiers such as polysorbate 80. The incorporation of β-cyclodextrins (β-CD) proved beneficial in increasing the extraction yield and serving as an effective emulsifying agent for Pickering emulsions. Extracts formulated with β-CD showed greater stability of emulsion forms compared to those prepared without this excipient, highlighting the multiple benefits of including such molecules in extraction and formulation processes.

By integrating red clover extract with liquorice root extract and nutmeg essential oil through methods like spray-drying, lyophilisation, and extrusion, the study successfully produces microcapsules according to the quality requirements, from which lyophilised microcapsules have the best parameters and could be used in further pharmaceutical forms. Extruded microcapsules with emulsions, prepared using only red clover extract and excipients, are a great base for supplementing with water- and oil-soluble vitamins.

The development of chitosan and alginate-based microcapsules in a hard capsule and the introduction of sugar-free chewable tablets using pectin and natural fruit sweetness represent an innovative approach to creating attractive and healthier nutritional options.

THE LAYOUT OF THE DISSERTATION

The dissertation is structured around a comprehensive analysis presented through a collection of seven scientific articles, illustrated in Fig. 1.

Evaluation of extraction parameters on isoflavones yields and extracts biological properties:

- Influence of extraction methods and excipients on isoflavone yiels (*Paper 1 and 2*).
- Excipients' effect on isoflavone, phenolic, and flavonoid content (*Paper 3 and 6*).
- Asessment of technological modifications on antioxidant activity (*Paper 3 and 6*).
- Antibacterial and antiviral properties of the extracts (*Paper 3 and 6*).

Preparation of emulsions and their parameters:

- Formation and evaluation of red clover extract emulsions with various oils (*Paper 7*).
- Creation and study of emulsions containing *Myristica fragrans* Houtt. essential oil and plant extracts (*Paper 4 and 5*).

Microcapsules formation and parameters:

- Preparation of stomach and intestine-soluble microcapsules via extrusion method (*Paper 7*).
- Preparation of intestine-soluble microcapsules with essential oils and added extracts via extrusion method (*Paper 4*).
- Preparation of microcapsules using freeze drying and spray drying techniques (*Paper 5*).

Product development:

- Development of extruded microcapsules in hard capsule form, assessing active compound release (*Paper 7*).
- Preparation of powder microcapsules, exploring technological parameters and active compound release (*Paper 5*).
- Study of microcapsules in chewable get tablet form for release properties (*Paper 4*).

Fig. 1. *Scheme of the thesis with published scientific papers*

The thesis material is organised into four principal sections:

1. Examination of extraction parameters' impact on isoflavone yields and the biological properties of extracts.
2. Evaluation of the effects of isoflavones, extracts, and emulsifiers on emulsion quality and properties.
3. Investigation into the influence of technological factors and isoflavones on the formation and quality of microcapsules.
4. Exploration of the interplay between technological parameters, product development, and the release of active compounds in microcapsules.

PhD CANDIDATE'S CONTRIBUTION

Author Jurga Andrėja Kazlauskaitė substantially contributed to the work published in papers 1–7 and was responsible for the original draft preparation of all papers. The specific contributions in each publication are outlined below:

Paper 1: Conceptualization, methodology, investigation (extracts preparation, analysis of isoflavones), data curation, writing–review and editing, visualisation.

Paper 2: Conceptualization methodology, investigation (extracts preparation, analysis of isoflavones), data curation, writing–review and editing, visualisation.

Paper 3: Conceptualization, methodology, investigation (extract preparation, antioxidant activity determination, total phenolic and flavonoid content determination and analysis of isoflavones), data curation, writing–review and editing, visualisation.

Paper 4: Conceptualization, methodology, investigation (preparation of extracts, analysis of isoflavones, determination of phenolic and flavonoid content, antioxidant activity assessment, emulsion preparation, physical parameter determination, and microcapsules formation using extrusion method and quality evaluation), data curation, writing–review and editing, visualisation.

Paper 5: Conceptualization, methodology, investigation (extract preparation, emulsion preparation, determination of physical parameters, microcapsule formation, and *in vitro* release determination using total phenolic and flavonoid content, antioxidant activity determination, gelatin and pectin chewable tablets' preparation and evaluation), resource, data curation, writing–review and editing visualisation.

Paper 6: Conceptualization, methodology, investigation (extract preparation, antioxidant activity determination, total phenolic and flavonoid content determination, and isoflavones analysis), resources, data curation, writing–review and editing, visualisation.

Paper 7: Conceptualization, methodology, investigation (extracts preparation, emulsions preparation and physical parameters evaluation, microcapsules formation and evaluation), data curation, writing–review and editing, visualisation.

CO-AUTHORS' CONTRIBUTION

All co-authors actively participated in the writing, review, and editing processes of the papers they co-authored.

Prof. dr. Jurga Bernatonienė was the supervisor and coordinator of all investigations in Papers 1–7, contributed to the conception of all studies and organised resources for conducting experiments.

Prof. dr. Liudas Ivanauskas facilitated the resource organisation for experiments related to Papers 1, 2, and 3. Developed the method for quantifying daidzein and genistein in Papers 1, 2 and 3.

Lect. Mindaugas Marksa contributed to the methodology and qualitative analysis of daidzein and genistein in Papers 3–7. Additionally, provided the methodology for GC-MS analysis of essential oils in Papers 4 and 5, and developed and performed the ABTS post-column method in Paper 3.

Lect. dr. Inga Matulytė provided methodologies and prepared nutmeg essential oil; developed microencapsulation techniques using the extrusion method in Papers 4 and 7. Conducted firmness analysis using a texture analyser in Paper 5, and devised methodologies for the spray-drying method. Additionally, prepared chewable gel tablets with gelatin and performed texture analysis, as described in Paper 5.

Prof. dr. Alvydas Pavilonis contributed to the development of the methodology and conducted antimicrobial tests in Paper 4.

Prof. dr. Raimundas Lelešius contributed to the development of the methodology and conducted antiviral tests in Paper 4.

LIST OF SCIENTIFIC PAPERS

Research articles reporting the results of the dissertation include:

Paper 1: Kazlauskaitė, Jurga Andrėja; Ivanauskas, Liudas; Bernatonienė, Jurga. Novel Extraction Method Using Excipients to Enhance Yield of Genistein and Daidzein in *Trifolium pratensis* L // Pharmaceutics. Basel, Switzerland: MDPI, 2021, vol. 13, no. 6, p. 1-17, ISSN 1999-4923. doi:10.3390/pharmaceutics13060777. [Impact factor: 6.525, aggregate impact factor: 4.805, quartile: Q1 (2021. InCites JCR SCIE)];

Paper 2: Kazlauskaitė, Jurga Andrėja; Ivanauskas, Liudas; Bernatonienė, Jurga. Cyclodextrin Assisted Extraction Method as a Green Alternative to Increase the Isoflavone Yield from *Trifolium pratensis* L. Extract // Pharmaceutics. Basel, Switzerland: MDPI, 2021, vol. 13, no. 5, p. 1-14, ISSN 1999-4923. doi:10.3390/pharmaceutics13050620. [Impact factor: 6.525, aggregate impact factor: 4.805, quartile: Q1 (2021. InCites JCR SCIE)];

Paper 3: Kazlauskaitė, Jurga Andrėja; Ivanauskas, Liudas; Marksa, Mindaugas; Bernatonienė, Jurga. The Effect of Traditional and Cyclodextrin-Assisted Extraction Methods on *Trifolium pratense* L. (Red Clover) Extracts Antioxidant Potential // Antioxidants. Basel: MDPI, 2022, vol. 11, no. 2, p. 1-23, ISSN 2076-3921. doi:10.3390/antiox11020435. [Impact factor: 7, aggregate impact factor: 5.233, quartile: Q1 (2022. InCites JCR SCIE)];

Paper 4: Kazlauskaitė, Jurga Andrėja; Matulytė, Inga; Marksa, Mindaugas; Lelešius, Raimundas; Pavilonis, Alvydas; Bernatonienė, Jurga. Application of Antiviral, Antioxidant and Antibacterial *Glycyrrhiza glabra* L., *Trifolium pratense* L. Extracts and Myristica fragrans Houtt. Essential Oil in Microcapsules // Pharmaceutics. Basel: MDPI, 2023, vol. 15, no. 2, p. 1-20, ISSN 1999-4923, 1999-4923. doi:10.3390/pharmaceutics15020464. [Impact factor: 5.4, aggregate impact factor: 4.5, quartile: Q1 (2022. InCites JCR SCIE)];

Paper 5: Kazlauskaitė, Jurga Andrėja; Matulytė, Inga; Marksa, Mindaugas; Bernatonienė, Jurga. Nutmeg Essential Oil, Red Clover, and Liquorice Extracts Microencapsulation Method Selection for the Release of Active Compounds from Gel Tablets of Different Bases // Pharmaceutics. Basel: MDPI, 2023, vol. 15, no. 3, p. 1-17, ISSN 1999-4923, 1999-4923. doi:10.3390/pharmaceutics15030949. [Impact factor: 5.4, aggregate impact factor: 4.5, quartile: Q1 (2022. InCites JCR SCIE)];

Paper 6: Kazlauskaitė, Jurga Andrėja; Marksa, Mindaugas; Bernatonienė, Impact of Polyvinylpyrrolidone-Vinyl Acetate Copolymer and Sodium Starch Glycolate Excipients on Phenolic Extraction from Red Clover: Enhancing Biological Activity and Antioxidant Potential // Pharmaceutics.

Basel: MDPI, 2024, vol. 16, no. 3, p. 1-21, ISSN 1999-4923. doi:10.3390/pharmaceutics16030399. [Impact factor: 5.4, aggregate impact factor: 4.5, quartile: Q1 (2022. InCites JCR SCIE)];

Paper 7: Kazlauskaitė, Jurga Andrėja; Matulytė, Inga; Marksa, Mindaugas; Bernatonienė, Jurga. Technological Functionalisation of Microencapsulated Genistein and Daidzein Delivery Systems Soluble in the Stomach and Intestines // Pharmaceutics. Basel: MDPI, 2024, vol. 16, no. 4, p. 1-20, ISSN 1999-4923. doi:10.3390/pharmaceutics16040530. [Impact factor: 5.4, aggregate impact factor: 4.5, quartile: Q1 (2022. InCites JCR SCIE)].

CONFERENCE PRESENTATIONS

Conference presentations reporting the results of the dissertation include:

Conf. 1: Kazlauskaitė, Jurga Andrėja; Bernatonienė, Jurga. Identification of isoflavones aglycones in *Trifolium pratense* L. blossoms using different extraction methods // International conference "Contemporary Pharmacy: Issues, Challenges and Expectations 2020 Autumn": abstract book: 23rd October 2020, Kaunas, Lithuania ISBN 9789955156697, p. 45-45.

Conf. 2: Kazlauskaitė, Jurga Andrėja; Marksa, Mindaugas; Bernatonienė, Jurga. Quantification of isoflavones aglycones in the late harvest aerial parts of *Trifolium pratense* L. using different hydrolysis methods // Medicina: Abstracts accepted for the International Scientific Conference on Medicine organised within the frame of the 79th International Scientific Conference of the University of Latvia: 23–24 April, Riga, Latvia, 2021, vol. 57, suppl. 1, p. 280–280, ISSN 1010-660X, 1648-9144.

Conf. 3: Kazlauskaitė, Jurga Andrėja; Bernatonienė, Jurga. The Investigation of total phenolic content and antioxidant activity of the *Trifolium pratense* L. blossoms extracts // The (Extra)ordinary COINS 2021 – [16th] International conference of Life Sciences: book of abstracts: February 27, 2021, Vilnius, Lithuania, Vilnius University. p. 42-42.

Conf. 4: Kazlauskaitė, Jurga Andrėja; Bernatonienė, Jurga. The Influence of Excipients on Isoflavones Yield from *Trifolium pratensis* L. Extracts // The Joint International Pharmacy Symposium "Contemporary Pharmacy: Issues Challenges and Expectations 2021" and "11th Conference: Pharmacy Science and Practice", CPICE-PSP 2021: abstract book: October 22nd, 2021, Kaunas, Lithuania. ISBN 9789955157113, p. 39–39.

Conf. 5: Kazlauskaitė, Jurga Andrėja; Bernatonienė, Jurga. Relationship between antioxidant activity and isoflavones in *Trifolium pratense* L. extracts // Current approaches of pharmaceutical science in development and standardisation of medicines and dietary supplements that contain components of natural origin: The Proceedings of the III International Scientific and Practical Internet-Conference. Kharkiv, 2021, p. 20–21: lent, ISSN 2519-2655.

Conf. 6: Kazlauskaitė, Jurga Andrėja; Bernatonienė, Jurga. Antioxidant activity in *Trifolium pratense* L. blossoms using different extraction methods // 64th International Conference for Students of Physics and Natural Sciences Open Readings 2021: 16–19 March 2021, Vilnius, Lithuania: Vilnius University p. 449–449, ISBN 9786090705902.

Conf. 7: Kazlauskaitė, Jurga Andrėja; Matulytė, Inga; Bernatonienė, Jurga. The Influence of βCyclodextrin Amounton the Stability of Red Clover o/w Emulsion // Науково-технічний прогрес і оптимізація технологічних

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1. SUMMARY OF MATERIALS AND METHODS

1.1. Plant material

The floral buds and blossoms of *Trifolium pratense* L. (red clover) were gathered between September 25th and 26th, 2020 to 2023, from their natural habitat in Laičiai, Kupiškis district, Lithuania (coordinates: latitude 55°53′24.2″ N; longitude 25°19′36.0″ E). Following collection, the plant material was air-dried and stored at room temperature. Prior to utilisation, the red clover flowers were ground into a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). The moisture content of ground samples was determined by a moisture analyser KERN MLB apparatus (KERN & Sohn GmbH, Balingen, Germany), and all further results were expressed as dw. *A detailed description of plant raw materials and conditions of plant material processing are presented in Papers 1 and 2.*

In this study, additional plant material was used. *Glycyrrhiza glabra* L. roots (the country of origin is China) were bought from LSMU pharmacy (Kaunas, Lithuania). *Myristica fragrans* Houtt. seeds' country of origin was Grenada (supplier Spaisvilė, Pašaltuonys, Lithuania), and *Sinapis alba* milled seeds were bought at Saldva (Kabalda, Lithuania). *A detailed description of Glycyrrhiza glabra L. roots and Myristica fragrans Houtt. seeds are presented in Papers 4 and 5; information on Sinapis alba seeds are provided in Paper 7.*

1.2. Extraction and hydrodistillation

1.2.1. Traditional extraction methods

Various extraction methods, including maceration, reflux, and ultrasound-assisted extraction, were employed to maximise the extraction of isoflavones from red clover. Maceration involved natural hydrolysis over three days, while reflux was conducted at boiling temperatures of solvents using different processing times. Ultrasound-assisted extraction utilised an ultrasound bath operating at a frequency of 38 kHz, 100%. Following extraction, hydrolysis of the extracts was carried out using various methods: acidic hydrolysis with 37% HCl and subsequent neutralisation with 2M NaOH; alkaline hydrolysis with 25% NaOH and neutralisation with 25% acetic acid; and thermal hydrolysis. *Detailed descriptions of the extraction conditions, including plant amounts, extraction times, temperatures, solvents, and their concentrations, can be found in Papers 1 and 2.*

The *Glycyrrhiza glabra* L. extract was obtained from dried and milled plant root powder. Initially, maceration in water was conducted for 4 hours, followed by ultrasound-assisted extraction using an ultrasound bath operating at 38 kHz. *Paper 4 provides detailed descriptions of the extraction conditions employed for Glycyrrhiza glabra L. roots.*

White mustard extraction was conducted utilising an ultrasound bath. *Details regarding the extraction of Sinapis alba seeds can be found in Paper 7.*

1.2.2. Extraction methods with excipients

Various excipients and their concentrations were utilised in the extraction process of red clover. Samples were modified and prepared with PVP/VAC, SSG, croscarmellose sodium, magnesium aluminometasilicate, and α-, β-, or γ-CDs. The selection of conditions, solvents, and extraction parameters for employing these excipients was based on the isoflavones yield obtained from traditional extraction methods. Three conditions were chosen for extraction with excipients, followed by subsequent experiments to determine the optimal excipient concentrations: ultrasound processing (10 or 30 minutes) combined with reflux and reflux alone. *Detailed descriptions of the extraction conditions, including plant amounts, extraction times, temperatures, solvents, and their concentrations, can be found in Papers 1 and 2.*

1.2.3. Essential oil preparation

Myristica fragrans Houtt. essential oil was prepared by modified hydrodistillation using magnesium aluminometasilicate. The hydrodistillation was conducted with a Clevenger-type apparatus. *A detailed description of Glycyrrhiza glabra L. roots and Myristica fragrans Houtt. seeds are presented in Paper 4.*

1.3. Phytochemical characterisation methods

1.3.1. HPLC-PDA

Qualitative and quantitative analysis of isoflavones in red clover extracts was conducted using the validated HPLC-PDA method. Phenolic analysis was performed using an ACE 5 C18 250 × 4.6 mm column with a linear gradient elution profile, following the parameters and conditions outlined in *Papers 1–7*.

1.3.2. HPLC-PDA-MS

The compounds with the highest antioxidant activity were identified using the ABTS-post column method coupled with HPLC-PDA-MS. Following reversed-phase liquid chromatographic separation, a thorough qualitative profiling of the red clover extract was conducted using electrospray ionisation in both negative and positive modes, followed by MS analysis. Compound identification was achieved by comparing the obtained mass spectra with literature data and mechanisms provided in freely accessible databases. *For more comprehensive methodological details, refer to Papers 3 and 6.*

1.3.3. GC-MS

The analysis of *Myristica fragrans* Houtt. essential oil and the quantification of its chemical compounds in microcapsules were conducted using an RTX-5MS column measuring 30 m × 0.25 mm i.d. with a 0.25 µL film thickness. Identification of volatile compounds was achieved through a mass spectra library search (NIST 14). *For detailed method parameters, please refer to Papers 4 and 5.*

1.3.4. Spectrophotometric assays

The total phenolic content of plant extracts was determined using Folin–Ciocalteu's phenol reagent at a wavelength of 765 nm. Results were expressed as equivalent mg GA/g dw. *Further details of the method can be found in Papers 3–7.*

For the determination of the total flavonoid content of plant extracts, a mixture of reagents consisting of 1.0 mL 96% ethanol, 0.05 mL 33% acetic acid, 0.15 mL 10% aluminium chloride, and 2.0 mL 5% hexamethylenetetramine solutions was used, with measurements taken at a wavelength of 475 nm. Results were expressed as equivalent RU/g dw. *A detailed description of the method is provided in Papers 3–7.*

1.4. Antioxidant and biological activity methods, and application

1.4.1. Antioxidant activity

Antioxidant properties of red clover extracts evaluated by *in vitro* spectrophotometric assays. Free radical scavenging activity was assessed using ABTS (λ = 734 nm) and DPPH (λ = 517 nm) assays; the results were expressed as equivalent to µg TE/g dw. Reducing activity was assessed by FRAP (λ = 593 nm) assay. The results were expressed as equivalent of mg FE(II)/g dw, precisely as described in *Papers 3 and 6.*

The antioxidant properties of red clover extracts were also assessed using an HPLC-ABTS system equipped with a 3 m reaction loop (inner diameter: 0.25 mm, outer diameter: 1.58 mm). Test solutions were detected at a wavelength of 650 nm, with signal strength represented as peaks of negative active compounds. The antioxidant activity of the extract compounds was expressed as equivalents of mg Trolox per gram of dry weight (mg TE/g dw). *For a comprehensive understanding of the methodology, refer to Papers 3 and 6.*

1.4.2. Antimicrobial activity

The antimicrobial activity evaluation involved a variety of microorganisms obtained from the ATCC. These microorganisms comprised standard cultures of non-spore bacteria, such as *Staphylococcus aureus* (ATCC 25923; human nasal microbiota), *Staphylococcus epidermidis* (ATCC 12228; human skin microbiota), *Enterococcus faecalis* (ATCC 29212; human colonic microbiota), *Escherichia coli* (ATCC 25922; human colonic microbiota), *Klebsiella pneumoniae* (ATCC 13883; human microbiota), *Pseudomonas aeruginosa* (ATCC 27853; human microbiota), and *Proteus vulgaris* (ATCC8427; human microbiota). Standard spore bacteria cultures, including *Bacillus cereus* (ATCC 6633; soil microbiota) and the fungus Candida albicans (ATCC 10231; human microbiota), were utilised. The antimicrobial properties of *Trifolium pratense* L. and *Glycyrrhiza glabra* L. extracts, *Myristica fragrans* Houtt. essential oil and commonly used antibiotics were assessed by employing the agar well diffusion method as described in *Papers 4 and 6.*

1.4.3. Antiviral activity

For the antiviral assessment, the Vero cell line (ATCC CCL-81) was employed, along with the Vero-adapted Beaudette IBV strain. TCID50 determination of both control and treated IBV was conducted in 96-well plates using Vero cells. After 72 hours, CPE were assessed, and virus titres were calculated via the Kärber method. Additionally, the CC_{50} values for each extract were determined using an MTT assay on Vero cells. Four methods were employed to evaluate antiviral activity, each involving treatment with diluted extract before or after infection, followed by observation for inhibition of CPE after incubation. The antiviral properties of *Trifolium pratense* L. extract, *Glycyrrhiza glabra* L. extract, and *Myristica fragrans* Houtt. essential oil was assessed according to the procedures outlined in *Paper 4*.

1.5. Emulsion Preparation

1.5.1. Preparation of emulsions for microcapsules production via extrusion method

During the research, two types of emulsions were prepared: one designed to be soluble in the stomach (chitosan) and the other in the gut (alginate). Additionally, emulsions were formulated using extracts other than red clover (such as *Glycyrrhiza glabra* L.) and various oils (including soy, sweet almond, olive, jojoba, and sunflower), as well as nutmeg essential oil.

Samples of chitosan and alginate emulsions were prepared using both ethanol and water extracts. However, since the ethanol extract contains a higher concentration of isoflavones and phenolic compounds, only the ethanol extracts were utilised in further studies. Emulsions containing red clover extracts and various oils were formulated using either alginate or chitosan.

After experimentation, it was found that the most stable emulsions with the smallest particles were those formulated with sweet almond oil. As a result, sweet almond oil was selected as the sole oil component for the final formulations. The emulsion preparation process involved mixing a solution containing excipients (sweet almond oil, emulsifiers such as mustard extract or β-CD, and xanthan) with a 2% or 0.5% sodium alginate solution.

A 2% chitosan solution was utilised for chitosan-based emulsions, and emulsifiers (such as polysorbate 80 or β-CD) were added dropwise and mixed. Subsequently, the required amount of sweet almond oil and extract was added to the solution. Despite incorporating mustard extract alongside chitosan, the mixture failed to form a stable emulsion with the desired red clover extract concentration. Using polysorbate with alginate, the formulation would have to undergo significant adjustments to create emulsions successfully. That is why these emulsifiers were not used with said gelling agents. *For detailed methodology, refer to Paper 7*.

The formulations required modifications when transitioning from using oils to nutmeg essential oil and additional extract. A 4% sodium alginate solution was the base material for emulsion preparation throughout the experiment. Emulsions containing *Trifolium pratense* L., *Glycyrrhiza glabra* L. extracts, and *Myristica fragrans* Houtt. essential oil was prepared by mixing a solution containing excipients (such as maltodextrin, inulin, and/or gum Arabic) with the sodium alginate solution and adding the extracts and essential oil. The emulsions were formulated and numbered from E1 to E6, with variations in the quantities of maltodextrin, inulin, and gum Arabic used. Notably, the quantities of sodium alginate, essential oil, extracts, and water

remained consistent across all formulations. *Further details on the emulsion preparation can be found in Paper 4.*

1.5.2. Emulsion preparation for microcapsule preparation using freeze-drying and spray-drying

The emulsion for lyophilisation/spray-drying was formulated with maltodextrin, gum Arabic, and inulin as excipients. These excipients were dissolved in liquorice and mixed with red clover extract, and then the essential oil was added. *For more detailed information on the emulsion preparation process, refer to Paper 5.*

1.6. Emulsion parameters evaluation

Various parameters of the emulsion were evaluated as follows: emulsion stability was assessed using a centrifuge at different speeds. The size and distribution of oil particles were determined using the Mastersizer 3000 with a Hydro EV unit. Additionally, the dynamic viscosity of the emulsions was measured using a viscometer. *For more comprehensive details on the methods employed, including the apparatuses and their parameters, refer to Papers 4 and 7.*

1.7. Microcapsules formation

1.7.1. Microcapsules formation using extrusion method

Microcapsules were prepared using the extrusion method, employing a medical syringe and syringe pump to generate droplets for microcapsule formation. These microcapsules were derived from emulsions described in the preceding Section 1.5.1. The emulsion droplets were ejected from the needle into the crosslinker solution. *For specific details regarding needle height, pumping speed, and crosslinker types, refer to Papers 4 and 7.*

1.7.2. Microcapsules formation using freeze-drying and spray-drying methods

The identical emulsion formulation was utilised for both freeze-drying and spray-drying processes. Before undergoing lyophilisation, the samples were subjected to freezing. The lyophilisation process lasted for 48 hours.

For spray drying, the conditions employed were as follows: based on prior pilot studies, an inlet temperature of 160 °C was set, while the outlet temperature ranged between 80 °C and 90 °C. The spray flow feed rate was

maintained at 30 mL/min, with an air pressure of 6 bar and an aspirator setting of 100%. *For more comprehensive details regarding the methods utilised, refer to Paper 5*.

1.8. Microcapsules parameters evaluation

1.8.1. Assessment of microcapsules prepared via the extrusion technique

Microcapsules produced through the extrusion technique were subjected to evaluation: firmness was assessed using a texture analyser, size was measured using a micrometre, morphological features of the microcapsules were examined under a light microscope, and swelling characteristics were evaluated using either water or simulated intestinal media. *Detailed descriptions of these methods are provided in Papers 4 and 7.*

1.8.2. Evaluation of microcapsules prepared through freeze-drying and spray-drying methods

The microcapsules obtained via spray-drying and lyophilisation underwent analysis to determine their product yield, which is calculated as the percentage ratio between the final product's total mass and the emulsion's non-solvent mass. Additionally, moisture content was assessed using a moisture analyser, while bulk and tapped volumes were measured using a Sotax tap density tester. Solubility in water was also evaluated. *For detailed descriptions of these methods, please refer to Paper 5.*

1.9. Chewable tablet preparation with microcapsules

Various formulations of pectin gel tablets were prepared by mixing apple juice, puree, and other ingredients, followed by heating. Pectin was dissolved in the warm mixture, and citric acid was added before heating to 100 °C. Microcapsules (5% w/w) were incorporated into the pectin gel mass, which was then poured into silicone moulds and left to set for 24 hours at room temperature. The resulting pectin gel tablets were stored in a plastic container at room temperature. Tablets prepared using gelatin served as the comparison base. *Conditions and equipment used for tablets preparation are described in Paper 5.*

1.10. *In vitro* release of the final products

In vitro release testing of microcapsules prepared via extrusion, lyophilisation, and spray drying, as well as chewable gel tablets containing microcapsules, was conducted in both gastric and intestinal media. The experiments were carried out using the Sotax AT7 Smart Dissolution System, with a gastric medium prepared according to the European Pharmacopoeia guidelines.

Samples were incubated in gastric medium for 30–90 minutes before being transferred to intestinal medium for an additional 30–180 minutes. Subsequently, the samples were filtered and prepared for analysis using GC–MS to detect volatile compounds from essential oils and HPLC to quantify isoflavones daidzein, genistein, and glycyrrhizin. *For a detailed description of the methods and reagents used, please refer to Papers 4, 5, and 7.*

1.11. Data analysis and statistics

Data from Papers 1–7 was analysed and visualised using IBM SPSS Statistics version 20.0, Microsoft Office Excel version 2021, and GraphPad Prism 8 software. All experiments were conducted a minimum of three times, and results were expressed as mean values ± standard deviation (SD), where applicable.

Friedman and Wilcoxon's tests were utilised to compare three different measurements, while the Mann–Whitney U test was compared between two groups. Correlation and regression coefficients were calculated using the Spearman test. Statistical significance was determined at $p < 0.05$. *A comprehensive overview of the statistical methods employed in this study can be found in each respective paper (Papers 1–7).*

2. SUMMARY OF RESULTS

2.1. Evaluation of extraction parameters on isoflavones yields and extracts biological properties

2.1.1. Refining traditional extraction methods for isoflavone extraction efficiency

Red clover extracts were obtained through maceration, ultrasound-assisted and heat-reflux methods coupled with acidic, alkaline, and thermal hydrolysis techniques. The highest yields of isoflavones were achieved through ultrasound-assisted extraction with processing times of 10 or 30 minutes, followed by thermal hydrolysis and the heat-reflux method alone. Specifically, the isoflavone yields were as follows: 393.23 ± 19.66 μg/g daidzein and 171.57 ± 8.58 μg/g genistein (ultrasound processing for 10 minutes with a solvent of 50% ethanol, followed by reflux); 415.07 ± 20.75 μg/g daidzein and 150.57 ± 7.53 μg/g genistein (ultrasound processing for 10 minutes with a solvent of 50% ethanol, followed by reflux); and 432.30 ± 21.61 μg/g daidzein and 154.50 ± 7.72 μg/g genistein (reflux alone with 50% ethanol as the solvent). These conditions were applied across all excipients and fully described in *Paper 2*.

2.1.2. The impact of excipients on isoflavone yield, phenolic and flavonoid content in extraction processes

Seven different excipients were employed in the extraction of isoflavones genistein and daidzein: magnesium aluminometasilicate, croscarmellose sodium, SSG, PVP/VAC, and α-, β-, γ-CDs. The findings underscore the substantial enhancement in isoflavone yield facilitated by the inclusion of excipients during extraction processes.

The investigation into isoflavone yields was conducted across two groups of excipients: α-, β-, γ-CDs, and separately, magnesium aluminometasilicate, croscarmellose sodium, SSG, PVP/VAC (as detailed in *Papers 1 and 2*). Within the CDs group, the analysis showed that the highest genistein yield occurred in a sample prepared with 1% β-CD in purified water, resulting in a yield of 258.67 ± 10.34 μg/g (using the reflux extraction method). Furthermore, the highest daidzein yields were observed in two distinct samples. One sample, prepared in ethanol with 1% β-CD, yielded 697.16 ± 27.88 μg/g (employing ultrasound processing for 30 minutes followed by reflux). Additionally, another sample prepared in ethanol, with 1% γ-CD, exhibited a yield

of 682.90 ± 27.31 μg/g under identical extraction conditions as the β-CD sample.

Concentrations of CDs were increased to 5% to enhance isoflavone yields, but solely in water, as CDs exhibit better solubility in water than in ethanol. The highest ($p < 0.05$) quantities of isoflavones were obtained from β- and γ-CD samples, utilising reflux extraction. Sample β-CD yielded the highest of daidzein (417.1 ± 16.68 μg/g), while γ-CD yielded the highest genistein (222.133 ± 8.82 μg/g) amount. Nonetheless, the increase in CD concentration predominantly elevated the amount of daidzein over genistein. Daidzein yields increased on average by 1.06 (α-CDs), 1.4 (β-CDs), and 1.25 (γ-CDs) times when CDs were increased from 1 to 5%. Genistein yields surged using α- and β-CDs (1.28 and 1.12 times, respectively) but decreased using β-CD. However, similar to extracts prepared with 1% CDs in water, glycosides, daidzin, and genistin were not detected in extracts with 5% excipients.

Switching the solvent from water to 50% ethanol notably boosts the aglycone yield in the extract, increasing it by an average of 2–3 times. This enhancement was consistent across all CDs, except for β-CD, which decreased genistein yield by 1.17 times. Notably, utilising α-CD in the extraction amplified genistein yield by 3.32 times, while γ-CD increased it by 2.36 times. Daidzein amounts showed significant increases with all CDs – 3.64, 2.31, and 3.06 times (for α-, β-, and γ-CD, respectively).

Genistin and daidzin were solely detected in ethanolic samples, even with excipients, due to their poor water solubility and hydrolysis to aglycones during extraction. The highest detected genistin concentration was found in a sample prepared using 1% α-CD in ethanol, yielding 139.73 ± 4.13 μg/g (using the reflux extraction method). The highest daidzin concentration was observed in a similar sample prepared using 1% γ-CD as an excipient in ethanol, resulting in 27.67 ± 0.83 μg/g (using the reflux extraction method). Upon comparing samples prepared with excipients, it was observed that when using α-CDs, the average genistin and daidzin amounts were higher than using β-, γ-CD, or no excipients in the extracts (control samples).

Working with the second group of excipients, the same extraction conditions mentioned before were utilised. Purified water served as the solvent with the excipients sodium croscarmellose, SSG, or PVP/VAC. Significant increases in isoflavone yields were observed compared to control samples across all conditions. However, the use of excipients did not result in the extraction of glycosides (daidzin and genistin). Notably, the excipient magnesium aluminometasilicate was ineffective, as it reduced the amount of isoflavones compared to control samples prepared under identical conditions.

Using the excipients, statistically significant yields of aglycones were obtained with 1% PVP/VAC. Employing the reflux method, aglycones yields found in the water were 307.80 ± 15.39 μg/g daidzein and 121.40 ± 6.07 μg/g genistein with 1% PVP/VAC, and the results were the highest compared will all the samples with excipients in water.

The results indicate that, except for magnesium aluminometasilicate, which did not increase the yields of isoflavones daidzein and genistein, lower amounts of isoflavones were obtained with sodium croscarmellose and SSG compared to PVP/VAC. However, these amounts were statistically significantly higher compared to controls. Aqueous SSG samples, when subjected to reflux, exhibited statistically significant results compared to the control samples, and they showed higher daidzein yields compared to the corresponding sodium croscarmellose samples (55.83 ± 1.67 μg/g and 111.40 ± 5.57 μg/g, respectively, for SSG; 28.73 ± 0.86 μg/g and 97.9 ± 2.93 μg/g, respectively, for croscarmellose sodium). However, the aglycone yields were not as high as those obtained using PVP/VAC.

The concentration of PVP/VAC in water was elevated to 5% (w/v). However, this adjustment couldn't be made for the SSG sample because the samples became excessively thick. With the 5% concentration, the highest yields were achieved using the reflux method: 626.30 ± 18.78 μg/g for daidzein and 175.27 ± 5.25 μg/g for genistein. Nonetheless, even with this higher concentration of PVP/VAC, glycosides daidzin and genistin were not detected.

Changing the solvent to 50% ethanol for 1% PVP/VAC in extraction media yielded glycosides and the highest aglycone daidzein yield in this study – 820.50 ± 41.02 μg/g (using reflux and ultrasound processing for 10 minutes). Genistein yields using this method were lower (144.50 ± 4.33 μg/g) were lower but were statistically significant compared to the control sample. Although 50% ethanol increased the amount of daidzein compared to the 5% excipient used in water, there was no significant increase in genistein levels. Therefore, the use of excipient vinylpyrrolidone-vinyl acetate copolymer in ethanol mainly yielded glycosides and aglycone daidzein from plant material.

Switching the solvent to 50% ethanol for 1% PVP/VAC in the extraction media resulted in the extraction of glycosides and the highest aglycone daidzein yield in this study – 820.50 ± 41.02 μg/g (utilising reflux and ultrasound processing for 10 minutes). Genistein yields using this method were lower (144.50 ± 4.33 μg/g), but they were statistically significant compared to the control sample. Although 50% ethanol increased the amount of daidzein compared to the 5% excipient used in water, there was no significant increase in genistein levels. Therefore, the use of the excipient

vinylpyrrolidone-vinyl acetate copolymer in ethanol primarily yielded glycosides and aglycone daidzein from plant material.

The utilisation of various excipients significantly influences the extraction of isoflavone aglycones. When extracting genistein in water, the excipients were ranked in order of their ability to yield the highest possible amount as follows: magnesium aluminometasilicate < control < croscarmellose sodium < α-CD < SSG < γ-CD < PVP/VAC < β-CD. Conversely, when ethanol served as the solvent, the excipients were ranked in the following order: magnesium aluminometasilicate < PVP/VAC < α-CD < control < SSG < β-CD < γ-CD.

A different ranking was observed in the extraction of daidzein compared to genistein extraction. In water, the order of excipients' effectiveness was as follows: magnesium aluminometasilicate < control < croscarmellose sodium < SSG < α-CD < γ-CD < PVP/VAC < β-CD. Similarly, when using ethanol as the solvent, the ranking was: SSG < magnesium aluminometasilicate < control < α-CD < β-CD < γ-CD < PVP/VAC.

Considering total isoflavone aglycone and glycoside yields, the ranking of excipients in ethanol extraction would be: SSG < magnesium aluminometasilicate < control < α-CD < β-CD < PVP/VAC < γ-CD. Conversely, in water extraction, the ranking would be: magnesium aluminometasilicate < control < croscarmellose sodium < SSG < α-CD < γ-CD < PVP/VAC < β-CD.

This comparison sheds light on the varying effectiveness of excipients in different solvents, highlighting the importance of selecting the appropriate combination for optimising isoflavone extraction. The excipients PVP/VAC and β-CD demonstrate superior performance across various extraction conditions, consistently yielding higher amounts of isoflavones compared to other excipients. Their efficacy in enhancing both genistein and daidzein extraction in different solvents suggests their versatility and reliability in isoflavone extraction processes. *The precise impact of excipients on isoflavone yield in extraction processes is thoroughly reported in Papers 1 and 2.*

The total phenolic content in aqueous extracts prepared with CD excipients varied from 44.78 ± 0.92 (β-CD using the reflux method) to 32.31 ± 0.63 mg GA/g dw (β-CD using ultrasound processing for 30 minutes followed by reflux). Similarly, when the solvent was 50% (v/v) ethanol, the content ranged from 54.12 ± 0.46 (γ-CD using ultrasound processing for 30 minutes followed by reflux) to 34.38 ± 0.63 (α-CD using ultrasound processing for 10 minutes followed by reflux) mg GA/g dw. Most samples prepared with excipients, regardless of the solvent used, exhibited higher total phenolic compound contents compared to control samples prepared without CDs ($p < 0.05$). Notably, γ-CD proved to be the most effective excipient in ethanol compared to the results obtained with α- and β-CDs. The best results

for β-CD in ethanol were obtained under similar conditions to those used with water; the total amount of phenolic compounds was 50.11 ± 0.79 mg GA/g dw (β-CD in ethanol using the reflux method). Employing ethanol with α-CD, the best results were acquired using ultrasound processing for 30 minutes and thermal hydrolysis, yielding 48.46 ± 0.80 mg GA/g dw. It was determined that cyclodextrins significantly increased total phenolic content (compared with control) – using β-CD 20.29% (in aqueous samples); γ-CD 22.26% (in ethanolic samples).

The total flavonoid content ranged from 21.74 ± 0.12 to 17.95 ± 0.08 mg RU/g dw when excipients (CDs) and water were used. Using excipients with ethanol as the solvent, the total flavonoid content ranged from 21.18 to 20.82 mg RU/g dw. The total flavonoid content results were consistent with those of the total phenolic content, indicating that samples with a high level of phenolic content also contained a significant amount of flavonoids. The highest total flavonoid content was found in aqueous samples prepared with β-CD using either the reflux method or ultrasound processing for 30 minutes followed by reflux, resulting in 21.74 ± 0.12 and 21.47 ± 0.23 mg RU/g dw, respectively. Conversely, the lowest flavonoid yields were detected in samples prepared with γ-CD. When ethanol was used as a solvent, the highest flavonoid content was found in a sample prepared using α-CD with ultrasound processing for 10 minutes, followed by reflux, yielding 21.18 ± 0.24 mg RU/g dw. Similar quantities of flavonoids were observed in α-CD samples prepared in ethanol and using reflux (21.13 ± 0.13 mg RU/g dw), as well as in β-CD samples prepared under the same conditions as α-CD (21.11 ± 0.28 mg RU/g dw).

The utilisation of SSG and PVP/VAC excipients in the extraction process resulted in similar or even more significant increases in phenol and flavonoid contents compared to the use of CDs under certain conditions.

The total amount of phenolic compounds in aqueous samples varied from 35.79 ± 0.12 (prepared using 1% SSG and reflux) to 45.38 ± 0.19 mg GAE/g dw (prepared using 5% PVP/VAC, with ultrasound processing for 30 minutes following reflux). In ethanolic samples, the range was from 43.11 ± 0.24 (prepared using 1% SSG and reflux) to 52.48 ± 0.24 mg GAE/g dw (prepared using 1% PVP/VAC, with ultrasound processing for 30 minutes following reflux). Increasing the concentration of PVP/VAC excipient in the aqueous extraction media from 1% to 5% led to overall increases, with a statistically significant difference ($p < 0.05$) observed specifically in samples prepared through a combination of reflux and ultrasound processing. Across all samples prepared with excipients in both 50% ethanol and water solvents, there were statistically significant differences ($p < 0.05$) compared to the control samples.

PVP/VAC demonstrated greater extraction efficacy than the SSG excipient, even without increasing the concentration from 1% to 5%. Specifically, when employing PVP/VAC, there was an average increase of 11% in the amount of phenolic compounds compared to SSG. Nevertheless, using PVP/VAC in water increased phenol content by approximately 36% compared to controls, while in ethanol, it was around 24%. SSG increased phenol content by approximately 8.93% in ethanol and significantly higher (around 22.57%) in water compared to controls.

The content of total flavonoids ranged from 20.44 ± 0.12 (prepared using 1% SSG and reflux) to 23.10 ± 0.10 mg RU/g dw (prepared using 5% PVP/VAC, with ultrasound processing for 30 minutes following reflux) in aqueous extracts, and from 24.57 ± 0.13 (prepared using 1% SSG and reflux) to 26.98 ± 0.28 mg RU/g dw (prepared with PVP/VAC excipient using ultrasound processing for 30 minutes followed by reflux) in ethanolic extracts with excipients. It was determined that using PVP/VAC excipient, the lowest outcomes were observed with reflux alone, but an increase in flavonoid yield occurred when ultrasound was incorporated and processing time extended from 10 to 30 minutes. Similarly, a parallel pattern of increased flavonoid yield was observed when using the SSG excipient in the extraction process.

The investigation revealed a direct correlation between the concentration of PVP/VAC and the increase in flavonoid levels, specifically from 1% to 5%. A significant ($p < 0.05$) rise in flavonoid yield was noted when comparing samples with PVP/VAC concentrations of 1% and 5%. While a comparable effect was observed when comparing results from both excipients, it is noteworthy that adjusting the PVP/VAC concentration in the extraction media demonstrated the potential for further increases in flavonoid concentrations.

Using ethanol as a solvent, the total flavonoid amount was the highest in the sample prepared with PVP/VAC (5%) excipient using ultrasound processing for 30 minutes and reflux (26.98 ± 0.28 mg RU/g dw). The results with ethanol and excipients were better than with water. Still, the same trends remained: extracts prepared with PVP/VAC and SSG using only reflux yielded the lowest flavonoid concentrations, but when combining this method with ultrasound and increasing the processing time, the concentrations increased. The results were statistically significantly higher than controls prepared without excipients ($p < 0.05$).

Despite the total phenols and flavonoid content of samples with SSG and PVP/VAC under conditions like ultrasound processing for 30 minutes following reflux, showing greater results than using CDs, these excipients were much more challenging to clean from the extraction media. After cooling down, CDs required simple centrifugation and filtration and did not change the

thickness of extracts. However, SSG and PVP/VAC changed the thickness, and the cleaning process was much longer and more expensive. *The influence of excipients on phenolic and flavonoid content in extraction processes is comprehensively examined in Papers 3 and 6.*

2.1.3. Antioxidant effects of excipient-enhanced extracts and their correlation with phenols

The ABTS assay determined ethanolic samples' radical scavenging activity, ranging from 463.92 ± 6.46 to 385.56 ± 6.51 μg TE/g dw. The highest and lowest values were observed in the extract prepared using PVP/VAC with ultrasound processing for 30 minutes and in the control sample prepared without excipients but under identical conditions, respectively. Similar trends were noted in the DPPH assay. Specifically, employing PVP/VAC, ultrasound processing for 30 minutes and reflux resulted in the highest antioxidant activity, registering at 12.81 ± 0.05 μg TE/g dw, while the lowest activity was observed in the ethanolic control sample under identical conditions, measuring 10.49 ± 0.22 μg TE/g dw. FRAP method demonstrated that the highest ferric ion reducing power was observed in a sample prepared with γ-CD using only reflux (193.57 ± 1.94 mg FE(II)/g dw), whereas the lowest was observed in the control sample, consistent with the results obtained from the ABTS and DPPH methods (141.04 ± 7.16 mg FE(II)/g dw). Employing the post-column ABTS method revealed that the sample with α-CD prepared with reflux exhibited the highest antioxidant activity (32.68 ± 1.31 mg TE/g), while the lowest was detected in the control sample under the same conditions (9.38 ± 0.38 mg TE/g ethanolic sample). Importantly, all samples prepared with excipients exhibited statistically higher antioxidant values across all methods compared to the controls.

The experiments conducted on aqueous and ethanolic samples using ABTS and FRAP methods showed consistent results: the 5% PVP/VAC mixture, prepared with 30 minutes of ultrasound treatment followed by reflux, demonstrated the highest antioxidant activity. This method proved effective, with antioxidant capacities measuring 419.29 ± 7.00 μg TE/g dw and 167.45 ± 2.06 mg FE(II)/g dw according to the ABTS and FRAP methods, respectively. Nevertheless, the ABTS method indicated similar antioxidant activity between the γ-CD (under the same conditions as the previously mentioned PVP/VAC sample) and β-CD (using reflux) samples, comparable to the PVP/VAC sample, and the differences between all three samples were statistically insignificant. Notably, the DPPH method revealed a different outcome, with the β-CD sample treated with reflux showing the highest antioxidant capacity at 11.52 ± 0.12 μg TE/g dw. The ABTS post-column results

closely matched both the sample prepared using β-CD and reflux, as well as the 1% PVP/VAC sample prepared using 30 minutes of ultrasound followed by reflux, with values of 20.68 ± 0.83 and 20.9 ± 0.83 mg TE/g dw, respectively.

Upon observing the correlations among the samples, it was evident that all the samples prepared with excipients exhibited a robust relationship between total phenolic content and DPPH assay. Notably, SSG samples displayed the weakest correlation between the identified phenolic compounds and antioxidant activity, both in aqueous and ethanolic samples.

The most significant correlations between phenolic compounds and antioxidant methods were observed in PVP/VAC ethanolic samples, with coefficients of 0.959 (ABTS), 0.981 (DPPH), and 0.994 (FRAP). Similarly, in aqueous samples, the highest correlations were found with β-CD, scoring 0.979 (DPPH), 0.424 (ABTS), and 0.998 (FRAP). *The enhancement of antioxidant activity in samples prepared with excipients and their correlation with phenolic compound content is presented in Papers 3 and 6.*

The post-column ABTS method revealed the highest antioxidant activity in the ethanolic GE1 sample at 30.16 ± 1.21 mg TE/g, closely followed by PVP/VAC with 29.04 ± 1.16 mg TE/g dw, and the lowest in the BE1 ethanolic sample at 10.73 ± 0.43 mg TE/g. All samples demonstrated statistically significant increases in antioxidant levels compared to the controls. When using water as the solvent, the greatest radical scavenging activities were observed in samples extracted with PVP/VAC (20.90 ± 0.83 mg TE/g) and β-CD (20.68 ± 0.83 mg TE/g). The post-column ABTS method identified hyperoside, quercetin-O-hexoside-acetate, clovamide, and caffeoylmalic acid as contributing more to antioxidant responses than isoflavone aglycones daidzein, genistein, and their glycosides daidzin or genistin. *Detailed findings and compound analyses from the ABTS-post column method are discussed in Papers 3 and 6.*

2.1.4. Antimicrobial and antiviral activity of extracts

The antimicrobial activities of the samples were evaluated by monitoring their diffusion into solid nutrient agar. Various formulations, including samples containing excipients (PVP/VAC, SSG, and α-, β-, γ-CDs in water or ethanol), as well as controls prepared without excipients, were tested against pathogenic bacteria. Despite assessing all three different preparations, no significant difference was discerned between the samples prepared with excipients and those without. Therefore, only the results of the ethanolic and aqueous samples prepared using ultrasound processing and reflux are provided.

In this study, despite employing a maximum concentration of red clover extract at 210 mg/mL, no inhibitory effect was observed on *Enterococcus faecalis*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* in either the water or ethanolic samples. Furthermore, the ethanolic extract showed no inhibition against *Bacillus cereus*. Gram-positive bacteria were more susceptible to the aqueous extracts tested than Gram-negative bacteria. The aqueous samples inhibited five microorganisms: *Staphylococcus aureus* (125.51mg/mL), *Staphylococcus epidermidis* (65.92 mg/mL), *Bacillus cereus* (99.34 mg/mL), *Escherichia coli* (99.34 mg/mL), and *Candida albicans* (210.16 mg/mL) and the ethanol samples inhibited four: *Staphylococcus aureus* (34.23 mg/mL), *Staphylococcus epidermidis* (25.92 mg/mL), *Escherichia coli* (34.23 mg/mL), and *Candida albicans* (34.23 mg/mL). *The more detailed antimicrobial activity of red clover extracts is presented in Papers 4 and 6.*

The CC50 of *Trifolium pratense* L. against Vero cells was found to be 4.8 mg/mL. The solvent used ethanol, at concentrations of 8% (red clover at 4.8 mg/mL) and 6.67% (red clover at 4.0 mg/mL), exhibited a virucidal effect. Dilutions of 1:15 and 1:30 demonstrated a 90% reduction in viral titre, displaying moderate to contributory virucidal activity. At a 1:15 dilution (2 mg/mL), exhibited moderate virucidal activity, with a virus reduction of 2.75 $\log_{10}$. Similarly, at a 1:30 dilution (1 mg/mL), virus reductions ranged between 1.125 $\log_{10}$ and 1.75 $\log_{10}$. The virucidal activity of the extract was found to be dose-dependent ($p < 0.05$).

Antiviral testing of *Trifolium pratense* L. extracts revealed effectiveness against IBV, offering protection to Vero cells prior to or during infection. However, pretreatment of IBV with extracts post-infection and cell pretreatment before infection failed to inhibit 100% of the CPE completely. *The more detailed Antiviral Activity of red clover ethanolic extract is presented in Paper 4.*

2.2. Preparation of emulsions and their parameters

2.2.1. Formation and evaluation of red clover extract emulsion with different oils

Emulsions were formulated using red clover extracts as the active ingredient.

In the research to prepare emulsions, ethanolic and aqueous extracts of red clover prepared with β-CD were used. This decision was based on several properties: β-CD does not alter the viscosity of the extract, ensuring that the consistency remains favourable for application purposes; additionally, the excipient was fully separated from the final product. Importantly, the extract

enhanced the ease of emulsion formation with oil and significantly improved emulsion stability, a critical factor for its effectiveness. The extract had been concentrated to increase the potency further, doubling the concentration of active compounds.

Alginate or chitosan was employed in their creation to ensure solubility in both the stomach and intestines. Out of twelve chitosan-based formulations tested, only four (C1P, C2P, C1B, C2B) were deemed suitable for further investigation. Similarly, sixteen alginate-based formulations were explored, with four (A1M, A2M, A1B, A2B) progressing to further research. Emulsifiers utilised included mustard and β-CD. Various plant oils were tested for emulsion formation, with sweet almond oil yielding the smallest particles, thus becoming the preferred choice throughout the study.

Alginate emulsions demonstrated higher stability, ranging from 98.3 ± 1.2% (A2M) to 100 ± 0.8% (A1B), while chitosan emulsions exhibited lower stability, varying from 92.3 ± 0.5% (A2M) to 95.6 ± 0.6% (C2P). The droplet size in alginate emulsions ranged from 0.3 to 5.6 μm, whereas in chitosan emulsions, it ranged from 0.8 to 18.7 μm. Alginate-based emulsions were classified as microemulsions, while those with chitosan were categorised as macroemulsions based on droplet size.

Emulsions formulated with a 2% alginate solution exhibited significantly higher viscosity, with samples A1M and A1B measuring 3460.6 ± 51.2 and 4425.8 ± 78.4 mPa·s, respectively, compared to those with a 0.5% alginate solution (A2M and A2B – 2882.0 ± 87.8 and 3072.3 ± 47.1 mPa·s, respectively). Among chitosan samples, C1P with a polysorbate 80 emulsifier showed the highest viscosity at 5456.4 ± 63.6 mPa·s, while samples with a higher concentration of red clover extract (20%) in C2P and C2B exhibited the lowest viscosity (A2P – 4654.5 ± 81.9 and C2B 4284.8 ± 65.3 mPa·s). *Formulations and physical parameters of alginate and chitosan emulsions are presented in Paper 7.*

2.2.2. Formation and investigation of emulsions incorporating *Myristica fragrans* Houtt. essential oil and plant extracts

Various emulsion formulations were tested to determine the optimal concentration of extracts and essential oils. Consistent with prior approaches to emulsion preparation, red clover extract that had been prepared with β-CD and subsequently concentrated was used. Out of the formulations evaluated, six were selected for further investigation, wherein their stability was assessed through the addition and alteration of excipients aimed at enhancing stability. The stability of all six prepared emulsions varied. Notably, the E6 emulsion exhibited the highest stability at 100% (CI) when all three exci-

pients were utilised (6.2% maltodextrin, 4.3% inulin, and 1.5% gum Arabic). The stability comparison revealed that a single excipient (maltodextrin/inulin/gum Arabic) couldn't maintain stability, with CI below 50%. Incorporating two additional excipients significantly increased stability by about 46% compared to E1. Subsequently, this emulsion was employed in the production of microcapsules using the extrusion method.

The drop diameter of *Myristica fragrans* Houtt. essential oil within the E6 emulsion was measured at 0.571 μm. Key percentiles were assessed, revealing that D10 measured 0.328 ± 0.051 μm, D50 measured 0.46 ± 0.03 μm, and D90 measured 0.89 ± 0.094 μm. The dynamic density of this emulsion was 5532.4 ± 56.5 mPa·s. *For a deeper insight into both the emulsion preparation process and the investigation conducted, refer to Paper 4.*

A formulation E6, with specific adjustments, was chosen to prepare microcapsules using both freeze-drying and spray-drying methods. Challenges arose when the emulsion coalesced into a lump despite efforts to disperse it, rendering dilution ineffective. Consequently, alterations were made to the use of alginate in this formulation and the techniques employed, requiring a higher proportion of dry materials to yield sufficient product. As a result, alternative excipient amounts were selected to compensate for the absence of alginate. The final emulsion formulation comprised red clover extract (12.4%), liquorice extract (49.59%), nutmeg essential oil (0.83%), gum Arabic (5.58%), maltodextrin (18.6%), and inulin (13.2%). As the methods necessitate a more liquid formulation, the addition of water to the emulsion alters its physical parameters, making it impractical to measure them. *Refer to Paper 5 for details regarding the emulsion formulation process and the conducted analysis.*

2.3. Microcapsule formation and parameters

2.3.1. Characterising gastrointestinal-soluble, extruded red clover emulsion microcapsules

Microcapsules were formed from eight emulsions. Alginate microcapsules containing mustard (A1M, A2M) extract exhibited a darker colour, attributed to the extract's light brown shade and a subtle mustard aroma. Notably, when β-CD (A1B and A2B) was utilised as an emulsifier, the microcapsules displayed reduced aggregation and achieved a more consistent morphology, contrasting with those formulated using mustard extract as the emulsifier. Alginate microcapsules exhibited greater uniformity compared to those formed with chitosan. The sizes of freshly prepared microcapsules varied depending on alginate concentration – lower alginate concentrations were

bigger (A2M and A2B – 3.06 ± 0.03 mm and 2.46 ± 0.08 mm, respectively) compared to lower concentrations samples (A1M and A1B – 2.58 ± 0.06 mm and 2.18 ± 0.04 mm, respectively). The microcapsule samples underwent substantial shrinkage, reducing their original size by over 50%. Alginate samples incorporated with mustard displayed shrinkage up to 61.56% for A1M and 68.25% for A2M, while samples with β-CD exhibited a comparable reduction of roughly 75%. The swelling capacity of alginitic microcapsules was lower when gelling agent concentrations were lower (A2M 124.86 ± 1.56% and A2B 132.82 ± 1.11%) compared with microcapsules with higher alginate concentrations (A1M 135.37 ± 2.45% and A1B 146.11 ± 1.67%). Similarly, higher gel former concentration resulted in stronger microcapsules, and the A1M sample had the highest firmness of 4400.01 ± 159.66 g. The use of mustard extract increased the firmness compared to samples with an emulsifier β-CD.

All chitosan samples displayed a uniform light brown colour, unaffected by the emulsifiers used. Microcapsules with polysorbate 80 (C1P and C2P 1.79 ± 0.09 mm and 1.97 ± 0.05 mm, respectively) resulted in smaller microcapsules compared to β-CD (C1B and C2B 2.37 ± 0.05 mm and 2.15 ± 0.02 mm, respectively); additionally, higher concentrations of red clover (formulations C2) extract led to larger microcapsules with polysorbate 80, while with β-CD, the size decreased. Like alginate microcapsules, those made of chitosan also experienced shrinkage. Among them, the smallest was the chitosan microcapsules C1P, incorporating polysorbate 80, measuring 1.22 ± 0.08 mm. Microcapsules containing polysorbate 80 exhibited the least shrinkage due to their lower water content (C1P ~53.70% and C2P ~55.70%). Samples containing β-CD showed a comparable reduction of approximately 75%. Microcapsules containing higher concentrations of red clover extract exhibited significantly softer textures compared to those with lower red clover content. Specifically, the sample prepared with polysorbate 80 (C2P) displayed the lowest firmness, measuring 538.31 ± 20.32 g. Among the chitosan samples, C1B demonstrated the highest firmness at 1266.78 ± 97.97 g. However, overall, the chitosan samples were notably softer when compared to alginate microcapsules. *For further elaboration on the physical characteristics of microcapsules with alginate and chitosan, refer to Paper 7.*

2.3.2. Physical parameters of red clover, liquorice extracts and nutmeg essential oil microcapsules

Microcapsules were prepared using the E6 formulation containing nutmeg essential oil, red clover flowers and liquorice root extracts. The extrusion method used a 5% crosslinker (Ca_2Cl) solution, resulting in spherical micro-

capsules with a light brown colour. The yield of microcapsules obtained was 68.67 ± 3.08% according to phenolic compounds content, with microcapsules measuring 1.87 ± 0.35 mm in diameter and exhibiting softness, requiring a force of 3758.52 to 4135.87 g to crush. The diameter of microcapsules decreased to 0.76 ± 0.11 mm, surpassing the device's maximum force capacity of 6500 g. In gastric media, the swelling index ranged from 19.74% to 13.96%, with alginate microcapsules displaying no swelling. Conversely, in the intestinal medium, microcapsules swelled significantly, ranging from 25.22% to 121.59%. *A detailed description of microcapsule physical parameters is provided in Paper 4.*

2.3.3. Comparative analysis of freeze-dried versus spray-dried microcapsules with red clover, liquorice extracts and nutmeg oil essential oil

For the microcapsule samples prepared using lyophilisation (LM) and spray-drying (SDM), the exact formulations of emulsions were used, including red clover, liquorice extract, and nutmeg essential oil. The two preparation methods exhibited notable differences in yield, moisture content, physical characteristics, and the release of bioactive compounds.

The yield of microcapsules prepared using lyophilisation was 85.34% and 45.12% using spray-drying. However, the moisture content in the SDM (3.24 ± 0.36%) samples was significantly lower than that in the LM (4.45 ± 0.23%) samples. The powder form microcapsules were analysed according to the pharmacopoeia test to determine their quality. The flowability of LM and SDM samples was different. According to the compressibility index and Hausner ratio, the powder of LM microcapsules was of fair quality (20.83 and 1.263, respectively), but SDM had very poor flowability (compressibility index 38.71, Hausner ratio 1.632). Spray-dried microcapsules exhibited a solubility of 95.16%, whereas lyophilised microcapsules showed a solubility of 94.69%. Despite spray-dried microcapsules having slightly higher solubility, statistical analysis revealed an insignificant difference. It is conceivable that particle size may have influenced solubility. Encapsulated essential oil particles produced using lyophilisation and spray drying were 13.51 μm and 7.33 μm, respectively, expressed as D [4, 3]. LM sample uniformity was 0.533 and SDM 0.379. Therefore, LM essential oil particles' sizes (in microcapsules) had a wider distribution range compared to the SDM samples. A scanning electron microscope revealed larger LM sample shapes than SDM samples. While spray-dried microcapsule powder displayed small splinter-shaped particles alongside spherical shapes, freeze-dried microcapsule powder exhibited an irregular form resembling shattered glass.

All the results obtained with LM samples determining active compounds were significantly higher than SDM samples obtained using spray-drying. The total phenolic content measured in the LM sample was 11.47 ± 0.01 mg GA/g dw, whereas in the SDM sample – 8.56 ± 0.42 mg GA/g dw. It can be concluded that the freeze-drying process caused less phenolic compounds degradation than spray-drying. Similar trends were noted in total flavonoid content, mirroring the observations of the phenolic content. The LM formulation showed slightly higher levels of flavones (7.56 ± 0.02 mg RU/g dw) than the SDM sample (5.21 ± 0.22 mg RU/g dw). Additionally, there was a positive correlation between phenolic content measurements and antioxidant activity across three different methods (DPPH, ABTS, and FRAP). All the results of the antioxidant activity of the LM sample were statistically higher ($p < 0.05$) compared to SDM. Since the lyophilised powder sample showed better quality and had more beneficial compounds like phenols and flavonoids, as well as stronger antioxidant properties compared to the spray-dried sample, it was decided to research further and use only LM microcapsules powder. *For additional investigation and comparison of microcapsules prepared via lyophilisation and spray drying, see Paper 5.*

2.4. Product development

2.4.1. Development and investigation of extruded microcapsules in capsule form

Extruded microcapsules with essential oil and extracts could be an additional form to incorporate in other pharmaceutical forms or used alone in personalised dosages for therapeutic benefits. Swelling is essential for the controlled release of the encapsulated material; the microcapsule shell can swell, changing its permeability and controlling the encapsulated substance's release rate and target site. Extruded microcapsules' swelling index in gastric media was from 19.74% to 13.96%, and alginate microcapsules with extracts and essential oil did not swell in gastric fluid. Conversely, microcapsules swelled from 25.22% to 121.59%, and the maximum swelling index was reached after 24 hours. Nevertheless, after that, the microcapsule lost its shape and softened. These microcapsules were only soluble in gut media. *For further information about microcapsules with essential oil swelling, see Paper 4.*

Developing microcapsules using solely red clover extract and employing both alginate and chitosan as base materials with various emulsifiers' different behaviours in gastric and gut media. Specifically, microcapsules composed of chitosan (formulations C1 and C2) readily dissolved upon

exposure to gastric media. Meanwhile, alginate-based microcapsules (formulations A1 and A2) initially underwent swelling in the gastric environment before its dissolution within the intestinal media. Chitosan microcapsules were observed to fully solubilise within 90 minutes. Not all alginate microcapsules disintegrated after 270 minutes of *in vitro* testing, including 150 minutes in intestinal media. Specifically, the A1 formulation demonstrated notable resilience, attributable to its thicker shell derived from a higher alginate concentration of 2%. This contrasted with the A2 formulation, which had a lower alginate concentration of only 0.5% and showed a different rate of disintegration. Most phenolic compounds were successfully released *in vitro*; however, some losses were experienced during the microencapsulation process. Among the alginate microcapsules, the highest concentration of phenolic content was observed in sample A2M, with 22.71 ± 0.08 mg GAE/g released after 210 minutes. Conversely, the lowest concentration was found in sample A1B, measuring 17.92 ± 0.06 mg GAE/g after 270 minutes. Regarding chitosan samples, the highest release of phenols occurred in sample C2P, with 18.14 ± 0.19 mg GAE/g being released after 90 minutes. Among the isoflavones released, genistein exhibited a gradual release, reaching its highest release concentration in the A2B alginate sample. In contrast, the lowest concentration of genistein was observed in the A1M sample. Similar release kinetics were noted for biochanin A and daidzein across different alginate samples. In the case of chitosan microcapsules, especially in the C2 formulation, a progressive dissolution over time was observed, resulting in the release of nearly all isoflavone aglycones. Notably, the incorporation of β-CD significantly enhanced the release of isoflavones in chitosan microcapsules, as evidenced by sample C2B surpassing C2P in genistein release. All chitosan samples underwent complete solubilisation in stomach media, with the C2 formulations demonstrating the highest release rates, albeit with comparatively lower concentrations of released biochanin A. *Refer to Paper 7 for further information on soluble extruded microcapsules in hard capsule form and the in vitro release of active compounds.*

2.4.2. Determining parameters of chewable gel base tablets – incorporating lyophilised microcapsules and investigating *in vitro* release of bioactive ingredients

The lyophilised powder exhibited higher yields and more efficient encapsulation than the spray-dried powder. Consequently, subsequent studies focused solely on the powder of LM microcapsules. These microcapsules demonstrated complete dissolution after 30 minutes in intestinal media, remaining fully undissolved in the stomach for up to 90 minutes. The

concentrations of bioactive compounds after 120 min in the gut media of the LM sample were 428.25 μg/mL, 235.63 μg/g, and 91.36 μg/g (glycyrrhizin, daidzein, and genistein, respectively). The lowest concentration of all the compounds was genistein. Isoflavones, daidzein, and genistein were released gradually in the gastric and intestinal medium. Regardless, glycyrrhizin release was most intense between 60 and 90 min. The percentage of determined monoterpenes in microcapsules LM release is calculated based on the concentrations of compounds identified in the essential oil. The maximum number of essential oil compounds was determined after 30 minutes – later, their amounts decreased. Sabinene's released concentration was the highest compared to other compounds of the nutmeg essential oil –58.51% (after 30 minutes). These microcapsules alone could also be put in the hard capsule shell or compressed into tablets. *For further information on the release of LM microcapsules, refer to Paper 5.*

It was decided to select chewable gels as the base among all available pharmaceutical forms and integrate microcapsules into it. A gelatin base was selected as the control, while the test samples were prepared with pectin as a base. Five compositions (A-E) were formulated. The best composition was selected by the appearance and flavour as the tablet's basis didn't contain additional sugar of the tablets (E tablets were very hard, A and B were soft and were not taken out from the form without mass loss, and D lacked flavour intensity. Composition C was selected for incorporation into the microcapsules. The contrast in appearance between gelatin and pectin tablets was distinct: gelatin tablets had a smoother surface, whereas pectin tablets appeared rough. Unlike their pectin counterparts, gelatin tablets exhibited less firmness and hardness but greater elasticity. Pectin chewable gel tablets with lyophilised microcapsules were significantly firmer and harder (1303.3 ± 377.28 g and 798.09 ± 201.12 g, respectively) among all the samples, especially when compared to the control sample of gelatin with LM microcapsules (548.69 ± 12.63 g and 549.51 ± 11.36 g, respectively). The tablets containing microcapsules showed an increase in firmness and hardness by approximately 27% and 31%, respectively, in the pectin base. However, gelatin tablets remained soft, with an increase of about 63% in firmness and 34% in hardness. Incorporating microcapsules notably reduced gelatin-based tablets' stickiness by 68% but increased it by 10% in pectin samples. The correlation with springiness remained undetermined; however, it was evident that pectin tablets exhibited lower elasticity than gelatin tablets (compared to their respective control tablets). *For further information on the comparison of different chewable gel base preparations and physical parameters tests, see Paper 5.*

The final test sample for the *in vitro* release test was done after 360 minutes following the complete disintegration of the chewable tablets in the gut media. This was done to assess the effectiveness of gel tablets based on the bioactive compounds (glycyrrhizin, genistein and daidzein). The pectin-based chewable tablets demonstrated an encapsulation effectiveness of 71.36 ± 4.22% for glycyrrhizin, 72.03 ± 6.84% for daidzein, and 64.83 ± 2.37% for genistein. Conversely, in gelatin tablets, the release effectiveness of active compounds was 63.41 ± 3.64% for glycyrrhizin, 61.78 ± 4.23% for daidzein, and 60.11 ± 3.82% for genistein. Comparatively, the gelatin LM sample exhibited lower concentrations of active compounds and relatively diminished release effectiveness, suggesting performance limitations compared to pectin-based tablets. Most monoterpenes from microcapsules were released within 30 minutes, with β-terpinene being the most abundant volatile compound. Pectin-based tablets generally exhibited higher release amounts of compounds ($p < 0.05$), except for sabinene, which showed a maximum release from gelatin-based tablets. *See Paper 5 for further clarification of release profiles of chewable gel-base tablets with and without microcapsules.*

3. DISCUSSION

Trifolium pratense L. contains isoflavones, which are of considerable interest to the pharmaceutical industry because of their potential health benefits and applications [6, 52]. Despite the considerable interest, there is a notable gap in comprehensive information on the total phenolic content and antioxidant activities of red clover flower extracts. While the focus has historically been on leaf extracts – presumed to contain higher isoflavone concentrations – recent studies suggest that the flowers also offer significant amounts of these valuable compounds [53–56]. Selecting the appropriate extraction method for isolating isoflavones from red clover flowers is crucial to ensure that the process is cost-effective, environmentally friendly, and safe while also addressing the challenge of isoflavones' limited solubility.

Different parameters (solvent, temperature, extraction method and time, glycosides hydrolysis method) of the extraction process had to be refined during this study in order to enhance isoflavones aglycones, daidzein, and genistein yield in the obtained red clover flower extract and later selection of suitable excipient. Three extraction methods were employed to establish an ideal strategy for extracting isoflavones. These methods were combined with various hydrolysis techniques (including acidic, alkaline, thermal, natural fermentation, and no hydrolysis) to convert glycosides into aglycones. The extraction techniques utilised were ultrasound-assisted extraction, heat-reflux extraction, and maceration. Many of these methods have been reported in the literature for extracting phenols or isoflavones from various legumes [57–60].

This research revealed that ultrasound-assisted extraction, especially when conducted at a processing temperature of 40 °C for 10 or 30 minutes, along with the heat-reflux method or heat-reflux method alone, was significantly more efficient at yielding higher amounts of isoflavones compared to other methods. During ultrasound-assisted extraction, the intense conditions caused by the collapse of solvent vapour and dissolved gas bubbles damage the red clover cell walls through liquid medium penetration. This process promotes solvent entry and enhances the extraction of phenols, including isoflavones glycosides and aglycones. However, it was observed that prolonged sonication could lead to the degradation of the extract despite the minimal formation of reactive oxidising agents like hydroxyl radicals and hydrogen peroxide [61, 62]. This process was in agreement with our findings that increasing processing time can negatively affect isoflavone yields, emphasising the importance of sonication duration. Notably, our study was supported by the literature suggesting that the stability of isoflavones aglycones under different pH and temperature conditions heavily influences their

yield during extraction and glycosides hydrolysis. These conditions can lead to their degradation or transformation into derivatives, leaving most of the used hydrolysis methods ineffective, except for thermal hydrolysis [63]. Thermal hydrolysis stands out as it leverages heat to induce chemical changes within isoflavones. These changes include the decarboxylation of malonyl-glucosides into acetyl-glucosides and the breakdown of ester bonds, which often results in the formation of glucosides. High-temperature processes facilitate the conversion of glycosides to aglycones, highlighting the necessity for controlled thermal conditions in optimising isoflavone extraction [58, 64].

This study determined that ethanol, particularly at a 50% concentration, significantly outperformed water in extracting isoflavones like daidzein and genistein, a result that can be attributed to ethanol's balanced polarity, which not only facilitates the dissolution of these moderately polar compounds but also aligns well with their solubility characteristics, enhancing their release from the plant material [65]. These results align with those of L. Y. Yoshiara et al., who demonstrated that pure organic solvents were inefficient for isoflavone extraction [66]. Additionally, Rostagno et al. concluded that a 50% ethanol solution was the ideal solvent for isoflavone extraction using ultrasound processing [67]. This finding is significant for the production of nutraceuticals, as it suggests that ethanol not only optimises extraction yields but also meets safety and regulatory requirements for such products.

Conversely, the limited solubility of isoflavones in water highlights its inherent constraints as an extraction solvent. However, incorporating water-soluble excipients like magnesium aluminometasilicate, sodium croscarmellose, SSG, PVP/VAC, and α-, β-, γ-CDs enhance water's extraction performance, suggesting a sustainable, although less efficient, alternative to ethanol-based methods. The use of these excipients in the extraction media (ethanolic and aqueous) to enhance the yield of isoflavones from red clover was done for the first time. Excipients are mostly used in oral solid dosage forms to enhance the bioavailability of poorly water-soluble drugs [68]. Therefore, using these compounds can be beneficial even for extracting targeted compounds depending on the specific mechanism of action of each excipient. In this study, excipients were expected to absorb daidzein and genistein during extraction and increase their final yields. All excipients, except magnesium aluminometasilicate, contributed to an increase in isoflavone yields (both in water and 50% ethanol) compared to control samples prepared under identical conditions. Magnesium aluminometasilicate has been reported in the literature to boost the yield and quantities of various compounds in essential oil extractions but did not enhance isoflavone yields in our experiments [23]. This suggests that magnesium aluminometasilicate may preferentially absorb

terpenes rather than isoflavones from red clover extracts, highlighting the importance of selecting excipients that are compatible with the specific compounds targeted for extraction.

All three CDs enhance isoflavone extraction by improving their solubility and stability. They encapsulate the molecules' hydrophobic sections, boosting solubility and protecting the compounds from degradation through hydrolysis and oxidation [28]. CDs also easily release bioactive compounds after cooling down and are conveniently cleaned out from extraction media using filtration. Daruházi et al.'s investigation into genistein's interactions with different CDs revealed a minimal interaction with α-CD, suggesting a lower affinity and less stable complex formation [69]. Consistent with these findings, our study observed that the interaction between genistein and α-CD, which has the smallest cavity size, was considerably less pronounced compared to β- and γ-CDs. However, our results demonstrate that α-CD significantly enhances isoflavone yields compared to control samples, indicating its effectiveness in isoflavone extraction despite its smaller cavity size [70]. Moreover, for CDs to effectively form complexes with targeted bioactive compounds it should exhibit hydrophobic characteristics due to CDs' hydrophobic cavities. The affinity between the host molecule (CDs) and the guest molecules (in this study's case, isoflavones) plays an important role in modulating the hydrophobic characteristics of the guests. Daidzein and genistein, with their inherently low solubility in water, benefit from this interaction. Conversely, their glycosylated counterparts exhibit enhanced hydrophilicity, a trait imparted by the polar sugar moieties attached to them, facilitating improved water molecule interactions [71]. This property leads to an increase in the yield of aglycones in extracts involving CDs, as the larger molecular structure of glycosides poses challenges for their incorporation into the CDs' cavities. Consequently, glycosides are either notably absent or present in low concentrations in extracts prepared with ethanol and water.

Increasing the concentration of CDs in the water-based extraction medium from 1% to 5% led to a notable enhancement in the levels of daidzin and genistein. Specifically, daidzin levels increased by approximately 1.06, 1.4, and 1.25 times when using α-, β-, and γ-CDs, respectively. For genistein, its concentration saw an uptick with the use of α- and γ-CDs (1.28 and 1.12 times, correspondingly) while observing a decline with β-CDs. Altering the extraction solvent to a 50% ethanol mixture resulted in a significant boost in aglycone yields, with an average increase of 2-3 times (compared with controls), except in the case of β-CD, where genistein's yield decreased by 17%. Particularly, α-CD utilisation in the extraction process increased genistein yield by 3.32 times and γ-CD by 2.36 times. Additionally, the quantity of daidzein experienced growth across all CD variants – 3.64, 2.31, and 3.06

times for α-, β-, and γ-CDs, respectively. The genistein yield decrease with β-CD could be explained based on Boonyarattanakalin et al.'s study results that highlight the impact of ethanol in CD complexation, demonstrating that ethanol can interfere with and compete for inclusion in CD complexes, particularly with β-CD [70]. This suggests a specific competitive interaction between genistein and ethanol for β-CD binding.

Isoflavone extraction using SSG and PVP/VAC showed promising outcomes, particularly when the concentration of PVP/VAC in water was increased to 5%. While SSG and PVP/VAC effectively extracted isoflavones, removing these substances completely from the extraction medium proved challenging. Furthermore, high levels of SSG led to an undesirable thickening effect within the extract, adversely affecting its characteristics.

The total phenolic and flavonoid contents, as well as the antioxidant potential of extracts prepared with various excipients, were determined. The excipients used in this study included SSG, PVP/VAC, and α-, β-, γ- CDs. The extraction conditions were selected based on the three most promising methods (ultrasound processing (10 or 30 min) followed by thermal hydrolysis or reflux alone) identified in previous research, which focused on isoflavone yield. Previously determined isoflavone yields were in agreement with determined phenols and flavonoid contents. The study demonstrated a notable increase in phenolic compounds when utilising excipients in aqueous extractions, significantly surpassing control samples without excipients ($p < 0.05$). Among the excipients, α- or β- CDs facilitated the most efficient phenolic extraction via reflux; however, integrating ultrasound with reflux reduced phenolic yield. Conversely, employing γ-CD, PVP/VAC, and SSG alongside ultrasound and reflux proved more advantageous, though γ-CD and SSG yielded lesser phenolics compared to α- or β-CDs. Aqueous extractions with β-CDs and PVP/VAC showed enhanced phenol and flavonoid levels, whereas ethanol extractions favoured α-, γ-CDs, SSG, and PVP/VAC for higher yields. Kaurinovic et al. indicated lower flavonoid levels in red clover with water or ethanol compared to this investigation [72]. Esmaeili et al. reported flavonoid yields *in vivo* red clover ranging from 27.57 to 46.88 mg GAE/g dw, aligning with our findings using non-toxic, green extraction methods [73]. Küçükboyaci et al.'s research showed higher active compound levels using methanol, a potent but toxic solvent [74]. The use of methanol and chloroform poses toxicity risks and is deemed unsafe for studies on living organisms. Despite solvent evaporation steps, the risk of residual toxicity highlights the safer alternative of employing non-toxic solvents and excipients, ensuring cleaner final extracts [75].

Antioxidant activities were measured through various methods due to the diverse array of antioxidants present in plant extracts, including ABTS, post-column-ABTS, DPPH, and FRAP test. The results highlighted that extraction conditions, particularly the use of excipients, significantly impact the extracts' phytochemical composition and, consequently, the antioxidant effectiveness [76]. The biological benefits of polyphenolics are linked to their antioxidant properties, such as neutralising free radicals. These include their roles in antibacterial, anti-inflammatory, antiallergic, antiviral, cancer prevention and others [77]. While there is considerable research on phenols in red clover, studies specifically targeting its flowers are limited, as most research focuses on the leaves or the entire plant. Jakubczyk et al.'s investigation involved extracting red clover flowers using a Soxhlet apparatus with 96% ethanol, finding its antioxidant activity (measured by DPPH) to be around 30% lower for controls and 33–39% lower for samples with excipients compared to this study [78]. Similarly, Horvat et al. prepared extracts from the leaves of different red clover strains, revealing that their DPPH inhibition was on average 18% lower than our controls and 24–27% lower than our samples with excipients [79]. Both the ABTS and DPPH methods showed similar results, which demonstrate that the results are reproducible and that these methods are complementary. Literature indicates that red clover flowers typically demonstrate low (0.0251 mg FE(II)/g dw) to undetectable reducing antioxidant power via FRAP method [78, 80]. Contrasting these findings, our samples showed statistically higher activities than the controls, with no significant variance observed between those prepared using PVP/VAC and SSG. Differences were noted with the use of CDs. Moreover, despite the use of different solvents and drying conditions for red clover, the results of this study exceeded those documented by Zawislak et al. in all instances involving excipient samples [81]. Numerous studies have also reported this correlation [79, 82]. This confirms that the phenolic compounds are the primary source of inherent antioxidant activity, which has additionally been stated in other publications [76, 83]. The correlations showed stronger results in ethanol samples for PVP/VAC and SSG than those in water when using CDs with varying mechanisms of action as excipients. However, employing excipients enhances flavonoid solubility in water and ethanol, enhancing extracts' antioxidant activity.

The ABTS post-column method is beneficial for analysing the antioxidant activities of specific compounds in complex mixtures [84]. This study assessed both an extract's overall antioxidant capability and a standard isoflavone mix (genistein, daidzein, genistin, and daidzin), known for contributing to radical scavenging activities [85]. However, isoflavones showed no antioxidant activity in this method, suggesting they require longer ABTS

assay incubation times to reach reaction equilibrium, a finding supported by existing literature [86]. Despite prolonged incubation, when tested via the ABTS spectrophotometric method, the isoflavone samples showed a minimal antioxidant response of 134.64 ± 5.87 μg TE/g. This minimal activity indicates that isoflavones might enhance the overall antioxidant effect of the extract through synergistic interactions with other compounds [87, 88]. In the extracts, the ABTS post-column chromatogram identified caffeic malic acid, hyperoside, and clovamide as having the highest antioxidant response compared to other compounds present.

Despite the literature suggesting limited antimicrobial and antivirus activity in red clover flowers, our findings reveal that red clover extract exhibits selective antimicrobial action, particularly in higher concentrations, especially against Gram-positive bacteria. Excipients do not significantly alter the antimicrobial activity of red clover, nor does it vary markedly between ethanolic and aqueous extracts, with both types showing effectiveness against a number of microorganisms. The main active compounds in red clover, daidzein and genistein, have been identified as potential antimicrobial agents, particularly against Gram-positive bacteria. These findings are consistent with the literature – Khan et al. reported that for the extracts of the species Trifolium, the studied extracts presented a greater efficiency against gram-positive bacteria rather than gram-negative bacteria [89].

The red clover extracts demonstrated a dose-dependent reduction in viral titers, achieving significant virucidal activity at certain dilutions. Specifically, higher concentrations were more effective, indicating potential for *Trifolium pratense* L. as a natural antiviral agent. Red clover extract could lead to effective, natural remedies against viral infections.

Various red clover extract emulsions were created using different oils and emulsifiers to identify the key components for further microcapsules development. Sweet almond oil produced the smallest particles with all emulsifiers (polysorbate 80, mustard extract and β-CD), enhancing emulsion stability through a robust barrier at the oil-water interface [90]. Smaller oil particles in emulsion result in tighter packing and uniform coverage in microcapsule formation, ensuring better encapsulation efficiency and controlled release – key for long-term stability and precise delivery in applications [91].

Four formulations were chosen (two with alginate (A1, A2) and two with chitosan (C1, C2)) with varying emulsifiers, and in the chitosan case, the extract was used. All of the emulsifiers were of different origins: acts as a natural emulsifier forming a protective layer around oil droplets; β-CD is a naturally derived cyclic oligosaccharide but can be obtained synthetically, it is a non-ionic surfactant and emulsifier forming Pickering emulsions and polysorbate 80 synthetic, non-ionic surfactant and emulsifier, facilitating the

dispersion of oil droplets within the aqueous phase [92–94]. Due to their distinct chemical properties, alginate and chitosan face challenges in forming emulsions with identical formulations. Therefore, the formulation between form emulsions differs, and the ones prepared with alginate were more stable as xanthan gum was used as a co-emulsifier to enhance the stability of the system, and it didn't make the samples as thick as with chitosan [95–97].

The alginate concentration positively affected emulsion stability, mirroring observations in the literature [98, 99]. However, higher concentrations of alginate led to higher viscosity within the samples. β-CD demonstrated superior emulsification in both alginate and chitosan samples without significantly altering their viscosity, unlike polysorbate 80 [100]. Furthermore, β-CD did not introduce any extraneous odours or flavours as a mustard extract. Instead, this compound can mask undesired tastes or smells, adding to its appeal as an emulsifying agent [101].

In this research, when emulsions were formed only with red clover concentrated extract, alginate-derived emulsions, with droplet sizes varied from 0.3 to 5.6 μm. It can qualify as microemulsions, typically defined by 100 to 1000 nm sizes and noted for their thermodynamic stability [102]. This form of stability is attributed to droplets forming spontaneously, reducing the system's free energy without needing external energy for the process. Meanwhile, emulsions made from chitosan, showing droplet sizes ranging from 0.8 to 18.7 μm, fall into the macroemulsion category. These are recognised for their kinetic stability, in contrast to the thermodynamic stability seen in microemulsions [102, 103]. Using additional extracts and *Myristica fragrans* Houtt. essential oil resulted in a drop diameter of 0.571 μm in the emulsion. In the literature, the particle size of the emulsion varied with different essential oils, ranging from 16.70 to 55.56 μm [104]. No similar studies were found that involved *Myristica fragrans* Houtt. essential oil, extracts, and combinations of gum Arabic, maltodextrin, and inulin.

Further forming the microcapsules from the obtained emulsions extrusion method indicated that the formulation and choice of emulsifiers significantly influence the characteristics of microcapsules, particularly in terms of colour, aroma, aggregation, morphology, size, shrinkage, swelling capacity, and firmness.

Comparison between alginate and chitosan microcapsules revealed that alginate microcapsules exhibited greater uniformity. Alginate's gel-forming ability stems from its interaction with calcium ions, yielding a stable gel network. This promotes uniformity and stability. In contrast, chitosan, derived from crustaceans' chitin, forms gels via protonation, resulting in less predictable structures due to intermolecular hydrogen bonds [105, 106]. Lower concentrations of the gelling agent resulted in lower swelling capa-

cities, indicating that the polymer concentration directly influences the water retention and structural integrity of the microcapsules [107].

Even though β-CD and polysorbate are used in emulsion preparations, little information exists about their influence on microcapsule formation using the extrusion method. Nevertheless, using β-CD as an emulsifier in alginitic samples (A1B and A2B) resulted in microcapsules with notably reduced aggregation and more consistent morphology than those formulated with mustard extract. This observation confirms that β-CD offers an increased stabilising effect on the emulsion, thereby promoting consistent microcapsule formation. Consistent with existing literature, β-CD used in alternative microcapsule preparation methods has demonstrated efficacy in enhancing the stability of active compounds, shielding them from oxidation, and extending shelf life [108, 109]. However, the greater firmness observed in microcapsules containing mustard extract compared to those with β-CD can be attributed to the calcium content in mustard extract [110]. Calcium acts as a polymerisation agent for alginate, thereby enhancing the firmness of the microcapsules.

Similar to alginate, the characteristics of chitosan microcapsules vary depending on the emulsifier employed. Polysorbate 80 produced smaller microcapsules than β-CD. Higher concentrations of red clover extract resulted in larger microcapsules when combined with polysorbate 80, while with β-CD, the size decreased. This aligns with P. Eslami et al., who found that β-CD significantly increased microcapsule size compared to polysorbate 80. Moreover, adjusting the amount of β-CD or polysorbate 80 in the formulation significantly impacted both encapsulation efficiency and particle size [111]. Using chitosan with polysorbate 80 yielded rounder microcapsules than β-CD. As observed in other studies, the higher viscosity of polysorbate 80 contributes to droplets' increased resistance to deformation, resulting in more uniform shapes [112].

Microcapsules formed with nutmeg essential oil, red clover, and liquorice extracts exhibited comparable firmness to previous alginate-based microcapsules containing only red clover extract and mustard as the emulsifier. This similarity in firmness can be attributed to an increased crosslinker concentration of 5%, which provides more calcium ions to interact with the guluronic acid blocks of the alginate [113]. This denser crosslinking network, facilitated by the elevated concentration of crosslinker, aims to protect volatile compounds from essential oils. A firmer alginate shell ensures a more sustained release of the active ingredient, safeguarding it against premature degradation or release [114].

The *in vitro* release profiles of phenolic compounds from extruded various microcapsule formulations filled capsule and their ability to release bioactive isoflavones–daidzein, genistein, and biochanin A – were done. Due to its positive charge arising from the amino functional group, Chitosan is soluble in acidic solutions such as gastric medium (pH value about 1.5–3.5). Therefore, the chitosan microcapsules dissolve in about 90 minutes, releasing the encapsulated compounds for absorption in the stomach [107]. In contrast, alginate microcapsules, which dissolve in alkaline conditions and swell in gastric environment, successfully release active compounds into the gut media [115]. Notably, even after 270 minutes, some alginate microcapsules, particularly those with the A1 formulation, did not completely disintegrate due to their thicker alginate-based shells compared to the A2 formulation (0.5% alginate), thus restricting the full release of phenols Structural differences were evident in phenolic release profiles. The A2 formulation, despite faster disintegration, released the highest amount of phenolic compounds among alginate microcapsules (22.71 ± 0.08 mg GAE/g) after 210 minutes. Thinner alginate shells enhance bioactive release efficiency, while the A1 formulation, with its robust shell, showed the lowest phenolic release, highlighting the balance between stability and release efficiency [113]. Chitosan microcapsules, especially with the C2 formulation, displayed gradual dissolution and proved highly efficient in releasing isoflavones. Integration of β-CD as an emulsifier significantly boosted solubility and release rates of isoflavones like genistein, with sample C2B surpassing C2P. This improvement stems from β-CD's capacity to enhance the water solubility of hydrophobic compounds such as isoflavones [108].

For the microcapsule samples prepared using lyophilisation (LM) and spray-drying (SDM), exactly the same formulations of emulsions were used (including red clover extract, liquorice extract and nutmeg essential oil) but resulted in different qualities. Each method significantly influences the physical characteristics, bioactive compound retention, and overall quality of the resulting microcapsules [116, 117].

The obtained yield from the lyophilisation was notably higher (85.34%) compared to just 45.12% for spray-drying. This discrepancy could be attributed to the inherent loss of material common in spray drying processes due to factors like adhesion to equipment surfaces, incomplete particle recovery, or the degradation of active compounds [117]. Lyophilisation outperformed spray-drying in preserving phenolic compounds, with concentrations significantly higher (11.47 *vs* 8.56 mg GA/g dw). This pattern extended to other key antioxidants like flavonoids. Preservation of these compounds is vital for microcapsule antioxidant activity, significantly higher in lyophilised samples across DPPH, ABTS, and FRAP assays. Despite the longer duration of

lyophilisation, its low temperature advantageously retains labile components compared to the high temperatures required in spray drying, as noted in the literature [118].

Physical properties, such as flowability and particle size distribution, varied significantly between the methods. SDM exhibited poorer flowability, as reflected by a higher compressibility index of 38.71, which could complicate handling and application in industrial settings. This finding aligns with the results of the literature comparing these two methods [119]. Nevertheless, particle size analysis showed that SDM tends to produce smaller and more uniformly shaped particles than lyophilisation methods, which could affect the release and solubility of the encapsulated compounds. Consistent with the literature, SDM typically results in spherical microcapsules, as in our research, whereas freeze-dried ones often appear as shattered glass [120].

LM microcapsules demonstrated better encapsulation efficiency and yield than their spray-dried counterparts, leading to their selection for incorporation into the chewable gel tablet base from pectin. Although LM microcapsules dissolved completely within 30 minutes in intestinal media, a notably faster rate compared to previously discussed microcapsules prepared via extrusion, the phenols extracted from liquorice and red clover (glycyrrhizin, daidzein and genistein) had higher concentrations of active ingredients than those found in extruded microcapsules. Key volatile compounds from nutmeg essential oil were detected in microcapsules: α-pinene, known for reducing antibiotic resistance [121]; sabinene, which combats skeletal muscle atrophy [122]; β-myrcene, offering a myriad of benefits such as analgesic, sedative, anti-diabetic, antioxidant, anti-inflammatory, antibacterial, and anticancer properties [123]; and β-terpinene, exhibiting anti-inflammatory effects by inhibiting the production of pro-inflammatory mediators [124].

A clear distinction was observed between gelatin and pectin bases in the formulation of chewable gels. Pectin-based gels, despite their rougher appearance and higher firmness and hardness, outperformed gelatin bases in encapsulating and releasing bioactive compounds. Gelatine melts near body temperature, which is unsuitable for vegetarians and vegans [125, 126]. The selected pectin base (composition C) demonstrated higher encapsulation effectiveness for all tested compounds and ensured a more controlled release, which is crucial for maximising therapeutic efficacy.

In vitro studies have demonstrated the advantages of pectin over gelatin for controlled-release applications. While gelatin-based tablets showed lower release efficiency and were stickier, pectin tablets provided a more stable release of bioactive compounds such as glycyrrhizin and daidzein, achieving significantly better release efficiency. Additional research corroborates the

effectiveness of pectin not only in tablet form but also in film-based formulations, highlighting its potential as a versatile gelling agent for controlled release systems [127]. The inclusion of lyophilised microcapsules in pectin gels also improved the mechanical properties of the tablets, suggesting that pectin is a more suitable matrix for the delivery of encapsulated compounds in therapeutic applications.

4. SUMMARY OF THE CONCLUSIONS

1. Ultrasound-assisted extraction (38 kHz, 100%; 40 °C; time 10 or 30 minutes), particularly when combined with thermal hydrolysis or reflux alone, was found to be the most effective method for enhancing isoflavones aglycones (daidzein and genistein) yields from red clover. Excipients significantly influenced the yield and quality of isoflavone extractions. The use of CDs and other excipients under previously mentioned conditions yields isoflavones and improves the phenolic and flavonoid content of the extracts.

For excipient-assisted extraction, excipients like PVP/VAC and CDs showed improved extract antioxidant activities, as demonstrated in various assays (ABTS, DPPH, FRAP). This enhancement correlates strongly with the increased phenolic contents, highlighting the potential of excipients to boost the health benefits of plant extracts. The antimicrobial and antiviral assessments revealed that red clover extracts obtained with solvents like ethanol effectively inhibit common pathogens, specifically Gram+ bacteria, and have potential antiviral activity. Nevertheless, excipients did not enhance antimicrobial and antiviral activity.

2. Emulsions' stability and particle size were influenced by the type of oil, gelling agent and emulsifiers used in emulsion formation. Alginate-based emulsions show higher stability and smaller particle sizes than those with chitosan. Formulations with alginate and β-CD as emulsifiers provided more stable emulsions with smaller droplet sizes, which are advantageous for consistent bioavailability and extended shelf life in chitosan and alginate emulsions.

Emulsions with incorporated nutmeg essential oil and plant extracts showed varying stability levels, significantly enhanced using multiple emulsifiers.

3. Technological factors such as the choice of polymer (alginate, chitosan), emulsifier (β-CD, mustard extract, polysorbate 80), and microencapsulation method (extrusion, freeze-drying or spray-drying) significantly affected the physical properties, stability, and quality of microcapsules.

Microcapsules formulated with alginate and chitosan (formulation 2) exhibited the highest potential among all samples produced by the extrusion method, demonstrating optimal swelling, release, and firmness. Incorporating β-CD enhances bioactive compound release, offering opportunities for improving delivery systems in both gelificators (alginate, chitosan) samples. Because of its chemical composition, mustard with alginate yields a stronger shell. Alginate microcapsules exhibit controlled release in both formulations,

while chitosan microcapsules dissolve in stomach media, suggesting targeted delivery potential.

To protect volatile compounds in extruded microcapsules from emulsions using nutmeg essential oil, a higher crosslinker concentration (5%) was needed.

The comparison between freeze-dried and spray-dried microcapsules highlighted differences in bioactive compound retention, solubility, and physical characteristics. LM showed higher yield and preserved more phenolics and flavonoids, offering better quality over SDM.

4. Developing extruded microcapsules into hard gelatin capsules demonstrated that material composition significantly affects the release and dissolution profiles in different gastrointestinal media, pointing to potential strategies for optimising drug delivery systems. This system needs improvement to enhance the concentration of bioactive compounds, as it was quite low in chitosan microcapsules.

Chewable gel tablets incorporating lyophilised microcapsules showed distinct release patterns for bioactive compounds influenced by the gel base (gelatin or pectin). The base material can significantly influence the release dynamics of encapsulated compounds, as demonstrated by pectin, which releases them more gradually due to its firmness compared to gelatin.

SUMMARY IN LITHUANIAN

Įvadas

Vaistiniai augalai visame pasaulyje yra neatsiejama medicinos dalis ir išlieka pagrindinis natūralių biologiškai aktyvių junginių šaltinis daugumai pasaulio gyventojų, ypač besivystančiose šalyse [1]. Mokslo metodų tobulėjimas paskatino vaistinių augalų farmakologinį vertinimą perkelti iš empirinių stebėjimų į mokslinius tyrimus ir įtraukti augalinės kilmės junginius į įrodymais grįstos medicinos sritį [2, 3]. Augaluose esantys biologiškai aktyvūs junginiai pasižymi tarpusavio sinergija, daugiafunkciškumu ir struktūriniu įvairumu, suteikiančiu jiems pranašumų, palyginti su vienkomponenčiais sintetiniais vaistiniais preparatais [4, 5]. Dėl šios priežasties fitocheminės medžiagos atlieka svarbų vaidmenį atradant ir kuriant naujus vaistus.

Raudonieji dobilai (*Trifolium pratense* L.) yra žinomi dėl savo gydomųjų savybių ir užima reikšmingą vietą tiek tradicinėje, tiek šiuolaikinėje fitoterapijoje [6, 7]. Šio augalo žiedai tyrimui pasirinkti dėl plataus panaudojimo ir biologinio aktyvumo, kurį lemia augale randami izoflavonai, kumarinai, flavonoidai ir fenolinės rūgštys. Dėl šių junginių padidėja raudonųjų dobilų žiedų teikiama nauda sveikatai ir jie yra vertingas maisto papildų komponentas [8, 9].

Pagrindiniai izoflavonai, randami *Fabaceae* šeimos ankštiniuose augaluose, įskaitant ir raudonuosius dobilus, yra genisteinas, daidzeinas, glicitinas, formononetinas ir biochaninas A [12]. Šie junginiai struktūriškai panašūs į estrogenus, pavyzdžiui, į 17-β-estradiolį, ir geba konkuruoti dėl prisijungimo prie estrogeno receptorių. Izoflavonai, sąveikaudami su estrogenų receptoriais, gali sukelti estrogeninį arba antiestrogeninį poveikį, priklausomai nuo dozės ir poveikio trukmės [9–12]. Dėl izoflavonų fitoestrogeninių savybių raudonieji dobilai tapo populiarus pasirinkimas siekiant sušvelninti menopauzės simptomus, kaip karščio bangos, nuotaikų svyravimas ir osteoporozė. Raudonieji dobilai veikia kaip pakaitinės hormonų terapijos alternatyva [13]. Be to, raudonųjų dobilų izoflavonai pasižymi antioksidacinėmis, priešvėžinėmis, priešuždegiminėmis ir kardioprotekcinėmis savybėmis, kurios gali padėti apsisaugoti nuo įvairių lėtinių ligų, taip pat padidina augalo patrauklumą produktų formulėse, skirtose sveikatai stiprinti [14–19]. Visgi minėtiems preparatams reikalingi išsamesni klinikiniai tyrimai.

Izoflavonai dažniausiai aptinkami glikozidų, tokių kaip daidzinas ir genistinas, forma. Šie konjuguoti izoflavonai yra neaktyvūs junginiai ir dėl struktūros ypatybių sunkiai absorbuojami žarnyne, tačiau tampa aktyvūs aglikono formoje (daidzeinas, genisteinas), kai gliukozės fragmentas pašalinamas iš molekulės. Dėl šios priežasties hidrolizė yra itin svarbus žingsnis

norint gauti biologiškai aktyvias ir lengvai organizme absorbuojamas izoflavonų formas. Tinkamo ekstrakcijos metodo ir parametrų pasirinkimas, siekiant sukurti inovatyvų produktą iš augalinės kilmės žaliavų, yra svarbus etapas [21, 22].

Pagalbinės medžiagos, tokios kaip (α-, β-, γ-) ciklodekstrinai (CD), polivinilpirolidono-vinilacetato kopolimeras (PVP/VAC), kroskarmeliozės natrio druska, karboksimetilinto krakmolo natrio druska (SSG) arba magnio aliuminio metasilikatas, gali būti naudojamos siekiant pagerinti izoflavonų ekstrakciją iš raudonųjų dobilų. Šie vaistų gamybai naudojami junginiai didina aktyviųjų junginių stabilumą ir biologinį prieinamumą [23–28]. Pagalbinių medžiagų naudojimas aktyviųjų junginių iš augalinės žaliavos ekstrakcijai yra mažai tirta sritis, kuri suteikia naujų galimybių šių metodų efektyvumui ir saugumui pagerinti. Nors šiuo metu siekiama ieškoti mažai toksiškų tirpiklių, kurie padėtų išgauti mažai tirpius vandenyje biologiškai aktyvius junginius, technologiniame procese mažai dėmesio skiriama pagalbinėms medžiagoms, kurios padidintų tikslinių junginių išeigas arba sumažintų reikalingo tirpiklio kiekį [29, 30]. Visgi literatūroje buvo rasta keletas tyrimų, susijusių su β-CD panaudojimu ekstrakcijos procese. Vienas jų – sėkmingai padidintas fenolinių junginių kiekis iš persikų išspaudų [27]. Pagalbinės medžiagos, kaip jūros vanduo, polisorbatas 20 ar magnio aliuminio metasilikatas, išbandytos augalinių žaliavų distiliavimo procese siekiant padidinti eterinio aliejaus kiekį [31–33]. Šiuo tyrimu buvo siekiama užpildyti esamą tyrimų spragą ir įvertinti pagalbines medžiagas izoflavonų ekstrakcijos iš raudonųjų dobilų žiedų procese. Atrinktos pagalbinės medžiagos padidino izoflavonų genisteino ir daidzeino išeigą ir ekstrakto antioksidacinį potencialą.

Genisteinas ir daidzeinas yra vandenyje mažai tirpūs, todėl jų pasisavinimas organizme yra gana nedidelis, palyginti su suvartojamu jų kiekiu, todėl nepakanka gauti izoflavonais papildytų ekstraktų. Dėl jų jautrumo aplinkos veiksniams, kaip šiluma ir šviesa, ir dėl jiems būdingų savybių, kaip mažas tirpumas vandenyje, greitas metabolizmas ir pašalinimas iš organizmo, formuojant tolesnes farmacines formas, būtina paruoštą ekstraktą inkorporuoti į emulsiją [34–36]. Emulsijos forma yra labai universali. Jos leidžia kurti mikro- arba nanokapsules, kurios padidina veikliųjų medžiagų stabilumą, biologinį prieinamumą, veiksmingumą ir sumažina galimą šalutinį poveikį, kontroliuojant inkapsuliuotų junginių išsiskyrimo greitį. Taip pat jos gali būti pritaikomos gaminant kietąsias farmacines formas [34].

Mikrokapsuliacijos technologija apima mažos molekulinės masės aktyviųjų junginių, dalelių įterpimą į apsauginę dangą, o tai leidžia kontroliuoti veikliųjų medžiagų absorbciją ir pasiskirstymą tiksliniame audinyje, užtikrinant ilgalaikį poveikį [38–41]. Mikrokapsuliavimo gamybos metodų parinkimas ir veiksmingumas priklauso nuo specifinių parametrų: norimo dalelių

dydžio, fizikomechaninio apvalkalo, veikliųjų medžiagų savybių ir absorbcijos vietos, procese naudojamų pagalbinių medžiagų parinkimo [39, 42]. Mikrokapsulės gali būti paruoštos liofilizacijos, purškiamojo džiovinimo arba ekstruzijos būdais, o kiekvienas iš metodų turi išskirtinių privalumų [43]. Liofilizacija išsaugo jautrių junginių, taip pat ir fenolių, struktūrą ir biologinį aktyvumą, kai vanduo pašalinamas esant žemai temperatūrai ir sumažintam slėgiui. Taip ribojamas terminis molekulės skilimas [44, 45]. Purškiamasis džiovinimas yra greitas ir ekonomiškas būdas inkapsuliuoti veikliąsias medžiagas, be to, jis suteikia papildomą naudą – galima gauti įvairių dydžių daleles, tinkamas geriamiesiems ir inhaliaciniams preparatams [45–47]. Ekstruzija leidžia nepertraukiamai gaminti vienodo dydžio mikrokapsules, kurios yra būtinos kontroliuojamojo atpalaidavimo preparatams [48, 49]. Strateginis polimerų, tirpstančių skrandyje arba žarnyne, naudojimas mikrokapsulių apvalkalų formavimui ypač naudingas gerinant izoflavonų organoleptines savybes, biologinį prieinamumą ir terapinį poveikį kartu su visais minėtais mikrokapsuliavimo metodais [50, 51]. Šiuos polimerus, pavyzdžiui, chitozaną ir alginatą, galima naudoti specifinėse formuluotėse, kad jie ištirptų konkrečiose vietose, priklausomai nuo pH vertės. Taip užtikrinama, kad inkapsuliuoti izoflavonai atpalaiduojami tikslinėje vietoje, kur jų įsisavinimas yra didžiausias [51].

Šio tyrimo metu buvo išplėtota mikrokapsuliuotos genisteino ir daidzeino pernašos sistema iš *Trifolium pratense* L. žiedų ir jos technologinis funkcionalizavimas nuo ekstraktų gamybos iki prototipų – emulsijų ir mikrokapsulių gamybos. Tyrimo metu iš plataus spektro mėginių buvo nustatytos veiksmingiausios formuluotės, įvertinta technologinių veiksnių įtaka galutinių produktų efektyvumui ir funkcionalumui.

Darbo tikslas:

Įvertinti technologinių veiksnių įtaką skirtingais metodais pagamintų mikrokapsulių su genisteinu ir daidzeinu kokybei bei veikliųjų junginių tirpimui *in vitro.*

Uždaviniai:

1. Nustatyti ekstrakcijos procesų įtaką genisteino ir daidzeino (izoflavonų) išeigai iš raudonųjų dobilų (*Trifolium pratense* L.) žiedų, įvertinti pagamintų ištraukų antioksidacinį ir antimikrobinį aktyvumą.
2. Įvertinti izoflavonų, pasirinktų ištraukų ir emulsiklių, formuojančių tiesioginio tipo emulsijas, įtaką emulsijų kokybei.

3. Įvertinti technologinių veiksnių ir izoflavonų įtaką skirtingais metodais pagamintų mikrokapsulių formavimui ir kokybei.
4. Įvertinti technologinių veiksnių įtaką produkto su mikrokapsulėmis formavimui, kokybei ir veikliųjų junginių išsiskyrimui *in vitro*.

Mokslinis naujumas ir praktinė reikšmė

Efektyviosios skysčių chromatografijos ir masių spektrometrijos (LC-MS) analitiniu metodu įvertinta kokybinė ir kiekybinė raudonųjų dobilų ištraukų sudėtis, kiekybiškai nustatytos izoflavonų (genisteino, daidzeino, daidzino ir genistino) išeigos. Taikyti spektrofotometriniai metodai bendram fenolinių junginių ir flavonoidų kiekiui įvertinti. Šio tyrimo metu buvo sukurti nauji izoflavonų (genisteino ir daidzeino) ekstrakcijos iš raudonųjų dobilų žiedų metodai, naudojant pagalbines medžiagas. Atlikti tyrimai atskleidė, kad pagalbinių medžiagų, tokių kaip SSG, PVP/VAC ir (α-, β-, γ-) CD, naudojimas ekstrakcijos procese gerokai padidina fenolinių junginių ir izoflavonų išgavimo efektyvumą (50 proc. etanolio tirpale ir vandenyje) ir sustiprina ekstraktų antioksidacinį potencialą. Pastebėta, kad pagalbinių medžiagų naudojimas neturėjo įtakos antimikrobinėms ekstraktų savybėms. Šiais metodais sprendžiama prasto izoflavonų tirpumo vandenyje problema, kartu užtikrinant, kad ekstrakcija išliktų ekonomiška, nekenksminga aplinkai ir atitiktų žaliosios chemijos principus.

Formuojant emulsijas naudoti skirtingi natūralūs ir sintetiniai emulsikliai. Ekstraktai, paruošti su β-CD ar garstyčių ekstraktu, pasižymėjo didesniu emulsijų stabilumu, palyginti su emulsijomis, kuriose buvo naudotas polisorbatas 80. Emulsijos mikrokapsulių gamybai buvo formuotos naudojant raudonųjų dobilų žiedų ekstraktą, kaip aktyvųjį komponentą, arba raudonųjų dobilų ekstrakto mišinį su saldymedžio šaknų ekstraktu ir muskatų eteriniu aliejumi.

Mikrokapsulėms formuoti buvo taikomi skirtingi metodai: purškiamasis džiovinimas, liofilizacija ir ekstruzija. Natrio alginatas ir chitozanas buvo panaudoti mikrokapsulių apvalkalų formavimuisi ir aktyviųjų junginių atsipalaidavimo kontrolei. Eksperimento metu sėkmingai pagamintos kokybės reikalavimus atitinkančios mikrokapsulės, iš kurių liofilizuotos mikrokapsulės pasižymėjo geriausiais technologiniais parametrais, palyginti su išpurškiamojo džiovinimo metu gautomis mikrokapsulėmis, ir galėjo būti naudojamos tolesnių farmacinių formų – kramtomųjų tablečių – gamybai. Liofilizuotos mikrokapsulės buvo įtrauktos į pektininę kramtomųjų tablečių bazę. Pektinas buvo naudojamas kaip gelifikuojamasis agentas, nenaudojant pridėtinių cukrų, o šioje mikrokapsulių formuluotėje buvo ne tik raudonųjų dobilų ekstrakto, bet ir saldymedžio šaknų ekstrakto bei muskatų eterinio aliejaus. Tyrimo

metu buvo sukurtos dvi ekstruzinių mikrokapsulių technologijos: skrandyje irios (chitozano apvalkalas) mikrokapsulės ir žarnyne tirpiosios kapsulės (alginato apvalkalas) su raudonųjų dobilų žiedų ekstraktu, pagalbinėmis medžiagomis ir migdolų aliejumi. Šios mikrokapsulės yra bazė papildomam vitaminų, tirpių vandenyje ir aliejuje, įtraukimui. Minėtos ekstruzinės mikrokapsulės buvo inkorporuotos kietojoje želatininėje kapsulėje. Kitoje ekstruzinių mikrokapsulių formuluotėje buvo naudojamas alginato apvalkalas, raudonųjų dobilų ekstraktas, saldymedžio šaknų ekstraktas bei muskatų eterinis aliejus. Atlikti tyrimai ir sukurti produktai suteikia vertingos informacijos farmacijos srityje ir atskleidžia raudonųjų dobilų komercinį potencialą.

Tyrimo objektas ir metodai

Tyrimo objektas – raudonųjų dobilų (*Trifolium pratense* L.) žiedai. Žaliava buvo renkama 2020−2023 m. rugsėjo 25−26 d. jų natūralioje augavietėje Laičiuose, Kupiškio rajone, Lietuvoje (koordinatės: 55°53′24,2″ šiaurės platumos; 25°19′36,0″ rytų ilgumos).

Fenolinių junginių, flavonoidų, izoflavonų, monoterpenų (iš eterinių aliejų) kiekis įvertintas taikant skysčių ir dujų chromatografiją, masių spektrometriją ir spektrofotometrinius metodus.

Paruoštų ekstraktų biologiniam poveikiui įvertinti naudoti antioksidantinio aktyvumo (ABTS, ABTS-pokolonelinis metodas, FRAP, DPPH) ir antimikrobinio aktyvumo (agaro šulinio difuzijos) metodai.

Emulsijos stabilumas buvo vertinamas centrifuguojant, aliejaus dalelių dydis ir pasiskirstymas buvo nustatomas naudojant „Mastersizer 3000“ su „Hydro EV“ įrenginį. Mikrokapsulių gamybai naudotas švirkštinis siurblys, liofilizacijos ir purškiamojo džiovinimo metodai. Mikrokapsulės buvo vertinamos naudojant tekstūros analizatorių, mikrometrą ir šviesinį mikroskopą, „Sotax AT7“ išmaniąją sistemą.

Tyrimų rezultatai apdoroti matematiniais statistiniais metodais su „IBM SPSS 20.0“, „Microsoft Office Excel 2021“ ir „GraphPad Prism 8“ programine įranga. *Naudotos medžiagos ir išsamūs taikytų metodų aprašymai pateikti straipsniuose 1–7.*

Diskusija ir rezultatų apžvalga

Raudonieji dobilai (*Trifolium pratense* L.) savo sudėtyje kaupia izoflavonus, todėl yra vertingi farmacijos pramonei dėl šių junginių naudos sveikatai. Literatūroje trūksta išsamių duomenų apie raudonųjų dobilų žieduose esančių fenolinių junginių kiekį ir antioksidacinį aktyvumą, o ankstesniuose tyrimuose daugiausia dėmesio skirta lapų ekstraktams, kuriuose, kaip manoma, yra didesnė izoflavonų koncentracija [53–56]. Vienas iš šio tyrimo

tikslų buvo pagerinti izoflavonų ekstrakciją iš raudonųjų dobilų žiedų, atsižvelgiant į įvairius parametrus, pavyzdžiui, tirpiklį, temperatūrą, ekstrakcijos metodą ir glikozidų hidrolizės metodus, siekiant padidinti aglikonų, kaip daidzeinas ir genisteinas, išeigą. Iš visų taikytų ekstrakcijos metodų (maceracija, ultragarsas, fermentacija, virinimas tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi ir šių metodų derinimas kartu su įvairiais hidrolizės metodais) ir parametrų (žaliavos apdorojimo laikas, tirpiklis, tirpiklio sudėtis ir kt.) geriausiais buvo išrinkti ultragarso apdorojimas (40 °C, 10 arba 30 minučių) derinant su termine hidrolize arba virinimas tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi. Kaip tirpikliai atrinkti vanduo ir 50 proc. etanolio tirpalas. Ultragarso apdorojimą (30 minučių) derinant su termine hidrolize, pavyko išgauti didžiausią izoflavonų koncentraciją – 393,23 ± 19,66 μg/g daidzeino ir 171,57 ± 8,58 μg/g genisteino, naudojant 50 proc. etanolio tirpalą. Šis metodas buvo pranašesnis už tradicinį kaitinimą tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi, kurį taikant buvo pasiekta 432,30 ± 21,61 μg/g daidzeino ir 154,50 ± 7,72 μg/g genisteino koncentracijos. Ekstrakcija ultragarsu (10 ar 30 minučių) kartu su terminės hidrolizės metodu palengvino tirpiklio skverbimąsi į augalo ląsteles, padidino ekstrakcijos išeigą, tačiau pastebėta, kad padidėjo ir izoflavonų aglikonų skilimo rizika, kai ultragarsinio apdorojimo trukmė buvo ilgesnė [61, 62]. Terminė hidrolizė ekstrakcijos procese buvo svarbus žingsnis gerinant ekstrakcijos efektyvumą siekiant padidinti genisteino ir daidzeino kiekį, kadangi jos metu glikozidai buvo hidrolizuoti iki aglikonų [58, 64]. Nustatyta, kad 50 proc. etanolio tirpalas buvo efektyviausias tirpiklis izoflavonų ekstrakcijai, kadangi juo buvo subalansuotas aktyviųjų junginių tirpumas, o ekstrakcijos efektyvumas geresnis nei naudojant vandenį ar kitus toksiškus tirpiklius (pvz., metanolį, chloroformą) [65–67]. Tai patvirtino ankstesni tyrimai [63], kurie parodė, kad etanolis padidina ekstrakcijos išeigas ir atitinka maisto produktų, papildų ir nutraceutikų saugos ir reguliavimo reikalavimus.

Šiame tyrime į ekstrakcijos mišinį pridėjus vandenyje tirpių pagalbinių medžiagų, tokių kaip magnio aliuminio metasilikato, kroskarmeliozės natrio druskos, SSG, PVP/VAC ir α-, β-, γ-CD, pagerintas izoflavonų ekstrakcijos vandenyje efektyvumas. Ekstrakcijai su pagalbinėmis medžiagomis buvo taikyti prieš tai atrinkti metodai ir parametrai. Taip buvo sukurta tvari, nors ir mažiau efektyvi alternatyva etanoliui [68]. Visgi ekstrakcijos procese pagalbines medžiagas galima naudoti ir su etanolio tirpalu. Ciklodekstrinai turi standžią kūginę struktūrą, kurios vidinė dalis sudaryta iš hidrofobinio aktyviojo centro su hidrofiliniu išorės elementu. Ekstrakcijos proceso metu ciklodekstrinai sudaro kompleksą su tiksliniais junginiais dėl atsirandančių silpnų nekovalentinių sąveikų – Van der Valso jėgų, hidrofobinės sąveikos ar vandenilinių ryšių [28]. Dėl šios priežasties padidėja izoflavonų tirpumas ir stabi-

lumas ekstrakcijos ar reakcijos mišinyje (tirpale). Be to, mišiniui atvėsus, CD lengvai atpalaiduoja biologiškai aktyvius junginius ir gali būti lengvai pašalinami filtruojant.

Naudojant CD vandenyje, didžiausia genisteino išeiga buvo nustatyta naudojant 1 proc. β-CD – 258,67 ± 7,76 μg/g, o daidzeino – 290,33 ± 8,71 μg/g, taikant ekstrakciją tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi. Padidinus CD koncentraciją iki 5 proc., daidzeino kiekis tarp skirtingų CD padidėjo 1,06–1,40 karto. Genisteino kiekis padidėjo mažiau nei daidzeino, tačiau statistiškai reikšmingas padidėjimas buvo matomas su α- ir β-CD, bet junginio koncentracija sumažėjo, kai buvo naudojama didesnė – 5 proc. γ-CD koncentracija. Nepaisant to, kad buvo naudojamas didesnis CD kiekis vandenyje, glikozidų, daidzino ir genistino neaptikta.

Didžiausia daidzeino išeiga nustatyta etanolyje su 1 proc. β-CD – 697,16 ± 20,91 μg/g, ir su 1 proc. γ-CD – 682,90 ± 20,49 μg/g, taikant ultragarsinį apdorojimą (30 min.) su termine hidrolize. Nustatytas genisteino kiekis naudojant CD etanolyje buvo mažesnis nei vandenyje, tačiau didesnis nei kontroliniuose mėginiuose. Kontroliniai mėginiai − tai mėginiai, kurie buvo gaminti tokiomis pat sąlygomis, naudojant tuos pačius tirpiklius, tačiau be pagalbinių medžiagų. Visgi, tarp etanolinių mėginių didžiausias kiekis nustatytas naudojant 1 proc. β-CD tirpiklio virinimo temperatūroje su grįžtamuoju kondensatoriumi – 207,83 ± 6,23 μg/g. Pakeitus tirpiklį į 50 proc. etanolio tirpalą, aglikonų išeigos vidutiniškai padidėjo 2–3 kartus, palyginti su kontroliniais mėginiais, nors su β-CD genisteino išeiga sumažėjo 1,17 karto. Naudojant CD su 50 proc. etanolio tirpalu ekstraktuose buvo nustatyti ir glikozidai.

Ekstrakcijai vandenyje naudojant kitas pagalbinių medžiagų grupes – kroskarmeliozės natrio druską, SSG ir PVP/VAC, buvo taikytos tokios pat ekstrakcijos sąlygos kaip ir su CD. Visuose mėginiuose izoflavonų išeiga statistiškai reikšmingai padidėjo, išskyrus mėginius, kuriuose naudotas magnio aliuminio metasilikatas, dėl kurio izoflavonų kiekis sumažėjo. Magnio aliuminio metasilikatas selektyviai absorbuoja terpenus, o ne izoflavonus, ir tai pabrėžia pagalbinės medžiagos suderinamumo su tiksliniais junginiais svarbą [70, 71]. Naudojant 1 proc. PVP/VAC vandenyje ir taikant ekstrakciją tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi, gauta didžiausia daidzeino išeiga buvo 307,80 ± 9,23 μg/g, o genisteino – 121,40 ± 3,64 μg/g. PVP/VAC koncentraciją vandenyje padidinus iki 5 proc., gautas dar didesnis daidzeino ir genisteino kiekis. Pakeitus tirpiklį į 50 proc. etanolio tirpalą, esant tai pačiai 1 proc. PVP/VAC koncentracijai, tyrime gauta didžiausia daidzeino išeiga – 820,50 ± 24,61 μg/g. Sėkmingai išskirti glikozidai naudojant tiek ultragarsą (10 arba 30 minučių) derinant su termine hidrolize, tiek taikant ekstrakciją tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi.

Analizuojant fenolinių junginių kiekį ekstraktuose naudojant ciklodekstrinus, nustatyta, kad γ-CD ir β-CD reikšmingai padidino bendrą fenolinių junginių kiekį raudonųjų dobilų žiedų ekstraktuose. Didžiausias fenolinių junginių kiekis buvo nustatytas naudojant 50 proc. etanolio tirpalą su γ-CD – 54,12 mg GA/g dw (ultragarsinis apdorojimas 30 minučių kartu su termine hidrolize). Naudojant β-CD su vandeniu gauta 44,78 mg GA/g dw, o su 50 proc. etanolio tirpalu 50,11 mg GA/g dw fenolinių junginių, taikant tas pačias ekstrakcijos sąlygas – tirpiklio virimo temperatūroje su grįžtamuoju kondensatoriumi. Naudojant SSG ir PVP/VAC pagalbines medžiagas, fenolinių medžiagų koncentracijos padidėjo ir tam tikromis sąlygomis prilygo arba net viršijo išeigą, gautą naudojant CD. Ypač išsiskyrė rezultatai, gauti naudojant 5 proc. PVP/VAC vandenyje ir etanolyje, kai gautas didžiausias fenolinių medžiagų kiekis buvo 45,38 mg GAE/g dw ir 52,48 mg GAE/g dw (ultragarsinis apdorojimas 30 minučių kartu su termine hidrolize). Didesnės, palyginti su kontroliniais mėginiais, flavonoidų koncentracijos nustatytos su visomis naudotomis pagalbinėmis medžiagomis. Naudojant β-CD vandenyje, gauta 21,74 mg RU/g dw flavonoidų koncentracija. Flavonoidų kiekis padidėjo ir mėginiuose su 1 proc. PVP/VAC 23,10 mg RU/g dw vandeniniuose ir 26,98 mg RU/g dw etanoliniuose ekstraktuose. Visgi, SSG ir PVP/VAC pagalbines medžiagas buvo daug sunkiau išvalyti iš ekstrakcijos mišinio nei CD. Naudojant SSG ir PVP/VAC, mėginiai tapo tirštesni, o gryninimo procesas buvo sudėtingesnis, ilgesnis ir brangesnis.

Ekstrakcijos sąlygos turėjo didelę įtaką ekstraktų antioksidaciniam potencialui, o pagalbinių medžiagų naudojimas ekstrakcijos terpėje turėjo reikšmingą vaidmenį didinant fenolinių junginių ir flavonoidų kiekį dobilų ekstraktuose [75–77]. Tyrime gauti rezultatai patvirtino ekstrahavimo metodų, pagalbinių medžiagų naudojimo ir antioksidacinio aktyvumo, išmatuoto ABTS, DPPH ir FRAP metodais, koreliaciją [76, 78, 79]. Pažymėtina, kad tokių pagalbinių medžiagų kaip PVP/VAC ir CD naudojimas reikšmingai padidino ekstraktų antioksidacinį aktyvumą tiek vandeniniuose, tiek etanoliniuose mėginiuose, palyginti su kontrolinių mėginių rezultatais. Taikant pokolonėlinį ABTS metodą buvo nustatyta, kad didžiausiu antioksidaciniu poveikiu raudonųjų dobilų žiedų ekstraktuose pasižymi kavos rūgšties maleino esteris, hiperozidas ir klovamidas. Minėtų junginių antiradikalinis aktyvumas nustelbė izoflavonų, daidzeino ir genisteino, aktyvumą.

Nustatyta teigiamoji koreliacija tarp fenolinių junginių kiekio ir antioksidacinio aktyvumo. ABTS ir DPPH metodais nustatyta, kad didžiausiu antioksidaciniu aktyvumu pasižymėjo etanoliniai mėginiai, paruošti su γ-CD ir PVP/VAC. Be to, šie rezultatai atitiko literatūroje paskelbtų tyrimų, įrodžiusių, kad fenoliniai junginiai yra pagrindinė junginių grupė, lemianti raudonųjų dobilų ekstraktų antioksidacinį aktyvumą, rezultatus [76, 83].

Nors literatūroje teigiama, kad raudonųjų dobilų žiedų antimikrobinis ir antivirusinis aktyvumas yra ribotas, šios disertacijos tyrimo rezultatai parodė, kad raudonųjų dobilų ekstraktas pasižymi selektyviu antimikrobiniu poveikiu prieš gramteigiamas bakterijas, tokias kaip *Staphylococcus aureus* ir *Staphylococcus epidermidis*. Visgi pagalbinių medžiagų naudojimas ekstrakcijos procese neturėjo reikšmingo antimikrobinio poveikio patogeninėms *Enterococcus faecalis* ir *Pseudomonas aeruginosa* bakterijoms. Pagalbinės medžiagos nepakeičia raudonųjų dobilų antimikrobinio aktyvumo, taip pat jis reikšmingai nesiskiria tarp etanolinių ir vandeninių ekstraktų. Raudonųjų dobilų ekstraktai išsiskiria nuo dozės priklausančiu antivirusiniu poveikiu prieš IBV (paukščių infekcinio bronchito virusas) ir suteikia apsaugą Vero ląstelėms. Nustatytas didžiausias titras buvo 99,82 proc., o tai rodo viruso kiekio sumažėjimą. Raudonųjų dobilų ekstraktas gali būti veiksminga natūrali priemonė nuo virusinių infekcijų.

Tyrime buvo nagrinėjamas emulsijų, formuotų su raudonųjų dobilų žiedų ekstraktais, stabilumas ir savybės. Daugiausia dėmesio skirta įvairių pagalbinių medžiagų ir emulsiklių, tokių kaip alginatas, chitozanas, β-CD, garstyčių ekstraktas ir polisorbatas 80, daromai įtakai. Šioje tyrimo dalyje buvo pasirinktos keturios formuluotės – dvi su alginatu (A1, A2) ir dvi su chitozanu (C1, C2), naudotos skirtingos emulsiklių ir raudonųjų dobilų ekstrakto koncentracijos, siekiant gauti emulsijas, kurios vėliau bus naudojamos mikrokapsuliavimui. Emulsijose naudoti etanoliniai ir vandeniniai raudonųjų dobilų ekstraktai, paruošti naudojant β-CD. Visgi vėliau tyrimai atlikti tik su etanoliniais ekstraktais, kadangi juose nustatytos didesnės aktyviųjų junginių koncentracijos. Ekstraktų su β-CD klampa buvo nepakitusi, juose buvo nustatyta didelė izoflavonų, fenolinių junginių ir flavonoidų išeiga. Šie ekstraktai maišėsi su naudotais aliejais geriau nei kontroliniai mėginiai, pagaminti be pagalbinių medžiagų. Tai rodo, kad ekstraktai pasižymėjo emulguojančiomis savybėmis, ir tai naudinga emulsijų stabilumui. Tyrimui pasirinktas saldžiųjų migdolų aliejus, kuris su visais emulsikliais (polisorbatu 80, garstyčių ekstraktu ir β-CD) pasiskirstė tolygiai, formavo mažiausius lašus, didinančius emulsijos stabilumą dėl tvirto barjero tarp ekstrakto ir aliejaus [90].

Emulsijos, formuotos su natrio alginato tirpalu, pasižymėjo didesniu stabilumu – nuo 98,3 ± 1,2 iki 100 ± 0,8 proc. Dėl mažesnio lašelių dydžio (nuo 0,3 iki 5,6 μm) šios emulsijos buvo priskirtos mikroemulsijų kategorijai. Chitozano pagrindu pagamintos emulsijos, priskiriamos makroemulsijoms, priešingai – pasižymėjo didesniu lašelių dydžiu (nuo 0,8 iki 18,7 μm) ir mažesniu stabilumu (nuo 92,3 ± 0,5 iki 95,6 ± 0,6 proc.). Tyrimuose naudoti ekstraktai, pagaminti su β-CD, ne tik padidino emulsijų stabilumą, nekeisdami klampos, bet β-CD, naudotas kaip emulsiklis, padidino aktyviųjų jungi-

nių stabilumą, nesuteikė produktui specifiško skonio ir kvapo kaip garstyčių ekstraktas, todėl jį galima rinktis maisto ir farmacijos srityse [100, 101].

Iš natrio alginato tirpalo ir chitozano pagamintoms ekstruduotoms mikrokapsulėms buvo būdingos skirtingos savybės, kurioms įtakos turėjo atitinkami emulsikliai. Alginato mikrokapsulės, ypač tos, kuriose kaip emulsiklis naudotas β-CD (A1B ir A2B), pasižymėjo didesniu vienodumu ir mažesne agregacija nei tos, kuriose naudotas garstyčių ekstraktas. Alginato mikrokapsulės su raudonųjų dobilų ekstraktu buvo didesnės, išdžiūvusios susitraukė mažiau ir buvo tvirtesnės, kai buvo naudojama didesnė alginato tirpalo koncentracija (2 proc.) ir garstyčių ekstraktas. Alginatas sudaro skersinius joninius ryšius su tam tikrais dvivalenčiais katijonais (Ba^{2+}, Ca^{2+}, Zn^{2+}). Kalcio jonų yra aptinkama garstyčių ekstrakte, todėl jo įtraukimas prisideda prie papildomų ryšių sudarymo gelyje ir tvirtesnės plėvelės susiformavimo [110]. O chitozano mikrokapsulės, priešingai, nors ir išlaikydamos vienodą šviesiai rudą spalvą, nepaisant naudojamo emulsiklio, buvo minkštesnės ir mažesnės (išdžiūvusios), palyginti su alginato kapsulėmis [105, 106]. Chitozano mikrokapsulės, ypač su polisorbatu 80 (C1P ir C2P), buvo mažesnės, o susitraukimas minimalus dėl formuluotėse esančio mažesnio vandens kiekio. Didesnės raudonojo dobilo ekstrakto koncentracijos preparatuose su polisorbatu 80 formavo didesnes mikrokapsules, o preparatuose su β-CD – mažesnes. Abu emulsikliai formavo skirtingo tipo emulsijas (tradicines ir *Pickering* tipo), todėl tai darė įtaką susidariusių mikrokapsulių fizinėms savybėms [111]. Skirtingų gelifikuojančių medžiagų naudojimas išryškino skirtumus tarp gelifikacijos mechanizmų ir sąveikos su naudotais emulsikliais, kurie darė įtaką susiformavusių mikrokapsulių savybėms.

Fenolinių junginių išsiskyrimo iš skrandyje irių mikrokapsulių, pagamintų naudojant kaip pagrindines medžiagas alginatą ir chitozaną bei raudonųjų dobilų ekstraktą, *in vitro* profiliai atskleidė skirtingas savybes. Chitozano mikrokapsulės greitai ištirpsta rūgštinėse skrandžio sąlygose ir dėl savo funkcinių aminogrupių veiksmingai išskiria izoflavonus – daidzeiną, genisteiną ir biochaniną A [107]. β-CD chitozano mikrokapsulės pagerino hidrofobinių junginių, tokių kaip izoflavonai, tirpumą ir išsiskyrimo greitį – genisteino išsiskyrimas C2B mėginyje buvo daug didesnis, palyginti su C2P [108]. Alginato mikrokapsulės yra stabilios rūgštinėje aplinkoje, išbrinksta, bet neištirpsta. Ekstruzinių mikrokapsulių brinkimas daro lemiamą įtaką atpalaidavimo mechanizmams ir veiksmingumui skirtingose virškinimo terpėse. Per didelis brinkimas gali paskatinti staigų medžiagų atsipalaidavimą, kurio gali nepavykti kontroliuoti [107]. Fenolinių medžiagų išsiskyrimas ir alginato mikrokapsulių tirpimas vyko žarnyno terpėje [115]. Pažymėtina, kad iš didesnės alginato tirpalo koncentracijos (2 proc.) formuotų mikrokapsulių fenolinių junginių atsipalaidavimas buvo lėtesnis nei iš mažesnės alginato tirpalo

koncentracijos (0,5 proc.) formuotų mikrokapsulių. Pastarųjų didžiausias fenolinių medžiagų kiekis (22,71 ± 0,08 mg GAE/g) atsipalaidavo po 210 minučių [113].

Tolesniuose eksperimentuose emulsijos buvo ruošiamos prie raudonųjų dobilų žiedų ekstrakto pridedant saldymedžio šaknų ekstrakto ir aliejų keičiant *Myristica fragrans* Houtt eteriniu aliejumi. Stabiliausia emulsija E6, kurios stabilumas siekė 100 proc., buvo sudaryta iš trijų pagalbinių medžiagų – maltodekstrino, inulino ir gumiarabiko. Formuluotėse naudojant tik vieną iš minėtų pagalbinių medžiagų arba dviejų derinį didžiausias stabilumas nebuvo pasiektas. Nustatyta, kad eterinio aliejaus lašo skersmuo emulsijoje buvo 0,571 μm. Literatūroje nurodoma, kad emulsijose esančių dalelių dydis gali labai skirtis, jei naudojami skirtingi eteriniai aliejai, paprastai jis svyruoja nuo 16,70 iki 55,56 μm [104]. Nė viename ankstesniame tyrime nebuvo nagrinėtos emulsijos, kuriose *Myristica fragrans* Houtt eterinis aliejus būtų derinamas su raudonųjų dobilų ir saldymedžio ekstraktu, naudojant specifinį gumiarabiko, maltodekstrino ir inulino mišinį. E6 emulsija toliau buvo naudojama mikrokapsulių gamybai ekstruzijos metodu.

Mikrokapsulės buvo suformuotos naudojant ekstruzijos metodą su 5 proc. kalcio chloridu, kuris naudojamas kaip polimerizacijos reakcijos iniciatorius. Susidariusių mikrokapsulių išeiga pagal fenolinių junginių kiekį buvo 68,67 ± 3,08 proc. Padidinta polimerizacijos reakcijos iniciatoriaus koncentracija sustiprino alginato skersinių ryšių tinklą, sustiprindama eterinių aliejų lakiųjų junginių apsaugą ir užtikrindama ilgalaikį nepertraukiamą veikliųjų junginių atsipalaidavimą [113]. Šių kapsulių suspaudimas siekė 4135,87 g. Mikrokapsulių plėvelę sudarė alginatas, todėl jos labai mažai brinko skrandžio terpėje (nuo 13,96 iki 19,74 proc.), tačiau išbrinko žarnyno terpėje (nuo 25,22 iki 121,59 proc.).

Ekstruduotos mikrokapsulės, kuriose yra eterinių aliejų ir ekstraktų, yra universalus produktas, kurį galima įtraukti į kitus farmacijos produktus (tabletes, kapsules, kramtomąsias gelines tabletes) arba naudoti individualiomis terapinėmis dozėmis. Mikrokapsulių kontroliuojamą atpalaidavimą palengvina mikrokapsulės apvalkalo išbrinkimas, kuris keičia pralaidumą ir lemia atpalaidavimo greitį bei tikslinę pernašos vietą. Skrandžio terpėje šių mikrokapsulių brinkimo indeksas svyravo nuo 19,74 iki 13,96 proc., tačiau skrandžio terpėje mikrokapsulės iki galo neišbrinko, o žarnyno terpėje, priešingai, brinkimas gerokai padidėjo nuo 25,22 iki 121,59 proc. Brinkdamos mikrokapsulės prarado formą ir suminkštėjo, o tai rodo jų tirpumą žarnyno terpėje ir kontroliuojamą aktyviųjų medžiagų atsipalaidavimą.

E6 emulsijos sudėties koregavimas buvo būtinas liofilizacijai ir purškiamajam džiovinimui, kad mikrokapsuliavimas būtų veiksmingas. Buvo modifikuota sudėtis, keičiant sausųjų ir pagalbinių medžiagų santykį dėl iššūkių,

susijusių su koalescencija ir praskiedimu. Tyrime liofilizacijos ir purškiamojo džiovinimo metodams buvo naudojama ta pati modifikuota E6 formuluotė, kurioje yra raudonųjų dobilų ekstrakto, saldymedžio ekstrakto ir muskatų eterinio aliejaus. Tam tikrais aspektais liofilizacijos metodas (LM) buvo pranašesnis už purškiamąjį džiovinimą (SDM): gauta 85,34 proc. išeiga, palyginti su 45,12 proc. SDM. Mažesnė išeiga gauta dėl purškiamajam džiovinimui būdingų medžiagų nuostolių, pavyzdžiui, miltelių sukibimo su įranga ir veikliųjų junginių skilimo aukštoje adatos išpurškimo temperatūroje [117]. LM išsaugojo didesnį fenolinių junginių kiekį – 11,47 (LM) ir 8,56 mg GA/g dw (SDM), pasižymėjo geresniu antioksidaciniu aktyvumu, o tai lėmė žemoje temperatūroje vykęs procesas, kurio metu geriau išsaugomi labilūs komponentai [118]. Nustatyta, kad LM išsaugojo lakiuosius junginius ir didesnius jų kiekius iš eterinio aliejaus (α-pineną ir β-mirceną). LM mikrokapsulės buvo įvairių formų ir dydžių, primenančios sudužusio stiklo gabalėlius. SDM nors ir sferinės formos išsiskyrė labai prastu suberiamumu, tad tokių mikrokapsulių naudojimas pramonėje keltų sunkumų ir jų savybes reiktų gerinti [119]. Panašūs skirtumai tarp mikrokapsulių buvo aprašyti literatūroje [120]. Geresnis įkapsuliavimo efektyvumas ir didesnė biologiškai aktyvių junginių koncentracija LM mėginiuose rodo didesnį jų veiksmingumą, todėl jie yra tinkamesni kramtomosioms gelinėms tabletėms.

Kramtomųjų tablečių, pagamintų naudojant skirtingus gelio pagrindus (želatiną ir pektiną), fizinės savybės ir biologiškai aktyvių junginių atsipalaidavimas labai skyrėsi, o pektino pagrindo tabletės buvo pranašesnės už želatinos. Pektinui buvo teikiama pirmenybė dėl didesnio tvirtumo, kietumo ir kontroliuojamojo junginių atsipalaidavimo. Šios tabletės taip pat buvo pasirinktos dėl geresnių skonio savybių, nepridėto papildomo cukraus. Želatininės tabletės pasižymėjo mažesniu junginių atpalaidavimo efektyvumu ir fiziniu tvirtumu.

Pektinas pranašesnis už želatiną dėl tvirtesnės gelinės tabletės bazės struktūros, kuri užtikrina mikrokapsulių lakiųjų junginių apsaugą ir kontroliuojamąjį atsipalaidavimą [127]. Į pektino matricą įtraukus liofilizuotas mikrokapsules, dar labiau pagerėjo tablečių mechaninės savybės ir stabilumas, o tai leidžia manyti, kad jos geriau tinka gydymo tikslams, kai labai svarbu tiksliai dozuoti ir tolygiai atpalaiduoti aktyviąsias medžiagas. Pektino kramtomos tabletės buvo veiksmingos apsaugant ir atpalaiduojant biologiškai aktyvius junginius, tokius kaip daidzeinas ir glicirizinas, pasiekiant iki 72,03 ir 71,36 proc. junginių atsipalaidavimo efektyvumą [125, 126]. Monoterpenai iš mikrokapsulių efektyviausiai išsiskyrė per pirmąsias 30 minučių, nors sabinenas buvo išimtis – jis geriau išsiskyrė želatinos pagrindu pagamintose tabletėse. Gauti duomenys pabrėžia pagrindo medžiagos ir mikrokapsulės sudėties svarbą optimizuojant įkapsuliuotų medžiagų išsiskyrimą ir stabilu-

mą. Pektino gebėjimas tirpinimo metu išlaikyti didesnį biologiškai aktyvių junginių kiekį rodo jo potencialą kuriant veiksmingesnes farmacines formas, kurioms reikalinga kontroliuojama ir tikslinga pernašos sistema.

Išvados

1. Ultragarsinė ekstrakcija (38 kHz, 100 proc.; 40 °C; 10 ar 30 min.), derinama su termine hidrolize arba augalinės žaliavos ekstrakcija tirpiklio virimo temperatūroje naudojant grįžtamąjį kondensatorių, yra efektyviausi metodai izoflavonų aglikonų (daidzeino ir genisteino) kiekiui iš raudonųjų dobilų žiedų padidinti. Ekstrakcijos modifikavimas pagalbinėmis medžiagomis turėjo teigiamą įtaką fenolinių junginių, flavonoidų ir izoflavonų išeigos padidėjimui.

Taikant antioksidacinio aktyvumo nustatymo metodus (ABTS, DPPH, FRAP) nustatyta, kad pagalbinių medžiagų (PVP/VAC ir CD) naudojimas ekstrakcijos procese padidina ekstraktų antioksidacinį aktyvumą. Tai tiesiogiai susiję su padidėjusiu fenolinių junginių kiekiu ekstrakcijos terpėje. Tai rodo, kad pagalbinės medžiagos gali padidinti augalinių ekstraktų terapinį potencialą. Antimikrobinio ir antivirusinio vertinimo metu paaiškėjo, kad raudonųjų dobilų ekstraktai, pagaminti naudojant etanolį kaip tirpiklį, efektyviai slopina patogeninių bakterijų, ypač Gram+, dauginimąsi, ir turi antivirusinių savybių. Ekstrakcijos modifikavimas pagalbinėmis medžiagomis šioms ekstraktų savybėms įtakos neturėjo.

2. Emulsijų stabilumui ir dalelių dydžiui įtakos turėjo emulsijos formavimui naudoto aliejaus, gelifikuojančios medžiagos ir emulsiklio kiekis. Emulsijos, sukurtos naudojant alginatą, pasižymėjo didesniu stabilumu ir mažesniu dalelių dydžiu, palyginti su chitozano emulsijomis. Naudojant alginatą ir β-CD kaip emulsiklį, tyrimo metu buvo sukurtos stabiliausios emulsijos.

Emulsijų su muskatų eteriniu aliejumi ir augaliniais ekstraktais stabilumas skyrėsi, o naudojant kelis emulsiklius jis buvo gerokai didesnis.

3. Technologiniai veiksniai, tokie kaip polimero (alginatas, chitozanas), emulsiklio (β-CD, garstyčių ekstraktas, polisorbatas 80) ir mikrokapsuliavimo metodo (ekstruzijos, liofilizacijos ar purškiamojo džiovinimo) pasirinkimas, turėjo įtakos mikrokapsulių fizinėms savybėms, stabilumui ir kokybei.

Iš visų mėginių (ekstruzijos metodo) didžiausią potencialą turėjo A2 ir C2 sudėties mikrokapsulės, kurių brinkimas, atsipalaidavimas simuliacinėje *in vitro* terpėje ir tvirtumas buvo geriausias, palyginti su literatūros duomenimis ir mėginiais, tirtais šiame tyrime. Formuluotėse naudojant β-CD ir priklausomai nuo naudoto gelifikatoriaus (alginato ar chitozano) pagerėja biologiškai aktyvių junginių atpalaidavimas skrandžio ir žarnyno terpėse.

Garstyčių ekstraktas savo sudėtyje turi dvivalenčių jonų, todėl su alginatu suformuoja stipresnius mikrokapsulių apvalkalus. Mikrokapsulės su alginato plėvele pasižymi kontroliuojamuoju atsipalaidavimu žarnyne, o chitozano apvalkalo mikrokapsulės visiškai ištirpsta skrandžio terpėje.

Siekiant apsaugoti lakiuosius muskatų eterinio aliejaus junginius, mikrokapsulių formavimui buvo reikalingos didesnės polimerinimo iniciatoriaus koncentracijos (5 proc.), palyginti su ekstraktų įkapsuliavimui reikalingais kiekiais.

Lyginant liofilizuotas ir išpurškiamojo džiovinimo būdu gautas mikrokapsules, išryškėjo biologiškai aktyvių junginių išlaikymo, tirpumo ir fizinių savybių skirtumų. LM buvo didesnės išeigos ir išsaugota daugiau fenolinių junginių bei flavonoidų, todėl buvo geresnės kokybės nei SDM.

4. Ekstruzinių mikrokapsulių kūrimas, tobulinimas ir inkorporavimas į kietąją farmacinę formą – kapsulę padėjo suprasti, kad formuluotėse naudojamos medžiagos reikšmingai veikia biologiškai aktyvių junginių atsipalaidavimą žarnyno ir skrandžio terpėse, kuris yra svarbus veiksmingai vaistų pernašos sistemai. Sukurta sistema su alginatu yra veiksminga, tačiau sistemą su chitozanu reikėtų tobulinti dėl joje esančio nedidelio izoflavonų kiekio.

Kramtomosiose gelinėse tabletėse, kuriose yra liofilizuotų mikrokapsulių, biologiškai aktyvių junginių atpalaidavimo profiliai skyrėsi atsižvelgiant į gelio pagrindą (želatina ar pektinas). Kramtomosios gelinės tabletės gelifikuojanti medžiaga turi įtakos inkapsuliuotų junginių išsiskyrimo dinamikai – iš pektininių gelinių tablečių junginiai atsipalaidavo laipsniškai, o dėl gelio kietumo želatinos tabletėse buvo matomas priešingas efektas.

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Paper 1

Title: Cyclodextrin-Assisted Extraction Method as a Green Alternative to Increase the Isoflavone Yield from *Trifolium pratensis* L. Extract

Authors: Jurga Andreja Kazlauskaite, Liudas Ivanauskas, Jurga Bernatoniene

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Article

Cyclodextrin-Assisted Extraction Method as a Green Alternative to Increase the Isoflavone Yield from *Trifolium pratensis* L. Extract

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Abstract: *Trifolium pratense* L. is receiving increasing attention due to the isoflavones it contains, which have been studied for their benefits to human health. A common problem with isoflavone aglycones is a rather low water solubility and limited pharmaceutical applications. The use of excipients, such as cyclodextrins in the production of isoflavone rich extracts, could become one of the new strategies for the extraction of target compounds. The aim of this study was to evaluate an eco-friendly method using the effects of α-, β- and γ-cyclodextrins for isoflavone solubilization in plant extracts in comparison to a standard extract without excipients. Extractions of red clover were prepared using ultrasound-assisted combined with thermal hydrolysis and heat reflux. It was determined that cyclodextrins significantly increased the isoflavones aglycone yields. By increasing cyclodextrins in the extraction media from 1 to 5%, the daidzin concentration increased on average by 1.06 (α-cyclodextrins), 1.4 (β-cyclodextrins) and 1.25 (γ-cyclodextrins) times. Genistein concentration increased using α- and γ-cyclodextrins (1.28 and 1.12 times, α- and γ-cyclodextrins, respectively), but decreased using β-cyclodextrins. The results showed that the cyclodextrin-assisted extraction enhanced the yields of isoflavones from red clover, which suggests using cyclodextrins as a green alternative and a cost-effective method to increase its pharmaceutical application.

Keywords: innovative strategies; *Trifolium pratensis* L.; red clover; isoflavones; aglycones; excipients; cyclodextrins; extractions

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1. Introduction

Trifolium pratense L. (red clover) (Figure 1) is a member of the family Leguminosae or Fabaceae. It is a short-lived biennial plant which has been used for its wide health benefits, which come from isoflavones [1]. The main isoflavones found in red clover are daidzein, genistein, formononetin, and biochanin A, as well as their glycosides (such as genistin, daidzin) [2,3].

Isoflavones are part of a large family of secondary plant metabolites called flavonoids [4]. Daidzin and genistein are the aglycones belonging to the phytoestrogen class and possessing a broad range of pharmacological properties that reduce tumors, menopausal symptoms, osteoporosis, and signs of aging. Isoflavones have also been reported to improve learning skills and memory in menopausal women and to aid in the prevention and treatment of heart disease and diabetes [5–7]. Phytoestrogens structurally resemble naturally occurring estrogens in women and compete with them for binding estrogen receptors. These compounds have a stronger affinity for binding ER-β than ER-α. By interacting with ERs, isoflavones can exert estrogenic effects in humans. However, depending on the dose or

the duration of exposure, isoflavones can also act as antiestrogenic agents [8]. They are currently heralded as offering potential alternative therapies for a range of hormone-dependent conditions, including cancer, menopausal symptoms, and cardiovascular disease [9,10].

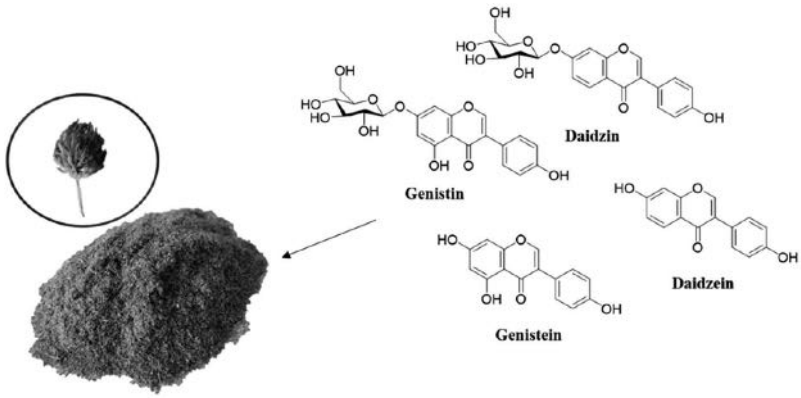

Figure 1. Milled red clover flower buds (0.5 mm) and the main isoflavones found in its extracts.

In plants, the most widely studied isoflavones, genistein and daidzein, are usually encountered as conjugates and are hydrolyzed to aglycones (biologically active form) in the human gut [11]. The isoflavone aglycones are absorbed faster and in greater amounts than their glucosides. Therefore, hydrolysis of the sugar moiety is an essential prerequisite for the bioavailability of isoflavones [12].

The estrogenic and antioxidant activities of daidzein and genistein, as mentioned before, are conditioned to the aglycone form, which presents reduced solubility in aqueous media [13,14]. Therefore, to preserve bioactive molecules and improve certain properties, they need to be used with finishing formulation with the capacity to improve needed characteristics. Additional materials, such as excipients, may be used to extract isoflavone aglycones in water or another solvent and thus increase their yield in extract. Cyclodextrins (CDs) can be suitable compounds for increasing the solubility of isoflavones. CDs can interact with appropriately sized molecules to result in the formation of inclusion complexes. These noncovalent complexes offer a variety of physicochemical advantages compared to the unmanipulated drugs, including the possibility for increased water solubility and solution stability [15].

There has been an increasing demand in recent years for cheaper, safer, and more eco-friendly alternatives to organic solvents. CD-based extraction is an emerging "green" technology of great potential [16]. CDs are being explored in the extraction of phenolic compounds, such as phenolic acids, flavonoids and stilbenes from various types of natural sources. The green extraction of phenolic compounds using CDs provides the possibility for a more effective exploitation of natural plant resources and wider use of phenolic compounds in the food and nutraceutical industries [17].

CDs are cyclic oligosaccharides composed of 6, 7, or 8 glucose units, named α-, β-, or γ-cyclodextrin, respectively (Figure 2) [18]. They have a rigid conical molecular structure with a hydrophobic interior and hydrophilic exterior. The internal cavity of these molecules can include a wide range of guest molecules, ranging from polar compounds, such as alcohols, acids, amines, and small inorganic anions, to non-polar compounds, such as aliphatic and aromatic hydrocarbons, while the hydrophilic exterior helps CDs to interact favorably with water [19].

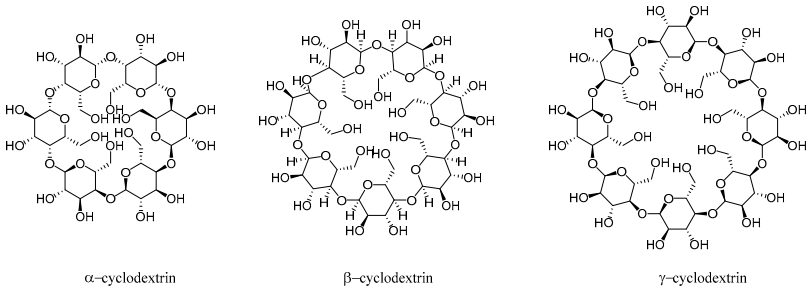

Figure 2. Chemical structures of α-, β- and γ-CD.

The molecular interaction of isoflavones (genistein and daidzein) and β-CD and its derivatives was studied by Shujing Li et al. [20] and V. Crupi et al. [21]. In these studies, it was concluded that CDs form host–guest complexes and improve the solubility of pure daidzein and genistein in water due to the formation of an inclusion complex [20,21]. Another published study investigated the formation of complexes of native cyclodextrins and pure genistein. Genistein was found to form the lowest number of complexes with α-CD. With β- and γ-CDs, genistein formed complexes, but at different rates. The resulting complexes were water soluble [22]. CDs can form dynamic solid inclusion complexes with a wide variety of solid, liquid, and gaseous compounds by the process of molecular complexation. There is no breakage or formation of covalent bonds during the formation of an inclusion complex [23]. The driving forces leading to the inclusion complex formation include the release of enthalpy-rich water molecules from the cavity, electrostatic interaction, Van der Waals interaction, hydrophobic interaction, hydrogen bonding, release of conformational strain, and charge–transfer interaction [24].

Choosing the right type of CD is important, since this factor can influence the extraction yield and the selection of compounds from the sample matrix. The diameter and volume of CDs increase with the increasing number of glucose units. Therefore, β-CD is widely used due in its appropriate cavity size; γ-CD is more suitable for moderate or larger sized compounds, while α-CD is limited to accommodate some small guest molecules [17].

To our knowledge, this is the first study using α-, β- and γ-CDs in *Trifolium pratensis* L. extracts to increase aglycones daidzein and genistein yield using water and 50% ethanol as a solvent. Thus, the present study was undertaken in order to evaluate the eco-friendly method of using the effects of α-, β-, and γ-CDs for isoflavone solubilization in red clover extracts in comparison to a standard extract without excipients.

2. Materials and Methods

2.1. Materials

Red clover samples were collected in red clover fields in Laičiai, Kupiškis district, Lithuania (latitude 55°53′24.2″ N; longitude 25°19′36.0″ E). The collections of flower buds and flowers were made on the 26th of September. Samples were dried and stored at room temperature. Before use, clover flowers were ground to a fine powder using Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Grinding was performed at 4025 g using a 0.5 mm trapezoid hole sieve.

HPLC grade and analytical-grade reagents were used: standards of genistein, genistin, daidzein and daidzin (Sigma Aldrich, Steinheim, Germany); hydrochloric acid, sodium hydroxide, acetic acid, methanol, acetonitrile, α-, β- and γ-CDs (Sigma Aldrich, Hamburg, Germany); ethanol (96%) (Vilniaus Degtine, Vilniaus, Lithuania); purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionized water was prepared with Milipore, SimPak 1 (Merck, Darmstadt, Germany).

2.2. Preparation and Extraction of Plant Material

2.2.1. Plant Material Moisture Determination

The moisture content of the milled red clover flowers was determined using a KERN MLB apparatus (KERN & Sohn GmbH, Balingen, Germany). Aa amount of 0.3 ± 0.01 g of the material was placed in the apparatus and heated to 105 °C. At the end of the operation, the device provided the moisture content of the material. The moisture of the plant material varied from 7 to 7.4%.

2.2.2. Ultrasound-Assisted Extraction with Thermal Hydrolysis (UAE)

Ultrasound-assisted extraction was performed using an ultrasound bath (frequency 38 kHz) (Grant Instruments™ XUB12 Digital, Cambridge, England). A sample of 0.3 ± 0.001 g of dried and milled flower heads was macerated in 10 mL of solvent. The extraction of isoflavones was performed by employing different extraction conditions: solvent (50% *v/v* ethanol and purified water) and extraction time (10 or 30 min), with the processing temperature of 40 ± 2 °C (the temperature is regulated automatically by the ultrasonic bath) [25,26].

Thermal hydrolysis was completed by transferring the extract to a 250 mL round bottom flask. It was refluxed in a sand bath at 100 °C for 1 h. After the procedure, the mixture was left to cool down and then centrifuged with Sigma 3-18K centrifuge (Sigma, Osterode am Harz, Germany) for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm, Frisenette, Knebel, Denmark) prior to HPLC analysis. Sample preparation conditions are listed in Table 1.

Table 1. Extraction conditions used for the experiment.

Sample Code	Extraction Method ***	Hydrolysis Method	Time, min	Solvent *	Excipient	Excipient:Extract Ratio
KV1 **	HR	-	60	Purified water	-	-
KV2 **	UA	thermal	10			
KV3 **			30			
KV4 **		-	10			
KV5 **			30			
KE1 **	HR	-	60	50% Ethanol	-	-
KE2 **	UA	thermal	10			
KE3 **			30			
KE4 **		-	10			
KE5 **			30			
A1	HR	-	60	Purified water	*α*-CD	1:100
A2	UA	thermal	10			
A3			30			
A4		-	10			
A5	HR	-	30			5:100
AP1	UA	thermal	60			
AP2			10			
AP3			30			
AP4		-	10			
AP5			30			
AE1	HR	-	60	50% Ethanol		1:100
AE2	UA	thermal	10			
AE4			30			
AE5		-	10			
AE6			30			
B1	HR	-	60	Purified water	*β*-CD	1:100
B2	UA	thermal	10			
B3			30			
B4		-	10			
B5	HR	-	30			5:100
BP1	UA	thermal	60			
BP2			10			
BP3			30			
BP4		-	10			
BP5			30			
BE1	HR	-	60	50% Ethanol		1:100
BE2	UA	thermal	10			
BE3			30			
BE4		-	10			
BE5			30			

Table 1. *Cont.*

Sample Code	Extraction Method ***	Hydrolysis Method	Time, min	Solvent *	Excipient	Excipient:Extract Ratio
G1	HR	-	60	Purified water	γ-CD	1:100
G2	UA	thermal	10			
G3			30			
G4		-	10			
G5			30			
GP1	HR	-	60			5:100
GP2	UA	thermal	10			
GP3			30			
GP4		-	10			
GP5			30			
GE1	HR	-	60	50% Ethanol		1:100
GE2	UA	thermal	10			
GE3			30			
GE4			10			
GE5			30			

* Solvent and herbal material ratio is the same in all the samples—10:0.3, respectively. ** Control samples prepared without excipients. *** HR—heat-refluxed method; UA—ultrasound-assisted method.

2.2.3. Heat-Reflux Extraction (HRE)

Amounts of 0.3 ± 0.001 g of dried and milled flower heads were mixed with 10 mL of solvent (50% ethanol *v/v* or purified water) in a 250 mL round bottom flask and refluxed in a sand bath at 100 °C for 1 h. After that, the mixture was left to cool at a temperature of 25 ± 2 °C. The samples were centrifuged for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm) prior to HPLC analysis. Sample preparation conditions are listed in Table 1.

2.3. The Use of CDs in the Preparation of Extracts

Samples were modified with *α*-, *β*- or *γ*-CDs. The extracts were made under the same conditions as previously listed. Purified water or 50% of ethanol (*v*/*v*) was used as the solvent, and the excipient was added to the extraction mixture. The same amount of 0.1 ± 0.001 g of *α*-, *β*- or *γ*-CDs was added to the extraction mixture samples (10 mL) to prepare samples with CD concentrations of 1% (*w*/*v*). Another amount of 0.5 ± 0.001 g of *α*-, *β*- or *γ*-CDs was added to the extraction mixture samples (10 mL) to prepare samples with CD concentrations of 5% (*w*/*v*). The excipient amount was based on the quantity of solvent in the extract. The samples were centrifuged for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm) prior to HPLC analysis. Sample preparation conditions are listed in Table 1.

2.4. HPLC–PDA Conditions

HPLC analyses were carried out using the Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan), consisting of a quaternary pump, an on-line degasser, a column temperature controller, the SIL-30AC autosampler (Shimadzu, Tokyo, Japan), equipped with the CTO-20AC thermostat (Shimadzu, Tokyo, Japan) as well as the SPD-M20A diode array detector (DAD). For the determination of polyphenols, an ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase consisted of solvent A (acetic acid/methanol/deionized water) (1:10:89 *v*/*v*/*v*) and solvent B (acetic acid/methanol) (1:99 *v*/*v*/*v*). The linear gradient elution profile was as follows: 80% A/20% B at 0 min; 30% A/70% B at 30 min; 90% A/10% B at 39 to 40 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Absorption was measured at 260 nm. Quantification of isoflavone compounds was performed using reference standards of daidzein, genistein, daidzin and genistin. The linear calibration curves were constructed (daidzein R^2 = 0.9999; genistein R^2 = 0.9999; daidzin R^2 = 0.9999; genistin R^2 = 0.9999); the peak areas were used for quantification. Linear calibration curve functions were as follows: f(x) = 59664.2x + 37164.6 (daidzein), f(x) = 73083.1x + 44202.9 (genistein), f(x) = 38202.1x + 19377.4 (daidzin), f(x) = 49602.9x + 24083.3

(genistin). The contents were expressed as μg/g dry weight (dw). The range of linearity of daidzein: 0.43–221 μg/mL; genistein: 0.43–218 μg/mL; daidzin: 0.32–165 μg/mL; genistin: 0.3–151.5 μg/mL. Isoflavone separation HPLC-DAD chromatogram is provided in Figure 3.

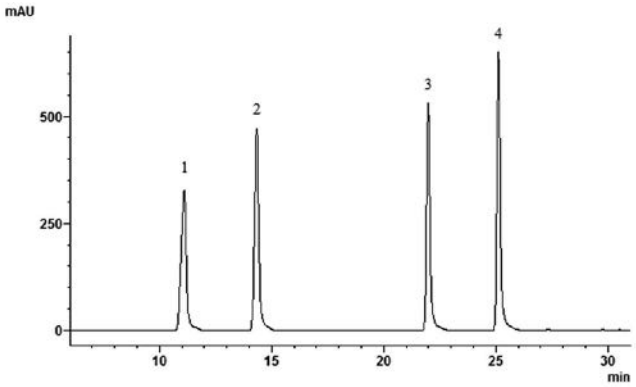

Figure 3. Isoflavone separation HPLC-DAD chromatogram: 1-daidzin; 2-genistin; 3-daidzein; 4-genistein.

2.4.1. Recovery Test of Isoflavones

The accuracy of HPLC-DAD was investigated using a modified Grazina et al. recovery test method [27]. Two selected ethanolic red clover extracts were prepared as described in Section 2.2.2. The UA sample was prepared using only ultrasound (processing time—10 min, at temperature of 40 ± 2 °C). The UAH sample was prepared using ultrasound for 10 min (at temperature of 40 ± 2 °C) and then heating the sample under reflux for 1 h. The samples were centrifuged for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size: 0.22 μm) prior to HPLC analysis. Each sample was injected 6 times (n = 6). The results of isoflavones recoveries are provided in Table 2.

Table 2. Recoveries (± RSD (%), n = 6) of daidzein, genistein, daidzin and genistin from red clover extracts.

Compound	± RSD *** (%), n = 6	
	UA *	UAH **
Daidzein	2.27	0.56
Genistein	3.10	3.61
Daidzin	0.82	0.65
Genistin	1.14	0.48

* Red clover sample sonicated for 10 min, at 40 °C; ** red clover sample sonicated for 10 min, at 40 °C and then heated under reflux for 1 h; *** recovery standard derivatization.

2.4.2. Isoflavones Obtained via CD-Assisted Extraction Release Analysis with HPLC-DAD

The isoflavone release from the CD complex was investigated using pure isoflavone standards (genistein, daidzein, genistin and daidzin). Test samples were prepared using 1 mL of isoflavones standards mixed with 9 mL of 50% *v*/*v* ethanol or purified water with 0.1 ± 0.001 g of α-, β- or γ-CD. The samples were sonicated for 10 min (at temperature of 40 ± 2 °C) and after that heated under reflux for 1 h. The samples were filtered through PVDF syringe filters (pore size 0.22 μm) prior to HPLC analysis. The results are shown in Table 3.

Table 3. Concentrations of isoflavones recovered from the samples with CDs.

Sample	Genistein, µg/mL	Daidzein, µg/mL	Genistin, µg/mL	Daidzin, µg/mL
Isoflavones without CDs	97.295	95.743	125.419	139.456
α-CDs prepared with isoflavones in purified water	96.701	95.487	125.271	139.222
β-CDs prepared with isoflavones in purified water	97.086	95.487	125.305	139.351
γ-CDs prepared with isoflavones in purified water	96.952	95.484	125.356	139.198
α-CDs prepared with isoflavones in 50% ethanol	97.053	95.487	125.386	139.382
β-CDs prepared with isoflavones in 50% ethanol	97.121	95.649	125.289	139.406
γ-CDs prepared with isoflavones in 50% ethanol	97.107	95.658	125.356	139.399

2.5. Statistical Analysis

Data are presented as mean ± standard deviation (SD). All experiments were performed in triplicate. Statistical analysis of the results was performed with SPSS 20.0 (IBM Corporation, Armonk, NY, USA). One-way ANOVA was used to investigate the differences between extractions. Post hoc comparisons of the means were performed according to Tukey's HSD test. The means of compared samples were considered significantly different when $p < 0.05$.

3. Results and Discussion

3.1. Quantification of the Aglycones Using Purified Water and CDs as an Excipient

Before the experiment optimal conditions were selected for this research, different concentrations of ethanol and two temperatures of ultrasound were selected in order to obtain higher amounts of isoflavones. By extending the ultrasound processing time from 10 to 30 min, daidzein yields decreased, but genistein using 50% of ethanol increased (EU1(KE4) and EU2(KE5) samples) (Table 4). By increasing ethanol concentrations and the ultrasound processing temperature, isoflavone yield decreased. This is why in this study, the ultrasound temperature was 40 °C (processing time 10–30 min, later used ethanol concentration 50%).

Table 4. Extraction conditions influence on yield of isoflavones.

Sample Code	Method	Solvent	Conditions	Daidzein, µg/mL	Genistein, µg/mL	Daidzin, µg/mL	Genistin, µg/mL
EU1(KE4)	UAE	50% EtOH	40 °C, 10 min	175.93	135.60	0.00	52.53
EU2(KE5)	UAE	50% EtOH	40 °C, 30 min	93.73	165.83	0.00	44.07
EU3	UAE	50% EtOH	60 °C, 10 min	98.89	121.21	0.00	41.22
EU4	UAE	50% EtOH	60 °C, 30 min	91.52	112.14	0.00	38.53
EU5	UAE	70% EtOH	40 °C, 10 min	56.15	69.02	0.00	79.33
EU6	UAE	70% EtOH	40 °C, 30 min	52.74	55.61	0.00	98.12
EU7	UAE	70% EtOH	60 °C, 10 min	84.12	106.35	0.00	84.62
EU8	UAE	70% EtOH	60 °C, 30 min	81.31	64.52	0.00	91.45
EU9	UAE	96% EtOH	40 °C, 10 min	21.22	147.33	0.00	89.24
EU10	UAE	96% EtOH	40 °C, 30 min	19.47	125.66	0.00	99.25
EU11	UAE	96% EtOH	60 °C, 10 min	73.54	119.41	0.00	92.17
EU12	UAE	96% EtOH	60 °C, 30 min	69.79	103.45	0.00	94.86
W1(KV4)	UAE	Purified water	40 °C, 10 min	51.06	1.23	0.00	0.00
W2(KV5)	UAE	Purified water	40 °C, 30 min	48.65	6.72	0.00	0.00
W3	UAE	Purified water	60 °C, 10 min	32.15	0.00	0.00	0.00
W4	UAE	Purified water	60 °C, 30 min	36.91	0.00	0.00	0.00

The yield of isoflavones was determined in aqueous extracts obtained from dried and milled Trifolium pratensis L. flower heads material by UAE and HRE. Isoflavones were determined using HPLC-PDA. To make sure that all the isoflavones obtained via CD-assisted extraction were released from the complex during HPLC-DAD analysis, we conducted an additional experiment (Section 2.4.2). The results showed (Table 2) that we obtained the same amounts of isoflavones as we had added. Therefore, all the isoflavones in the extracts, determined by HPLC-DAD analysis, were separated from the complexes with CDs, and there were no isoflavones still left in the complexes.

The use of HRE and UAE methods allows high yields of isoflavones to be obtained. UAE waves are transmitted through the liquid medium, damaging the plant wall, resulting in an improved solvent penetration. Therefore, bioactive components can be extracted in minutes [28]. The presence of heat during isoflavone extractions triggers chemical changes, with the decarboxylation of malonyl-glucosides to acetyl-glucosides and the breakdown of the ester bond being frequently observed, the latter leading to the formation of glucosides [26]. When using high temperatures, glycosides could convert to aglycones, if the pH is right for the hydrolysis. However, the temperature and processing time should be closely monitored and properly selected, because genistein and daidzein could degrade at high temperatures [29]. Due to their simplicity and efficiency, HRE and UAE with thermal hydrolysis have been used in the extraction of isoflavones in this study.

The use of CDs in an aqueous solution as extraction media can be considered a green extraction since water is the main solvent and the existence of CD hydrophobic cavity boosts the extraction of phenolic compounds, including isoflavones, due to the formation of the inclusion complex [17]. Comparing control (KV1-5) samples with samples prepared using CDs, it was determined that excipients enhanced aglycone yields (Figure 4).

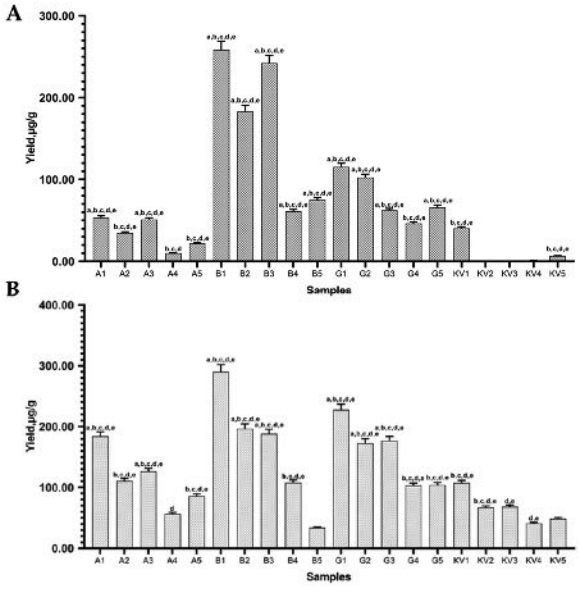

Figure 4. Quantitative yield of (**A**) genistein and (**B**) daidzein using excipients (1%). Control samples without excipients (KV1-5), samples with α-CD (A1-5), β-CD (B1-5), γ-CD (G1-5). [a] $p < 0.05$ vs. KV1, [b] $p < 0.05$ vs. KV2, [c] $p < 0.05$ vs. KV3, [d] $p < 0.05$ vs. KV4, [e] $p < 0.05$ vs. KV5. Sample codes are provided in Table 1.

The highest aglycone yields were detected in sample B1 (258.67 ± 10.34 genistein and 290.33 ± 11.61 μg/g daidzein); the results were statistically significant compared with control samples. It was observed that using α- and β-CDs (A2-5 and B2-5 samples using α- and β-CDs, respectively) during UAE and extending the sonication time from 10 to 30 min resulted in increased aglycone yields. However, using γ-CDs (G2-3 samples) during UAE (with additional thermal hydrolysis) prolonged the processing time and reduced aglycone amounts in extracts (Figure 4A,B). Almost all of the samples (excluding A2, A3, A4 and B5 samples) showed higher extraction results ($p < 0.05$) than the control samples without excipients, and the use of the HRE method produced higher isoflavones extraction results than UAE (Figure 4A,B). Using only UAE, without thermal hydrolysis (samples A, B; G 4-5), showed poor (Figure 4A,B) but statistically significant results (compared with controls) in extracting daidzein (Figure 4A). To obtain higher yields of daidzein and genistein, thermal hydrolysis is a necessary step.

The increased yield of isoflavones in extracts with excipients can be explained by the structure of CDs. The CD encapsulation of active compounds changes the compounds' physicochemical properties, such as their aqueous solubility and chemical stability. The CD molecule forms a hydrophilic shield around the appropriate lipophilic moiety of the drug molecule. This increases the apparent aqueous solubility of the active compounds. The CD can also protect chemically labile drug molecules from potentially corrosive environments and, in this way, reduce or even prevent drug hydrolysis, oxidation, racemization and enzymatic decomposition [17,30]. A. The studies of Daruházi et al. focused on the pure genistein and native, chemically non-modified CDs [22]. Their findings were similar to those obtained in this study: the interaction between genistein and α-CD with the smallest cavity size was found to be negligible compared to β- and γ-CDs. Therefore, genistein has the lowest affinity and cannot form a stable complex with α-CD. However, in this study, the results obtained with α-CD were statistically significant compared to the control samples (KV1-5) (Figure 4).

Additionally, the target compounds (in the case of this study, isoflavones) must be hydrophobic to form a complex with CDs due to the hydrophobic cavity of the CD. The more hydrophobic the guest is, the stronger the interaction is between the host and guest. Genistein and daidzein have poor water solubility, but daidzein is more lipophilic than genistein [31,32]. However, glycosides are naturally polar due to the availability of at least one sugar moiety in the molecular structure [33]. This physical property of isoflavones explains why the aglycone yield in the extracts with CDs increased and no glycosides were found.

3.2. Influence of Cyclodextrin Increase on Aglycone Yield

To enhance isoflavone yields, CD concentrations were increased to 5%. The highest ($p < 0.05$) amounts of isoflavones were obtained from BP1 and GP1 samples, which were prepared using β-CDs and γ-CDs, respectively (Figure 5). Sample BP1 yielded the highest amount of daidzein (417.1 ± 16.68 μg/g) and GP1 genistein (222.133 ± 8.82 μg/g).

However, the increase in CDs increased the amount of daidzein more than genistein. Daidzein yields increased on average by 1.06 (α-CDs), 1.4 (β-CDs) and 1.25 (γ-CDs) times when the CDs were increased from 1 to 5%. Genistein yields increased using α- and γ-CDs (1.28 and 1.12 times, α- and γ-CDs, respectively), but decreased using β-CD. Nevertheless, as in extracts with 1% CDs, the glycosides, daidzin and genistin were not detected in the extracts with 5% of excipients.

As previously mentioned, CD forms complexes with hydrophobic compounds. According to lipophilicity, expressed as the logarithm of the octanol–water partition coefficient log P o/w, genistein has 2.04 and daidzein 2.24, which means, that daidzein is more hydrophobic than genistein and as a result can form host–guest complexes with the CDs more actively [34]. The increase in the daidzein yield indicates that in high temperatures it can form more stable complexes with β-CDs and γ-CDs compared with genistein.

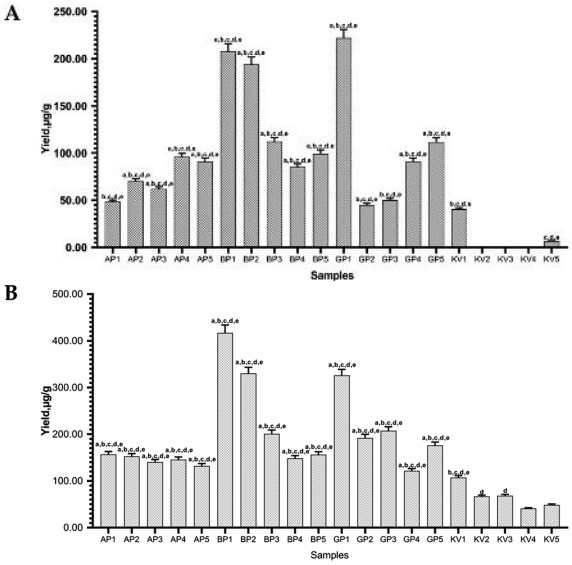

Figure 5. Quantitative yield of (**A**) genistein and (**B**) daidzein using excipients (5%). Control samples without excipients (KV1-5), samples with α-CD (AP1-5), β-CD (BP1-5), γ-CD (GP1-5). [a] $p < 0.05$ vs. KV1, [b] $p < 0.05$ vs. KV2, [c] $p < 0.05$ vs. KV3, [d] $p < 0.05$ vs. KV4, [e] $p < 0.05$ vs. KV5. Sample codes are provided in Table 1.

In this study, a temperature of up to 100 °C was employed. In the literature, phase-solubility studies of genistein and daidzein with CDs complexes were performed at lower temperatures (24–45 °C). Therefore, there is no information about isoflavones and CD phase solubility at high temperatures. Based on phase solubility diagrams found in the literature, it was observed that when performed at lower temperatures, increasing α- and γ-CD concentrations up to 10% in samples, the quantities of dissolved genistein increased. When using β-CDs, the results showed that genistein solubility increased. However, the results presented in the literature showed genistein solubility only with 2% concentration β-CD on a phase solubility diagram [22]. The solubility of daidzein with β-CDs also increased when the temperature was increased. Therefore, it is possible that daidzein complexes are formed more intensively at temperatures of up to 40 °C. In the literature, the formation of the genistein complex with cyclodextrins decreases with the increasing temperature [35,36].

3.3. Effect of 50% Ethanolic and CD-Assisted Extractions on the Aglycone Yield

In order to increase the isoflavone yield in extract, it was decided to use organic solvent as a co-solvent with CDs, because aglycones are compounds, which are more hydrophobic than hydrophilic; therefore, their solubility in water is limited. After the complex with CDs degrades, a precipitate of isoflavones can form in the water. Using organic solvent can prevent this process.

In the literature, other organic solvents were studied in relation to cyclodextrins, and their role as a co-solvent was observed. In the study of H. Yoshii et al., it was determined that only ethanol, methanol, acetonitrile, isopropyl alcohol and n-propyl alcohol can act as solvents and cannot fully compete with the drug for inclusion [37]. Conventional solid–liquid extraction commonly uses hydroalcoholic mixtures, and an increasing

popularity of ethanol can be noticed in the literature due to its food-grade and less toxic nature [38]. L. Y. Yoshiara et al. optimized soy isoflavone extraction with different solvents and reported that using pure organic solvents for isoflavone extraction was not efficient, suggesting that the use of these extraction solvents in binary or ternary mixtures with water could be more advantageous [39]. In this study, 50% ethanol was selected as a safe solvent to use, so that the extracted isoflavones could later be used in nutraceutical production. Additionally, these results were supported by results presented in Table 4.

When using 50% of ethanol as a solvent, the highest yields of daidzein ($p < 0.05$) were detected in BE3 and GE3 samples using β-CDs and γ-CDs, respectively (Figure 6B). The highest yield of genistein was observed in sample GE3 (226.97 $\pm$ 9.08 μg/g) (Figure 6A), although similar results were obtained in samples GE1-2 and BE1. The genistein and daidzein contents in the BE3 sample were 184.30 $\pm$ 7.37 μg/g and 697.167$\pm$ 27.88 μg/g; in the GE3 sample, they were 226.96 $\pm$ 9.07 μg/g and 682.90 $\pm$ 27.31 μg/g.

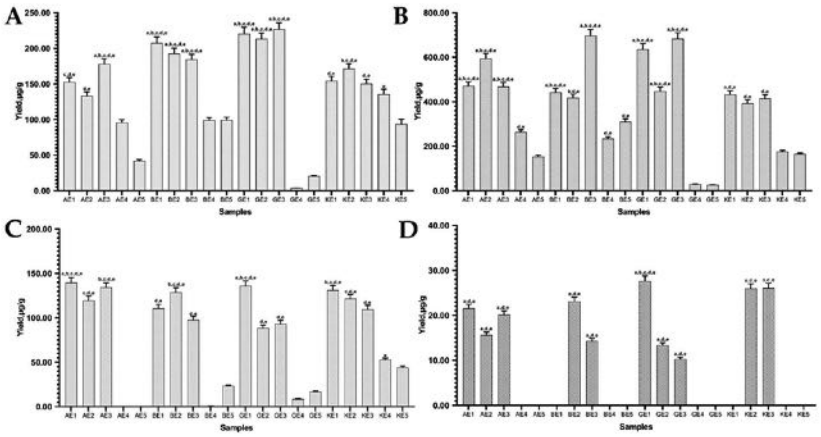

Figure 6. Quantitative yield of isoflavone aglycones ((**A**) genistein and (**B**) daidzein) and glycosides ((**C**) genistin and (**D**) daidzin) using excipients (1%) and 50% ethanol as a solvent. Control samples without excipients (KE1-5), samples with α-CD (AE1-5), β-CD (BE1-5), γ-CD (GE1-5). [a] $p < 0.05$ vs. KE1, [b] $p < 0.05$ vs. KE2, [c] $p < 0.05$ vs. KE3, [d] $p < 0.05$ vs. KE4, [e] $p < 0.05$ vs. KE5. Sample codes are provided in Table 1.

By changing the solvent from water (Figure 3) to 50% ethanol (Figure 6), the aglycone yield in the extract increases on average by 2–3 times. This was observed using all CDs, except β-CD, which reduced genistein yield by 1.17 times. Using α-CD in the extraction increased genistein yield by 3.32 times, and γ-CD increased the yield by 2.36 times. Daidzein amounts increased with all CDs—3.64, 2.31 and 3.06 times (α-, β- and γ-CD, respectively). As in the previous results, by using water as a solvent, the concentrations of isoflavones, obtained using only ultrasound without thermal hydrolysis, were low.

Boonyarattanakalin et al. studied the role of ethanol as a co-solvent in the cyclodextrin inclusion complexation. In the study, it was determined that ethanol has an effect on the formation of the cyclodextrin inclusion complex. It can act as a guest molecule, interfere with complex formation and compete with other guest molecules, but due to the different α-, β- and γ-CD cavity sizes, it interacts differently with each CD. Ethanol mainly forms complexes with β-CD [40]. Therefore, genistein and ethanol compete for β-CD. Due to the ethanol and CD interaction, the yield of isoflavones did not differ significantly compared with the control samples (KE1-5) as it did when CDs were used with purified water.

High yields of genistin and daidzin (Figure 6A,B) in the extracts showed that they were not fully hydrolyzed. The boiling point of 50% ethanol is lower than that of purified water. CDs form inclusion complexes more intensively at higher temperatures, so it is important to choose the right temperature [41]. Isoflavone aglycones can be unstable at high temperatures and break down [42]. The degradation reaction rate of isoflavones reaches its peak at ∼95 °C for genistein and at ∼98 °C for daidzein [43]. The boiling temperature of ethanol (50%) is 92 °C, and purified water has a boiling point of 100 °C. Therefore, the 8 °C difference can affect aglycones. Consequently, it can be assumed that glycosides are completely hydrolyzed to aglycones in purified water, using HRE or thermal hydrolysis, but may degrade due to exposure to high temperatures, yet some of them could be protected by the cyclodextrin–aglycone complex [17]. This is why using 50% ethanol results in more aglycones and their glycosides being recovered.

As mentioned before, not all the glycosides were hydrolyzed. The highest yields ($p < 0.05$) of genistin and daidzin were found in GE1 and AE1 samples (Figure 6A,B). Comparing the samples prepared with excipients, it was observed that when using α-CDs, the average genistin and daidzin amounts were higher than when using β-, γ-CD or no excipients in the extracts (control samples).

4. Conclusions

The results indicate that the use of α-, β- and γ-CDs during extractions increases the yield of isoflavones significantly. The use of purified water with CDs enhanced the isoflavone yields compared with samples without excipients.

The recovery of isoflavone aglycones from plant materials is influenced by the solvent, extraction time and temperature. Long extraction times and high temperatures increase the chance of the oxidation of CDs and the degradation of isoflavone aglycones, which decrease the yield of isoflavones in the extracts. To obtain a high amount of isoflavones, the water temperature should be closely monitored to avoid the degradation of compounds.

When using purified water as a solvent and increasing concentrations of CDs in the extraction media from 1 to 5%, daidzin yield increased on average by 1.06 (α-CDs), 1.4 (β-CDs) and 1.25 (γ-CDs) times. The amount of genistein increased using α- and γ-CDs (1.28 and 1.12 times α- and γ-CDs, respectively), but decreased using β-CD.

The highest genistein yield was detected in sample B1 258.67 $\pm$ 10.34 μg/g, which was prepared using β-CD and purified water. The highest daidzin yields were detected in BE3 and GE3–697.16 $\pm$ 27.88 and 682.90 $\pm$ 27.31 μg/g (using β- and γ-CD in 50% ethanol, respectively).

Our study demonstrated that the α-, β- and γ-CD-assisted extraction of *Trifolium pratense* L. enhanced the isoflavone yield compared to extraction using the same conditions without excipients. Increasing the levels of naturally occurring isoflavone aglycones from herbal materials can create new opportunities for the application of these bioactive compounds in food, nutraceutical and medical fields.

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Paper 2

Title: Novel Extraction Method Using Excipients to Enhance Yield of Genistein and Daidzein in *Trifolium pratensis* L.

Authors: Jurga Andreja Kazlauskaite, Liudas Ivanauskas, Jurga Bernatoniene

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Article

Novel Extraction Method Using Excipients to Enhance Yield of Genistein and Daidzein in *Trifolium pratensis* L.

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Abstract: Isoflavones can be found in different chemical forms, but the health beneficial effects mainly appear in their free forms—aglycones. Their yield in red clover (*Trifolium pratensis* L.) extracts differs due to different extraction and hydrolysis methodologies. The main aim of this study was to obtain the highest yields of daidzein and genistein from red clover blossoms through the various extraction and hydrolysis methods and to increase their quantities using additional excipients. Extracts were obtained by ultrasound-assisted, heat-reflux and maceration methods combining them with acidic, alkaline, and thermal hydrolysis. Using ultrasound-assisted extraction with optimal conditions and heat-reflux method highest yields of isoflavones were obtained in UTE510 (393.23 ± 19.66 μg/g daidzein and 171.57 ± 8.58 μg/g genistein); UTE530 (415.07 ± 20.75 μg/g daidzein and 150.57 ± 7.53 μg/g genistein) and HNE5 (432.30 ± 21.61 μg/g daidzein and 154.50 ± 7.72 μg/g genistein) samples. These conditions were used with excipients: magnesium aluminometasilicate, croscarmellose sodium, sodium carboxymethyl starch and vinylpyrrolidone-vinyl acetate copolymer. This is the first study reporting the ability of the vinylpyrrolidone-vinyl acetate copolymer to promote solubilization and availability of active compounds from a herbal extract, resulting in enhanced isoflavones yield. The results of the present study showing increased solubility and availability provided by the vinylpyrrolidone-vinyl acetate copolymer suggest that this preparation could in principle also reduce variability due to limited water solubility of isoflavones.

Keywords: *Trifolium pratensis* L.; red clover; isoflavones; aglycones; excipients; extractions

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1. Introduction

Red clover (*Trifolium pratense* L.) is a perennial legume that is widely grown organically and conventionally in many countries because of its agriculture value or for their use in traditional medicine [1,2]. In the past decade, red clover has received a considerable amount of interest as an alternative valuable source of isoflavones with variety of health protective effects [3].

The main isoflavones found in the red clover are formononetin, biochanin A, daidzein and genistein [4]. These isoflavones possess estrogenic [5] and antiproliferative effects [6]. Various products from semi-purified isoflavones and their free forms have been studied for the possible preventive role of breast and prostate cancer, maintenance of bone health, improvement of cardiovascular health and benefits related to menopausal problems [5,7–10].

Natural isoflavone glycosides, such as daidzin and genistin are not easily absorbed in intestinal absorptive cells because of their large hydrophilic structures. These conjugated isoflavones are inactive compounds but become active in aglycone (daidzein and genistein) form when the glucose molecule is removed from the structure (Figure 1). The hydrolysis

of glycosides is an important step to obtain biologically active and easily absorbed forms of isoflavones [11,12].

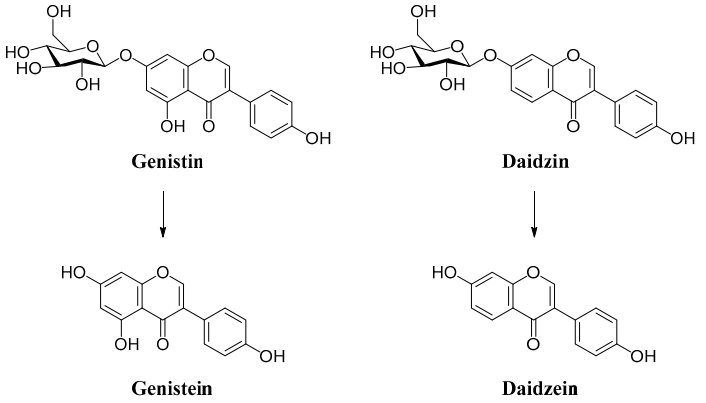

Figure 1. Chemical structures of genistin, genistein, daidzin and daidzein.

Choosing the right extraction method and parameters are the most important stages in the development of nutraceuticals from natural resources. Different extraction processes contribute to the extraction efficiency of the active ingredient from the solid matrix [13,14]. The extraction method of isoflavones daidzein and genistein from plant material should be simple, safe, inexpensive, and suitable for industrial applications. Isoflavonoids in plant material are mostly present as glycosides. Therefore, to extract the aglycone forms from plants, drastic methods (ultrasound/microwave-assisted extractions) or mild extraction techniques (maceration/percolation) followed by hydrolysis must be performed [15–17].

The conventional extraction methods, such as maceration, percolation and soxhlet extractions, have been employed for decades, but it is not economical because of relatively large quantities of solvent and the required long extraction times [18]. To reduce the time of extraction, many measures have been investigated, either separately or combined. Ultrasound-assisted extraction has been explored, changing various parameters, and it has shown a great increase of isoflavones yield, reduced quantities of solvents and shortened the time of extraction [15].

Additional transformation of isoflavone glycosides to aglycones can be achieved using an extraction method combined with hydrolysis. Using chemical (a base/an acid) or thermal (high temperatures) hydrolysis can increase aglycones content [19]. Using hydrolysis, glycosides convert to aglycones, but the temperatures and processing time should be closely monitored and properly selected because genistein and daidzein could degrade at high temperatures [20].

Excipients are the compounds that are added to the formulation along with pharmacologically active substances. The main purpose of adding them in drugs is to increase the bulk of the formulation along with imparting desired properties. Almost all drug dosage forms include an excipient to guarantee the dosage, stability and bioavailability [21,22]. Excipients can also be used for improving extraction and changing environmental conditions. For example, salts that change ion voltage, surfactants, emulsifiers (sorbitan esters (Spans®), polysorbates (Tweens®)), and pH-adjusting substances are used [23]. Croscarmellose sodium, sodium carboxymethyl starch and vinylpyrrolidone-vinyl acetate copolymer has been used for the first time as an excipient for chemical compound extractions; previously, it was used as an excipient in solid dosage forms [24–26]. Magnesium aluminometasilicate was used in previous studies to increase essential oil yield from nutmeg seeds [23].

So, the aim of this study was to establish an optimal extraction method, hydrolysis, and parameters to produce isoflavones from red clover and to use excipients on said extracts to increase the isoflavones yield.

2. Materials and Methods

2.1. Materials

Red clover samples were collected at Laičiai, Kupiškis district, northeast Lithuania. Flower buds and flowers (average color, light red, dark red and bright red) collections were made on 26 September. Samples were dried and stored at room temperature. Before use, clover flowers were grounded to a fine powder using Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Grinding was performed at 4025 g using a 0.5 mm trapezoid holes sieve.

HPLC-grade and analytical-grade reagents were used: hydrochloric acid, sodium hydroxide, acetic acid, methanol, acetonitrile (Sigma Aldrich, Hamburg, Germany); standards of genistein, genistin, daidzein and daidzein (Sigma Aldrich, Steinheim, Germany); and ethanol (96%) (Vilniaus Degtine, Vilniaus, Lithuania). Purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionized water was prepared with Milipore, SimPak 1 (Merck, Darmstadt, Germany). Excipients included croscarmellose sodium, sodium carboxymethyl starch and vinylpyrrolidone-vinyl acetate copolymer (JRSPharma & Gujarat Microwax Pvt Ltd., Ahmedabad, India) and magnesium aluminometasilicate (Neusilin®) (US2, Fuji Chemical Industries Co., Ltd., Toyoma, Japan).

2.2. Extraction of Plant Material

2.2.1. Moisture Determination of Red Clover Plant Matearial

The moisture content of the milled red clover flowers was determined using a KERN MLB apparatus (KERN & Sohn GmbH, Balingen, Germany). A total of 0.3 ± 0.01 g grams of the material was placed in the apparatus and heated to 105 °C. At the end of the operation, the device provided a calculated moisture content of the material [27]. The moisture of the red clover plant material humidity ranged from 7% to 7.4%.

2.2.2. Maceration Extraction (ME)

Maceration extraction was carried out using a modified method of Krähmer et al., 2013 [28]. A total of 0.3 ± 0.001 g of dried and milled flower heads were macerated in 10 mL ethanol (70 or 50% v/v). The samples were centrifuged for 10 min at 3382 g, followed by the decantation of the supernatant. The extracts were hydrolyzed using alkaline hydrolysis and then filtered through PVDF syringe filters (pore size 0.22 µm) for further HPLC analysis. The extraction conditions are displayed in Table 1 and decoding of the samples are provided in Figure 2.

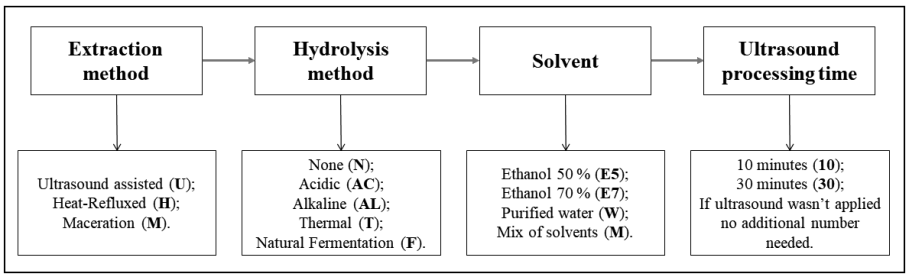

Figure 2. Decoding of the samples provided in Table 1.

Table 1. Extraction conditions used for the experiment.

Sample Code	Method *	Hydrolysis Method	Solvent	Processing/Extraction Time, min
UNE710	U	-	ethanol 70%	10
UACE710		acidic		10
UALE710		alkaline		10
UNE730		-		30
UACE730		acidic		30
UALE730		alkaline		30
UNE510	U	-	ethanol 50%	10
UACE510		acidic		10
UALE510		alkaline		10
UTE510		thermal		10
UNE530		-		30
UACE530		acidic		30
UALE530		alkaline		30
UTE530		thermal		30
HNW	H	-	purified water	60
UTW10	U	thermal	purified water	10
UTW30				30
MNE5	M	-	ethanol 50%	1080
MNE7		-	ethanol 70%	
MFM	M	natural fermentation	70%; 96% ethanol, deionized water	2880
HNE5	H	-	ethanol 50%	60

* U—Ultrasound-assisted extraction; H—Heat-reflux extraction; M—maceration.

2.2.3. Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction was performed using an ultrasound bath (frequency 38 kHz) (Cambridge, UK, Grant Instruments™ XUB12 Digital). A total of 0.3 ± 0.001 g of dried and milled flower heads was macerated in 10 mL of solvent. The extraction of isoflavones was performed by employing different extraction conditions—solvent (70 or 50% ethanol and purified water v/v) and extraction time: 10 to 30 min, processing temperature 40 ± 2 °C [17,29]. The samples were centrifuged for 10 min at 3382 g, followed by the decantation of the supernatant. The extracts were hydrolyzed and then filtered through PVDF syringe filters (pore size 0.22 µm) for further HPLC analysis. The extraction conditions are displayed in Table 1 and decoding of the samples are provided in Figure 2.

For easier comprehension, the samples are coded according to their conditions (Figure 2). The first letter of the sample indicates extraction method; the second, the hydrolysis method; and the third, the solvent and ultrasound processing time (if ultrasound was not applied, no additional number was added).

Some of the samples were modified and prepared with vinylpyrrolidone-vinyl acetate copolymer, croscarmellose sodium, sodium carboxymethyl starch or magnesium aluminometasilicate. The extracts were made in the same conditions, which were listed earlier. Sample preparation conditions are listed in Table 2 and decoding of the samples are provided in Figure 3.

Table 2. Extraction conditions using excipients for the experiment.

Sample Code	Extraction Method *	Hydrolysis Method	Solvent	Excipient	Excipient: Extract Ratio	Processing Time, min
UTEMO10	U	thermal	ethanol 50%	Magnesium aluminometasilicate	1:100	10
UTEMO30						30
HNEMO	H	-				60
UTWMO10	U	thermal	purified water		1:100	10
UTWMO30						30
HNWMO	H	-				60
UHWCO10	U	thermal	purified water	Croscarmellose sodium	1:100	10
UHWCO30						30
HNWCO	H	-				60
UTWSO10	U	thermal	purified water	Sodium carboxymethyl starch	1:100	10
UTWSO30						30
HNWSO	H	-				60
UTWVO10	U	thermal	purified water	Vinylpyrrolidone-vinyl acetate copolymer	1:100	10
UTWVO30						30
HNWVO	H	-				60
UTWVT10	U	thermal	purified water		2.5:100	10
UTWVT30						30
HNWVT	H	-				60
UTWVF10	U	thermal	purified water		5:100	10
UTWVF30						30
HNWVF	H	-				60
UTEVO10	U	thermal	ethanol 50%		1:100	10
UTEVO30						30
HNEVO	H	-				60

* U—ultrasound-assisted extraction; H—heat-reflux extraction; M—maceration.

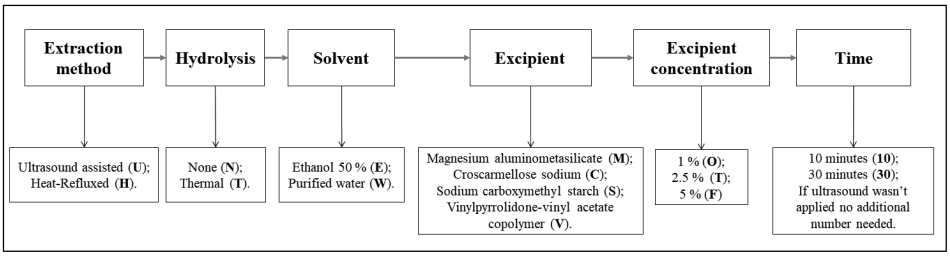

Figure 3. Decoding of the samples provided in Table 2.

The samples in Table 2 are coded according to their conditions (Figure 3). The first letter of the sample indicates the extraction method; the second, the hydrolysis method; the third, the solvent; the fourth, the excipient; and the fifth, the excipient concentration and the number show ultrasound processing time (if ultrasound was not applied, no additional number was added).

Purified water or 50% of ethanol (v/v) was used as the solvent and the excipient was added to the extraction mixture. The excipients concentration in the extract were 1% (v/w) (0.1 ± 0.001 g was added to the extraction mixture of 10 mL); for vinylpyrrolidone-vinyl

acetate copolymer, it was from 1 to 5% (v/w) (0.1 ± 0.001–0.5 ± 0.001 g were added to the extraction mixture of 10 mL). The excipient amount was based on solvent quantity. The samples were centrifuged for 10 min at 3382 g, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm) prior to HPLC analysis.

2.2.4. Heat-Reflux Extraction (HRE)

A total of 0.3 ± 0.001 g of dried and milled flower heads were mixed with 10 mL of used solvent (70%, 50% ethanol or purified water v/v) in a 250 mL round bottom flask and it was refluxed in the sand bath at 100 °C for 1 h. Consequently, the mixture was left to cool at 25 ± 2 °C temperature. The samples were centrifuged for 10 min at 3382 g, followed by decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm) prior to HPLC analysis. The extraction conditions are displayed in Table 1.

2.3. Hydrolysis and Neutralization

2.3.1. Acidic Hydrolysis and Neutralization

For the acid hydrolysis modified method of Zgórka, 2009 was used [4]. Extracts were transferred to a 250 mL round-bottom flask. A total of 37% HCl was added to the whole medium ratio 1:12 (v/v) and finally the flask was placed in a heating mantle under a reflux condenser. Sample hydrolysis was performed from the beginning of liquid boiling for a period of 1 h. Then, hydrolyzed extracts were cooled down to 25 ± 2 °C then neutralized to pH∼2.5 by adding aqueous solution of 2 M NaOH while stirring. The neutralized extracts were filtered. The neutralized solution was prepared for further HPLC analysis.

2.3.2. Alkaline Hydrolysis and Neutralization

Alkali hydrolysis was carried out using 25% NaOH. The pH was changed to 10.5 and then the extracts were sonicated at 45 ± 2 °C for 10 min. After hydrolysis, the sample was neutralized to pH 5.7 using 25% acetic acid. The neutralized extracts were filtered. The neutralized solution was prepared for further HPLC analysis.

2.3.3. Thermal Hydrolysis

Thermal hydrolysis was carried out by transferring the extract to a 250 mL round-bottom flask. It was refluxed in the sand bath at 100 °C for 1 h. After that, the mixture was left to cool at 25 ± 2 °C temperature. The samples were centrifuged for 10 min at 3382 g, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 µm) prior to HPLC analysis.

2.3.4. Maceration Extraction (ME) with Natural Hydrolysis

The maceration was carried out using 0.3 ± 0.001 g of dried and milled flower heads, which were weighed and covered completely with 30 mL deionized water and kept overnight. The next day, extracts were filtered using a Buchner funnel and filtrates were collected. A second overnight water extraction was carried out using 20 mL of deionized water. The spent plant material was extracted again overnight with 40 mL of 96% ethanol and the fourth time with 70% aqueous ethanol. All four filtrates from each sample were combined as one extract [1]. The extraction conditions are displayed in Table 1.

2.4. HPLC–PDA Conditions

HPLC analyses have been carried out using the Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan), consisting of a quaternary pump, an on-line de-gasser, a column temperature controller, the SIL-30AC autosampler (Shimadzu, Tokyo, Japan) equipped with the CTO-20AC thermostat (Shimadzu, Tokyo, Japan) as well as the SPD-M20A diode array detector (DAD). For determination of polyphenols, an ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland)

was used. The mobile phase consisted of solvent A (acetic acid/methanol/deionized water) (1:10:89 $v/v/v$) and solvent B (acetic acid/methanol) (1:99 $v/v/v$). The linear gradient elution profile was as follows: 80% A/20% B at 0 min, 30% A/70% B at 30 min, 90% A/10% B at 39 to 40 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Absorption was measured at 260 nm. Quantification of isoflavone compounds was performed using reference standards of daidzein, genistein, daidzin, and genistin. The range of linearity of daidzein was 0.43 to 221 µg/mL, genistein was 0.43 to 218 µg/mL, daidzin was 0.32 to 165 µg/mL, and genistin was 0.3 to 151.5 µg/mL. The linearities of the calibration curves are provided in Table 3. The contents were expressed as µg/g dry weight (dw). Specificity is the ability to unequivocally assess the analyte in the presence of components, which may be expected to be present. In this study, standards (genistein, daidzein, genistin, daidzin) were analyzed and their retention time and spectra were compared with prepared extracts [27].

Table 3. The linearities of calibration curves of isoflavones.

Component	Calibration Equation	Coefficient of Determination R^2	Coefficient of Correlation R	LOD *	LOQ **
Daidzein	59,664.2× + 37,164.6	0.9999	0.9999	0.05	0.12
Genistein	73,083.1× + 44,202.9	0.9999	0.9999	0.05	0.12
Daidzin	38,202.1× + 19,377.4	0.9999	0.9999	0.08	0.31
Genistin	49,602.9× + 24,083.3	0.9999	0.9999	0.075	0.28

* LOD—limit of detection; ** LOQ—limit of quantification.

2.5. Statistical Analysis

Data is presented as the mean ± standard deviation (SD). All experiments were performed in triplicate. Statistical analysis of the results was performed with SPSS 20.0 (IBM Corporation, Armonk, NY, USA). One-way ANOVA was used to investigate the differences between extractions. Post hoc comparisons of the means were performed according to Tukey's HSD test. The means of compared samples were considered significantly different when $p < 0.05$.

3. Results and Discussion

3.1. Determination of Isoflavones Aglycones in Trifolium pratensis L. Extracts

3.1.1. Aglycones Extraction Using UAE Method

The yield of daidzein and genistein were determined in the different concentrations ethanolic extracts obtained from dried *Trifolium pratensis* L. flower heads material by UAE. Isoflavones were determined using HPLC-PDA. Conventional extraction methods are based on the use of chemical solvents and sample heating to maximize the solubility of the active principles and speed up mass transfer. The extraction yield depends on several factors, including the type, concentration and amount of solvent, its residence time and temperature [30]. Different processing times (10 and 30 min) and two ethanol concentrations (50% and 70%) were employed for flower heads extraction using UAE (temperature 40 °C). These extraction conditions were used to determine the effect of treatment time and solvent concentration on isoflavones content when the hydrolysis is not involved. In the study by L. Y. Yoshiara et al., it was determined that using pure organic solvents for isoflavone extraction was not efficient, suggesting that the use of these extraction solvents in binary or ternary mixtures with water could be more convenient [31]. Additionally, Rostagno et al.'s study concluded that the best solvent for ultrasound-assisted extraction of isoflavones is 50% ethanol [32]. Therefore, based on conducted and published studies, it was decided to use two ethanol concentrations—50% and 70%—with water (v/v) as a safe solvent, so that the extracted isoflavones could later be used in nutraceuticals production. The results of genistein and daidzein yields, using only UAE method without hydrolysis, are shown in Figure 4.

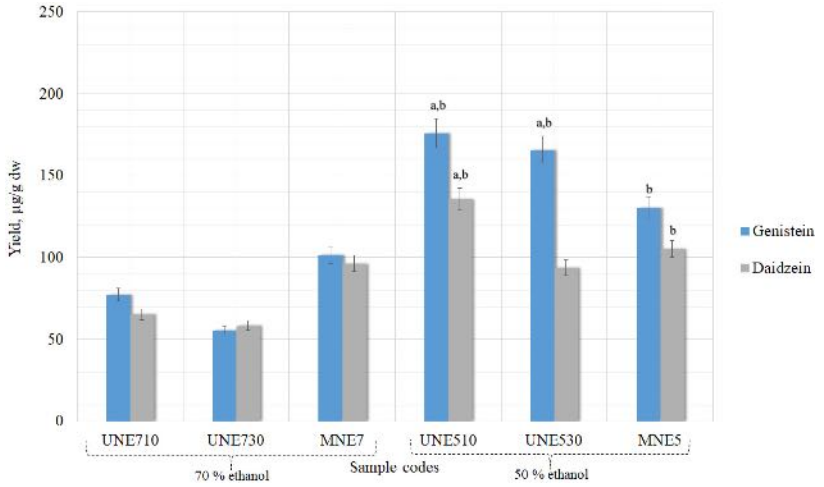

Figure 4. Influence of UAE treatment time and solvent concentration without hydrolysis on the maximal extraction yield of genistein and daidzein in extracts. [a] $p < 0.05$ vs maceration with 50% ethanol (MNE5); [b] $p < 0.05$ vs maceration with 70% ethanol (MNE7). Sample codes and preparation conditions are displayed in Table 1.

The highest genistein and daidzein amounts were obtained using 50% ethanol and 10 min ultrasound processing time −175.93 ± 8.7 and 135.60 ± 6.7 µg/g (genistein and daidzein, respectively). Extending the processing time from 10 to 30 min aglycones quantities decreased in all the samples. The driving force for UAE is cavitation. Decreasing yields of compounds when the extraction time is increased can be explained by cavitation bubbles collapse [33]. As soon as a bubble collapses near a surface (cell walls, herbal particles, or any suspended material in the liquid), it deforms, taking up a doughnut shape, impacting the wall with the potential to sweep particles away from the surface or indeed cause actual damage. During ultrasound extraction, the solvent vapors and any gases dissolved in the solvent that are in the bubble are exposed to the extreme conditions generated by collapse. If there is water vapor in the bubble, its collapse leads to the homolytic splitting of the water molecules to generate reactive HO· and hydrogen atoms. The radicals formed then undergo reactions to produce H_2O_2 and other active oxidizing agents [34]. The amount of generated oxidizing agents during processing is small, but it could cause some degradation of the extract if the sonication continued over a long period [35].

The differences between simple maceration (MNE5) and UAE samples when 50% concentration ethanol was used were statistically significant in the sample UNE510 and in the sample UNE530 only genistein yield was significant (Figure 4). The same results were precured when comparing UNE510 and UNE530 samples with maceration (MNE7) that was carried out using 70% concentration ethanol.

Low amounts of the glycosides genistin and daidzin were obtained in the samples; their yields were statistically insignificant ($p > 0.05$). Therefore, they are not shown in the graph.

3.1.2. Aglycones Extraction Using UAE Method with Acidic Hydrolysis

Combining UAE with acidic hydrolysis and using 70% or 50% concentration ethanol as a solvent, genistein was not found (Figure 5). The exception was sample UACE730, but the amount of genistein was low (10.67 ± 0.53 µg/g) and, compared to maceration, statistically insignificant ($p > 0.05$).

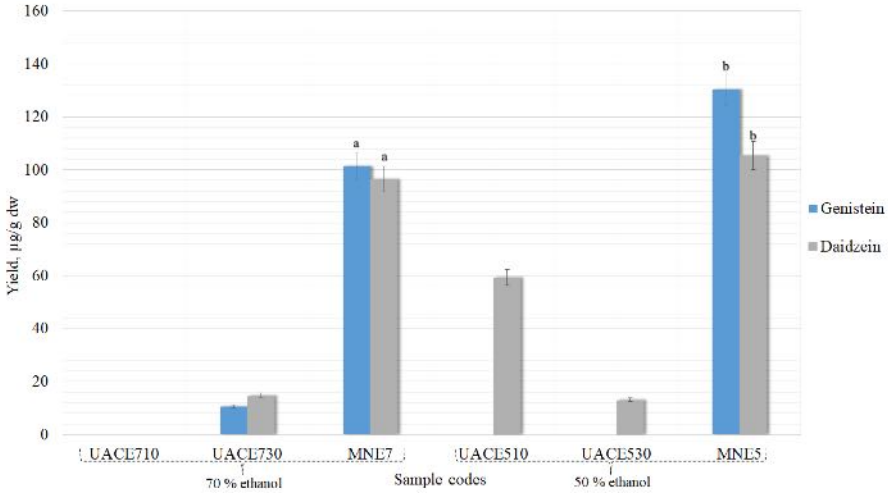

Figure 5. Influence of UAE treatment time and solvent concentration with acidic hydrolysis on the maximal extraction yield of genistein and daidzein in extracts. [a] $p < 0.05$ vs. maceration with 50% ethanol (MNE5); [b] $p < 0.05$ vs. maceration with 70% ethanol (MNE7). Sample codes and preparation conditions are displayed in Table 1.

During acidic hydrolysis, heating is required. Using hydrochloric acid, the samples were hydrolyzed from glycosides to aglycones; however, in high temperatures, genistein degrades [36]. Therefore, the heating in acidic conditions was too long, because no genistin or genistein was found in the samples (Table 4). Chemical structure of isoflavones dictates their stability under variable pH and temperature conditions. Genistein loss could be due to either a complete degradation or a transformation into isoflavone derivative [37]. Relatively large amounts of daidzin, compared to MNE5 and MNE7 samples, were found in the samples UACE710, UACE730, UACE510 and UACE530 (Table 4), indicating that the hydrolysis of these samples was not fully complete. Although no genistein remained during hydrolysis, it was not sufficient for the complete conversion of daidzin to daidzein; consequently, this method was not sufficient to obtain both aglycones.

Table 4. Isoflavone glycosides genistin and daidzin yields (µg/g) found in samples treated by acid hydrolysis.

Sample Code	Genistin, µg/g dw	Daidzin, µg/g dw
UACE710	0.00 ± 0.00	221.37 ± 11.06
UACE730	0.00 ± 0.00	94.37 ± 4.71
MNE7	95.67 ± 4.78	0.00 ± 0.00
UACE510	0.00 ± 0.00	15.33 ± 0.76
UACE530	0.00 ± 0.00	43.80 ± 2.34
MNE5	95.40 ± 4.77	0.00 ± 0.00

In the research by Gikas et al., 2008, red clover extraction was proceeded using HCl, but they did not perform neutralization [38]. Daidzein levels were similar, but genistein levels were different. The extracted amount of genistein was not very high (0.11 mg/g), but it did not degrade as in this experiment. The difference between these two experiments were UAE use. Ultrasound was not used in the study described in the article, so it can be speculated that a combination of ultrasound and heating in an acidic medium were too

harsh for genistein extraction. The paper also suggested that later-harvested clover has higher levels of daidzein and lower levels of genistein. This trend was also observed in this study.

3.1.3. Aglycones Extraction Using UAE Method with Alkaline Hydrolysis

During alkaline hydrolysis extending sonication time genistein yields increased (Figure 4). This tendency was also observed with daidzein when the solvent was 50% ethanol. Higher amounts of aglycones were obtained during alkaline than acid hydrolysis. Alkaline hydrolysis also yielded higher amounts of isoflavones than ultrasound alone. The sample that contained the most isoflavones was UALE530 (196.30 ± 9.8 and 173.10 ± 8.6 µg/g genistein and daidzein, respectively); it was significantly higher ($p < 0.05$) than when extracted using maceration (Figure 6). Lower but similar results were found in the UALE510 sample ($p < 0.05$).

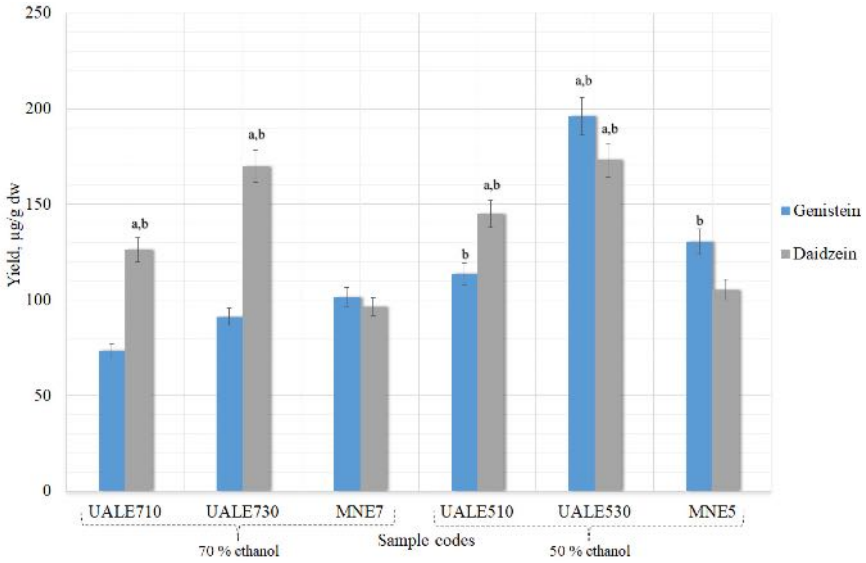

Figure 6. Influence of UAE treatment time and solvent concentration with alkaline hydrolysis on the maximal extraction yield of genistein and daidzein in extracts. [a] $p < 0.05$ vs. maceration with 50% ethanol (MNE5); [b] $p < 0.05$ vs. maceration with 70% ethanol (MNE7). Sample codes and preparation conditions are displayed in Table 1.

Evaluating the results obtained by the UAE method with or without chemical hydrolysis, it was found that statistically significant amounts of aglycones are present in the extracts when 50% concentration ethanol solvent is used. Therefore, only this ethanol concentration was used in further samples.

3.1.4. Aglycones Extraction Using UAE, HRE, ME and ME with Natural Fermentation

In sample MFM (Figure 7), extracted using ME with natural fermentation, no daidzein was detected, but a similar amount of genistein was found compared to other methods. This method was performed at 25 ± 2 °C, but the enzyme β-glucosidase that can be found in the red clover grounded powder converts glucosides to aglycones and possesses the highest activity at 45 °C. β-glucosidase is stable in high temperatures and does not denature for a long time [39]. During natural hydrolysis, the temperature was not high enough for

the enzymes to remove glucoside groups. In the literature, it was observed that not only can enzymes break the glycosides down, but it can also be achieved using heat and the reaction proceeds faster [40].

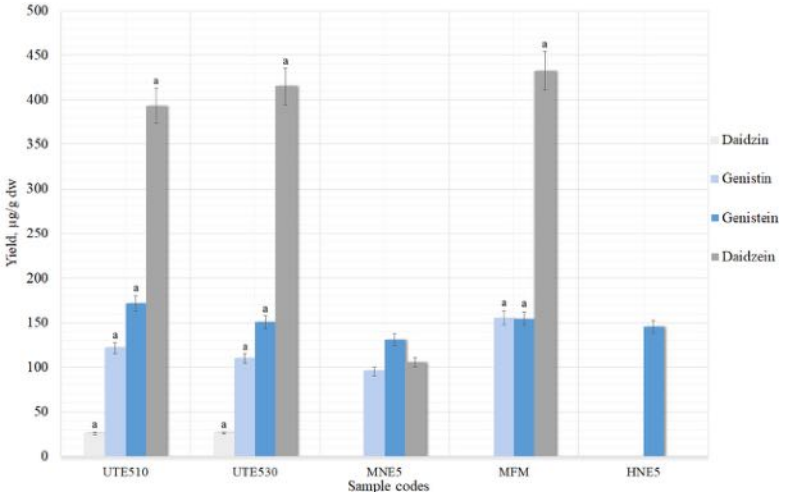

Figure 7. UAE treatment with thermal hydrolysis, HRE, ME and ME with natural hydrolysis on the maximal extraction yield of genistein and daidzein in extracts. [a] $p < 0.05$ vs. maceration with 50% ethanol (MNE5). Sample codes and preparation conditions are displayed in Table 1.

Thermal hydrolysis with the UAE method gave the best results compared with the UAE method without hydrolysis, with acidic or alkaline hydrolysis (Figure 7). Samples UTE510 and UTE530 yielded statistically significant results ($p < 0.05$) compared to the maceration (MNE5) sample. Glycosides daidzin and genistin were also present in these samples (Figure 7). Increasing the sonication time daidzein (from 393.23 ± 19.66 (UTE510) to 415.07 ± 20.75 (UTE530) µg/g) and daidzin (26.00 ± 1.30 (UTE510) to 28.1 ± 1.41 (UTE530) µg/g) amounts increased, but genistein (from 171.57 ± 8.57 (5.4) to 150.57 ± 7.52 (6.4) µg/g) and genistin (from 121.60 ± 6.08 (UTE510) to 109.70 ± 5.49 (UTE530) µg/g) decreased (Figure 7). The HRE method also yielded the highest results ($p < 0.05$) for daidzein in sample HNE5 (432.30 ± 21.6 µg/g) compared to all previous extraction methods and genistein did not degrade during heating (154.5 ± 7.7 µg/g).

In the research by Booth, Overk, Yao, Totura, et al., 2006, genistein and daidzein yields from red clover flower heads or aboveground parts extracts vary widely, but in very small amounts [1]. Comparing the yields of these two studies, the ranges of daidzein and genistein amounts obtained in this study are 10 times higher than the reported results. It was also observed that the amounts of isoflavones depends not only on the method of extraction, the growth stage of the plant or the part of the plant, but also on the growth conditions (growth temperature, humidity, soil fertility). As a result, the yield ranges of isoflavones can be very wide [3].

3.2. Selection of the Excipients

Excipients may improve the solubility of certain active substances in poorly water-soluble drugs [41]. Therefore, it was decided to use excipients during the extractions and to determine whether they could increase the yields of isoflavones. Most isoflavones were

obtained using ultrasound (40 °C, 10 to 30 min, 50% ethanol) in combination with thermal hydrolysis or using HRE alone. Therefore, these extraction conditions will be applied using additional compounds to improve the solubility of isoflavones. Samples prepared using UAE for 10 min (i.e., UTW10) or 30 min (i.e., UTW30) were combined with thermal hydrolysis or only HRE (i.e., HNW) alone. Experiment was carried out using excipients (1%) and purified water as a solvent (Figure 8).

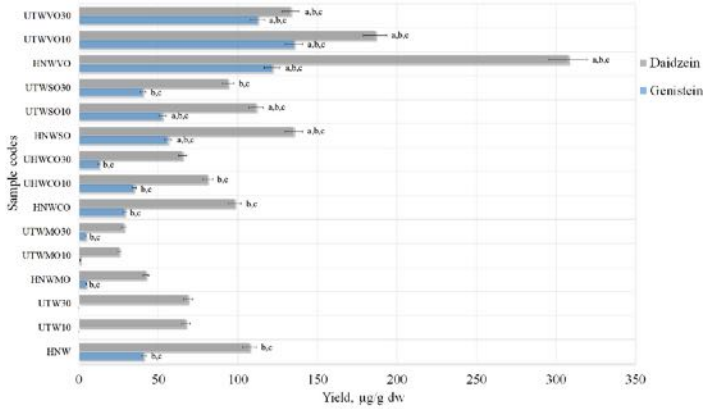

Figure 8. Quantitative yield of isoflavone aglycones using excipients (1%). Control samples without excipients (HNW; UTW10; UTW30), samples with magnesium aluminometasilicate (HNEMO; UTEMO10; UTEMO30), croscarmellose sodium (HNWCO; UHWCO10; UHWCO30), sodium carboxymethyl starch (HNWSO; UTWSO10; UTWSO30) and vinylpyrrolidone-vinyl acetate copolymer (HNWVO; UTWVO10; UTWVO30). [a] $p < 0.05$ vs. HNW, [b] $p < 0.05$ vs. UTW10, [c] $p < 0.05$ vs. UTW30. Sample codes and preparation conditions are displayed in Table 2.

Excipients can be natural, synthetic or semisynthetic compounds that play a vital part in pharmacological products [41]. Magnesium aluminometasilicate, croscarmellose sodium, sodium carboxymethyl starch and vinylpyrrolidone-vinyl acetate copolymer as excipients can improve the oral bioavailability of poorly water-soluble drugs by enhancing the solubility and drug release [42]. The use of modern carriers with a large specific surface area and high absorption capacity is a good way of incorporating higher doses of water-insoluble or poorly soluble compounds into liquid–solid systems and increase their bioavailability. The selected different excipients were expected to absorb isoflavones during extraction and increase their final yields.

When purified water was used as a solvent with the excipient croscarmellose sodium, sodium carboxymethyl starch or vinylpyrrolidone-vinyl acetate copolymer, the yield of isoflavones significantly increased compared to the control samples (Figure 8). Glycosides, daidzin and genistin were not obtained with the use of excipients. Excipient magnesium aluminometasilicate reduced the amount of isoflavones, compared to controls, prepared using the same conditions (Figure 8). In the literature, magnesium aluminometasilicate increased essential oil yield and quantities of various compounds in it [23]. Therefore, possibly, magnesium aluminometasilicate absorbed terpenes, but not isoflavones from red clover extracts.

Using the excipients, statistically significant yields of aglycones were obtained with 1% vinylpyrrolidone-vinyl acetate copolymer. Using the HRE method, aglycones yields found in HNWVO sample were 307.80 ± 15.39 μg/g daidzein and 121.40 ± 6.07 μg/g

genistein (Figure 8). Isoflavones yields decreased using UAE with thermal hydrolysis, and this correlation was observed in all the samples with excipients.

As the results show (Figure 8), lower amounts of isoflavones were obtained with croscarmellose sodium and sodium carboxymethyl starch compared to vinylpyrrolidone-vinyl acetate copolymer. Sodium carboxymethyl starch samples UTWSO10 (52.50 ± 2.63 μg/g genistein and 111.40 ± 5.57 μg/g daidzein) and UTWSO30 (40.23 ± 2.01 μg/g genistein and 93.57 ± 4.68 μg/g daidzein) showed statistically significant results compared to the control samples, but the yields of aglycones was not as high as using vinylpyrrolidone-vinyl acetate copolymer. Therefore, the use of excipients has great scope for improving the methodology of isoflavones extraction.

3.3. *Vinylpyrrolidone-Vinyl Acetate Copolymer Determination of the Optimal Concentration for Higher Amounts of Aglycones Using Purified Water*

When purified water is used as a solvent with an excipient vinylpyrrolidone-vinyl acetate copolymer, the same amounts of isoflavones can be obtained as with 50% ethanol under the same conditions. To test whether the yields of isoflavones obtained in water could be increased, the amounts of excipient added to the extract were increased. It was decided to use 1, 2.5 and 5% (*v*/*w*) vinylpyrrolidone-vinyl acetate copolymer for extractions.

After extractions with different amounts of vinylpyrrolidone-vinyl acetate copolymer, all data obtained were statistically significant compared to controls (HNW; UTW10; UTW30) (Figure 9). For this reason, the data was compared with the sample that yielded the highest amounts of isoflavones in this study, which was a HNE5 sample prepared using the HRE method.

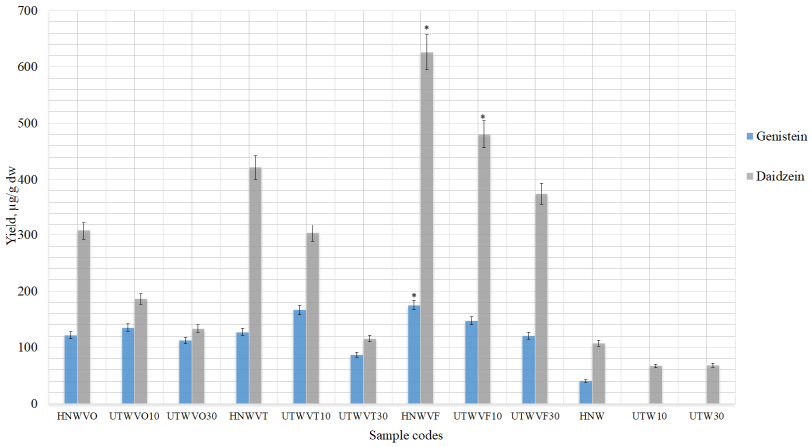

Figure 9. Quantitative yield of isoflavone aglycones using different amounts (1, 2.5 and 5%) of vinylpyrrolidone-vinyl acetate copolymer for extractions. Control samples without excipients (HNW; UTW10; UTW30), 1% of excipient (HNWVO; UTWVO10; UTWVO30), 2.5% (HNWVT; UTWVT10; UTWVT30) and 5% (HNWVF; UTWVF10; UTWVF30). * $p < 0.05$ vs. HNE5 sample prepared HRE method. Sample codes and preparation conditions are displayed in Table 2.

The best results were obtained with a sample using 5% excipient (Figure 9). The yields of isoflavones obtained were higher than the best result obtained in the whole study, which was previously determined in sample HNE5 (432.30 ± 21.6 μg/g and 154.5 ± 7.7 μg/g daidzein and genistein, respectively) (Figure 7). However, HNE5 sample was prepared using 50% ethanol, not purified water. The results were statistically significant when comparing with HNE5 sample in the HNWVF (626.10 ± 31.35 μg/g and 175.56 ± 8.7 μg/g

daidzein and genistein, respectively) (Figure 9). In the UTWVF10 sample, only the amount of daidzein was statistically significant (480.36 ± 24.01 μg/g and 147.23 ± 7.36 μg/g daidzein and genistein, respectively). Increasing the amounts of excipient, the solubility of the resulting isoflavones in the aqueous solvent increases. This could create an opportunity to use cheaper, safer solvents (such as purified water), but obtain the same amounts of isoflavones as using expensive solvents without excipients. Therefore, it would still be possible to try to increase the excipient concentrations in water and to set a maximum concentration at which maximum levels of aglycones could be obtained; this could be a future goal for further research.

3.4. Vinylpyrrolidone-Vinyl Acetate Copolymer Use with Ethanol

High concentrations of isoflavone aglycones were obtained using 5% vinylpyrrolidone-vinyl acetate copolymer in water extracts. Comparing the control samples PDV1-3 produced in water under the same conditions as HNE5, UTE510 and UTE530 using 50% ethanol, it was found that the samples obtained in water had statistically lower values (Figure 10). Therefore, due to the use of excipients with water, we decided to determine the amounts of isoflavones under the same conditions as 50% ethanol and 1% vinylpyrrolidone-vinyl acetate copolymer.

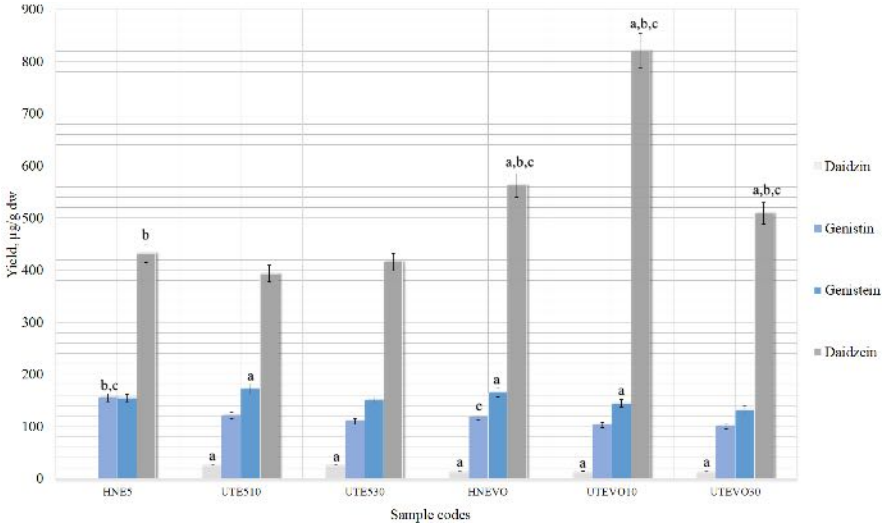

Figure 10. Quantitative yield of isoflavone aglycones using 1 of vinylpyrrolidone-vinyl acetate copolymer for extractions in 50% ethanol. Control samples without excipients (HNE5; UTE510; UTE530), 1% of excipient (HNEVO; UTEVO10; UTEVO30), [a] $p < 0.05$ vs. HNE5 sample; [b] $p < 0.05$ vs. UTE510, [c] $p < 0.05$ vs. UTE530. Sample codes and preparation conditions are displayed in Table 2.

As shown in Figure 10, no isoflavones glycosides were found in the aqueous extracts. Though, using 50% ethanol as a solvent in the extracts, both genistin and daidzin were detected (Figure 10). Daidzein yields in all three samples with vinylpyrrolidone-vinyl acetate copolymer (HNEVO; UTEVO10; UTEVO30) in ethanol were statistically significant when comparing them with controls. UTEVO10 contained the highest daidzein yield in this study—820.50 ± 41.02 μg/g. Genistein yields were lower, but the HNEVO and

UTEVO30 sample results were statistically significant compared to the control sample HNE5 (Figure 10). Samples prepared with excipient and ethanol had higher levels of isoflavones, both aglycones and glycosides. As mentioned before, samples prepared in water with the excipient showed similar yields of isoflavones as the samples prepared under the same conditions with ethanol, but without excipients. Although 50% ethanol increased the amount of daidzein compared to the 5% excipient used in water (Figure 9), there was no significant increase in genistein levels. Therefore, the use of excipient vinylpyrrolidone-vinyl acetate copolymer in ethanol mainly yielded glycosides (Figure 10) and aglycone daidzein from plant material.

4. Conclusions

After applying different extraction methods and hydrolysis, it was determined that, using UAE with optimal conditions (processing 10 or 30 min combined with thermal hydrolysis) and the HRE method, the highest extractions of isoflavones were obtained in samples UTE510 (393.23 ± 19.66 μg/g daidzein and 171.57 ± 8.58 μg/g genistein), UTE530 (415.07 ± 20.75 μg/g daidzein and 150.57 ± 7.53 μg/g genistein) and HNE5 (432.30 ± 21.61 μg/g daidzein and 154.50 ± 7.72 μg/g genistein). These conditions were used with excipients.

In this work, the use of different excipients during the extractions was performed as an effective strategy to enhance isoflavones yield in red clover extracts. This is the first study reporting the ability of the vinylpyrrolidone-vinyl acetate copolymer to promote solubilization and availability of active compounds from a herbal extract, resulting in enhanced isoflavones yield. Using 1% vinylpyrrolidone-vinyl acetate copolymer (HNWVO sample, 307.80 ± 15.39 μg/g and 121.40 ± 6.07 μg/g daidzein and genistein, respectively) in the production of copolymer extracts using water as solvent, it was determined that the amounts of genistein obtained were similar as using 50% ethanol as solvent. Increasing the amount of this excipient to 5%, isoflavone yield further increased (HNWVF sample) to 626.10 ± 31.35 μg/g daidzein and 175.56 ± 8.7 μg/g genistein. The results of the present study showing increased solubility and availability provided by the vinylpyrrolidone-vinyl acetate copolymer suggest that this preparation could in principle also reduce variability due to limited water solubility of isoflavones. By changing the solvent to 50% ethanol, the highest statistically significant yields of isoflavones in this study were obtained in the sample UTEVO10 820.50 ± 41.02 and 144 ± 7.22 μg/g daidzein and genistein, respectively.

The resulting isoflavone-rich extracts could be used in the production of various pharmaceutical forms, as recent studies suggest, with a possible preventive role in breast and prostate cancer, improvement of cardiovascular health or benefits related to menopausal problems.

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Paper 3

Title: The Effect of Traditional and Cyclodextrin-Assisted Extraction Methods on *Trifolium pratense* L. (Red Clover) Extracts Antioxidant Potential

Authors: Jurga Andreja Kazlauskaite, Liudas Ivanauskas, Mindaugas Marksa, Jurga Bernatoniene

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Article

The Effect of Traditional and Cyclodextrin-Assisted Extraction Methods on *Trifolium pratense* L. (Red Clover) Extracts Antioxidant Potential

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Abstract: Red clover is the subject of numerous studies because of its antioxidant properties, the positive influence of isoflavones on the health, and its potential use in the prevention and treatment of chronic diseases. The right excipients, such as cyclodextrins, can increase the profile of valuable phenolic compounds in extraction media to obtain rich in antioxidants, extracts that can be used in nutraceuticals production. The aim of this study was to investigate and compare the total phenolic content, flavonoid content, and antioxidant activity of red clover aerial parts, aqueous and ethanolic extracts prepared using traditional and cyclodextrins-assisted methods. The antioxidant activity of the extracts was established using ABTS, DPPH, FRAP, and ABTS-post column methods. It was determined that cyclodextrins significantly increased total phenolic content (compared with control)—using β-cyclodextrin 20.29% (in aqueous samples); γ-cyclodextrin 22.26% (in ethanolic samples). All the samples prepared with excipients demonstrated a strong relationship between total phenolic content and DPPH assay. Study showed that for extraction with water, the highest amounts of phenolic compounds, flavonoids and antioxidant activity will be achieved with β-cyclodextrin, but extractions with ethanol will give the best results with γ-cyclodextrin. Therefore, cyclodextrins are a great and safe tool for obtaining rich, red clover flower extracts that are high in antioxidant activity, which can be used in the pharmaceutical industry for nutraceuticals production.

Keywords: red clover; cyclodextrins; antioxidants; polyphenols; oxidative stress

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1. Introduction

Red clover (*Trifolium pratense* L.) has high concentrations of phenolic compounds, especially isoflavonoids, which are largely distributed in the *Leguminosae* family [1]. Its extracts are rich in phenolic compounds such as flavonoids, phenolic acids, clovamides, and saponins [2]. Scientific research determined, that red clover extracts consists of 35.54% isoflavones, 1.11% other flavonoids, 0.06% pterocarpans, $\leq$0.03% coumarins, and $\leq$0.03% tyramine [3]. Flavonoids are an important class of phenolic compounds that includes flavones, flavonols, and isoflavones, all characterized by a phenylbenzopyran moiety [4]. Isoflavones are a group of flavonoids typical of some legume species only. They exhibit estrogenic activity and represent the main phytoestrogens of current interest as nutraceuticals and dietary supplements. An antioxidant activity of possible physiological relevance was also reported for isoflavones such as genistein and daidzein [5–8]. The role of flavonoids (isoflavones) and other phenolic compounds as protective dietary constituents, with their estrogenic, antimicrobial, anti-inflammatory, antiallergenic, and antitumor activities, have become an increasingly important area of research. These compounds are

useful in nutraceuticals, cosmetics, medicine, and food additives, or used in the agri-food industry [9–11].

Phenolic compounds, including flavonoids, are antioxidants due to their ability to scavenge and/or reduce high-energy radicals. It is confirmed that total phenolic content correlates with antioxidant activity [12,13]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in neutralizing free radicals. Antioxidant properties stand to be a valuable mechanism of beneficial activity of plant-derived compounds and extracts [14–18].

There are numerous articles confirming antioxidant activity of *Trifolium pratense* L. The results of research suggested the great value of flowers and leaves of *Trifolium* species for their use in phytotherapy, due to their content of polyphenols responsible for the antioxidant activities; phenols in plants can also act as deterrents against herbivores, as antiviral and antimicrobial factors and photoprotectants against UV irradiation [19–21]. Using the right extraction method more phenolic compounds can be extracted, therefore, the antioxidant activity can be increased. Additional materials, such as excipients, may be used to extract insoluble polyphenols such as daidzein and genistein as well as enrich the extract with biologically active compounds [22].

The initial selection of herbal compounds possessing the antioxidant effects is carried out by in vitro methods. Spectrophotometric methods are widely applied [23–26]. They determine total amount of antioxidants and evaluate the total antioxidant activity in the researched complex samples. Most popular methods are scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and/or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) free radicals [27]. The most reliable and comprehensive evaluation of the radical-scavenging capacities of plant materials is possible only through the validation of highly efficient analytical methods. One highly promising method for the investigation of antioxidant activity is the HPLC post-column assay with various derivatization techniques. These methods exclude the interactional effects of compounds as the detection occurs with separated analytes. Most post-column assays use DPPH and ABTS radicals [28,29].

Excipients play important role in dosage form. These compounds are added to the formulation along with pharmacologically active substance. Almost all drug dosage forms include excipient to guarantee the dosage, stability, and bioavailability [30]. Cyclodextrins (CDs) can be suitable as excipients in excipient-assisted extraction [31]. It is generally accepted that CDs can form inclusion complex in aqueous solution where a lipophilic guest molecule or moiety locates in the inner cavity [32]. Due to this special property, CDs have been used extensively in pharmaceutical research; CDs can address solubility, stability, and bioavailability issues in a manner not possible with other inactive ingredients. CDs can also, for example, be used to convert liquid drugs into microcrystalline powders, prevent drug-drug or drug additive interactions, reduce or eliminate unpleasant taste and smell, and improve shelf-lives. The most common cyclodextrins are α-CD, β-CD, and γ-CD, which consist of six, seven, and eight glucopyranose units, respectively [33–35].

To our knowledge, this is the first article describing the effect of CDs on the antioxidant activity of red clover flowers extracts. Therefore, the aim of the study was to investigate and compare total phenolic content and antioxidant activity of red clover aerial parts extracts prepared using traditional methods and using CDs as excipients. We also seek to examine the effects of excipients on total flavonoids content.

2. Materials and Methods

2.1. Plant Material and Reagents

Red clover samples were collected in red clover fields in Laičiai, Kupiškis district, Lithuania (latitude 55°53024.2″ N; longitude 25°19036.0″ E). The collections of flower buds and flowers were made on the 26 September 2020. Samples were dried and stored at room temperature. Before use, clover flowers were ground to a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Grinding was performed at 4025 g using a 0.5 mm trapezoid hole sieve.

Purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionized water was prepared with Milipore, SimPak 1 (Merck, Darmstadt, Germany). The following reagents were used: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), aluminium chloride, hexaethylenetetraamine, acetic acid obtained from Sigma-Aldrich (Buchs, Switzerland); potassium persulfate from Alfa Aesar (Karlsruhe, Germany); monosodium phosphate, ferrous sulfate heptahydrate, saline phosphate buffer and hydrogen peroxide from Sigma Aldrich (Schnelldorf, Germany); disodium hydrogen phosphate obtained from Merck (Darmstadt, Germany); 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and α-, β-, and γ-CDs purchased from Sigma Aldrich (Hamburg, Germany); ethanol (96%) (Vilniaus Degtinė, Vilniaus, Lithuania).

2.2. Extracts Preparation

The raw material of red clover aerial parts was ground, and the moisture content of the milled red clover flowers was determined using a KERN MLB apparatus (KERN & Sohn GmbH, Balingen, Germany). The production of extracts was performed as described by Kazlauskaite et al. [22].

Heat-Reflux Extraction (HRE) was performed by using 0.3 ± 0.001 g of dried and milled flower heads. Plant material was mixed with 10 mL of solvent (50% ethanol *v/v* or purified water) in a round bottom flask and refluxed in a sand bath at 100 °C for 1 h. After reflux, the sample was left to cool down at a temperature of 25 ± 2 °C. After cooling down, the sample was centrifuged with Sigma 3-18K centrifuge (Sigma, Osterode am Harz, Germany) for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extract was filtered through PVDF syringe filters (pore size 0.22 μm).

Ultrasound-assisted extraction with thermal hydrolysis (UAE) was performed using an ultrasound bath (frequency 38 kHz) (Grant Instruments™ XUB12 Digital, Cambridge, England). The extraction was performed by using 0.3 ± 0.001 g dried and milled flower heads. Plant material was macerated in 10 mL of solvent, and the extraction of isoflavones using ultrasound was performed by employing different conditions: solvent (50% *v/v* ethanol and purified water) and extraction time (10 or 30 min), with the processing temperature of 40 ± 2 °C (the temperature is regulated automatically by the ultrasonic bath). After ultrasound processing the mixture was prepared for thermal hydrolysis. The sample was transferred to a 250 mL round bottom flask. It was refluxed in a sand bath at 100 °C for 1 h. After the procedure, the mixture was left to cool down and then centrifuged for 10 min at 3382× *g*, followed by the decantation of the supernatant. The extracts were filtered through PVDF syringe filters (pore size 0.22 μm, Frisenette, Knebel, Denmark).

Some of the samples were prepared with excipients: α-, β-, or γ-CDs. The extracts were made under the same conditions as previously listed (HRE or UAE). Purified water or 50% ethanol (*v*/*v*) was used as the solvent (10 mL), and the excipient (0.1 ± 0.001 g) was added to the extraction mixture with plant material (0.3 ± 0.001 g). CD concentrations in the sample was 1% (*w*/*v*). The excipient amount was based on the quantity of solvent in the extract. The samples were centrifuged for 10 min at 3382 × *g*, followed by the decantation of the supernatant. After that, the extracts were filtered through PVDF syringe filters (pore size 0.22 μm).

A list of samples prepared using different conditions and excipients is given in Table 1. For easier comprehension samples codes are based on their preparation conditions and decoding is shown in Figure 1.

Table 1. Samples list and preparation conditions.

Sample Name *	Method	Solvent	Excipients
AW1	Reflux (1)	water	α-CD
AW2	Ultrasound (10 min) combined with reflux (2)	water	α-CD
AW3	Ultrasound (30 min) combined with reflux (3)	water	α-CD
AE1	Reflux (1)	ethanol	α-CD
AE2	Ultrasound (10 min) combined with reflux (2)	ethanol	α-CD
AE3	Ultrasound (30 min) combined with reflux (3)	ethanol	α-CD
BW1	Reflux (1)	water	β-CD
BW2	Ultrasound (10 min) combined with reflux (2)	water	β-CD
BW3	Ultrasound (30 min) combined with reflux (3)	water	β-CD
BE1	Reflux (1)	ethanol	β-CD
BE2	Ultrasound (10 min) combined with reflux (2)	ethanol	β-CD
BE3	Ultrasound (30 min) combined with reflux (3)	ethanol	β-CD
GW1	Reflux (1)	water	γ-CD
GW2	Ultrasound (10 min) combined with reflux (2)	water	γ-CD
GW3	Ultrasound (30 min) combined with reflux (3)	water	γ-CD
GE1	Reflux (1)	ethanol	γ-CD
GE2	Ultrasound (10 min) combined with reflux (2)	ethanol	γ-CD
GE3	Ultrasound (30 min) combined with reflux (3)	ethanol	γ-CD
W1	Reflux (1)	water	-
W2	Ultrasound (10 min) combined with reflux (2)	water	-
W3	Ultrasound (30 min) combined with reflux (3)	water	-
E1	Reflux (1)	ethanol	-
E2	Ultrasound (10 min) combined with reflux (2)	ethanol	-
E3	Ultrasound (30 min) combined with reflux (3)	ethanol	-

* 1–3 means different conditions for sample preparation: (1) reflux; (2) ultrasound (10 min) combined with reflux; (3) ultrasound (30 min) combined with reflux (3).

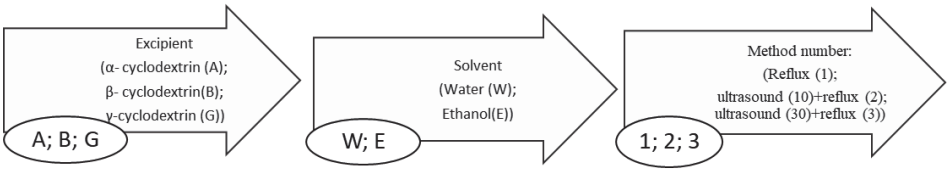

Figure 1. Sample codes generation scheme. If the excipient was not used in the preparation of the sample, the letter of excipient will be skipped, and the code will start from the solvent letter.

2.3. Determination of Total Phenolic Content

The content of total phenolic compounds in red clover samples was determined by using a slightly modified Saviranta et al. method [36], so 0.5 mL of extract was mixed with

2.5 mL of 1:9 diluted Folin–Ciocalteu's phenol reagent and 2.0 mL of 7% (*w*/*v*) sodium carbonate. Absorbance was measured at 765 nm after 1 h using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was obtained with a gallic acid (0–0.1 mg/g; y = 11.108; R^2 = 0.9981). The results were expressed as gallic acid equivalent per gram dry weight (mg GA/g dw).

2.4. Determination of Total Flavonoid Content

Analysis of total amount of flavonoids in extracts was performed by UV spectrophotometry, based on complexation of phenolic compounds with Al(III), as described in Urbonavičiute et al. with some modifications [37]. Accordingly, 0.1 mL of red clover aerial part extract was added to 1.0 mL 96% (*v*/*v*) of ethanol, 0.05 mL of 33% acetic acid, 0.15 mL 10% aluminium chloride, and 2.0 mL 5% hexaethylenetetraamine solutions. Spectrophotometric analysis was performed after 30 min at 475 nm wavelength using aspectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The results were expressed as rutin equivalent per gram dry weight (RE/g dw) and calculated by the following formula: TFC = C × V_e × F/M, where TFC is the total flavonoid content, mg RE/g dw; C is the concentration of the used standard, mg/L; V_e is the volume of the used solvent, L; F is the dilution coefficient of the sample; and M is the mass of the sample, g. The calibration curve was obtained with a rutin (0–0.5 mg/g; y = 5.0867; R^2 = 0.9985). The results were expressed as rutin equivalent per gram dry weight (RU/g dw).

2.5. Antioxidant Activity

2.5.1. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was prepared by mixing 0.3 M acetate buffer, 10 mM TPTZ solution with 40 mM HCl and 20 mM ferric chloride solution. Then, 10 µL of sample was mixed with 200 µL of FRAP reagent; the contents were mixed vigorously. The absorption was measured at 593 nm using spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was obtained with a ferrous sulfate (0–1 mg/g; y = 2.6272; R^2 = 0.9985). The results were expressed as ferrous sulfate equivalent per gram dry weight (FE(II)/g dw)

2.5.2. ABTS Radical Scavenging Activity Assay

The ABTS radical was generated through the oxidation of ABTS with potassium persulfate. Aqueous ABTS solution (7 mM) was mixed with potassium persulfate (2.45 mM) solution and stored in the dark for 12–16 h to produce a dark-colored solution containing ABTS radical cation. Before use, the ABTS radical cation was diluted with water with an initial absorbance of about 0.90 (±0.02) at 734 nm using spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). Free radical scavenging activity was evaluated by mixing 2.0 mL of ABTS working standard with 200 µL of test sample in cuvette. The samples ware incubated in the dark at room temperature for 30 min. The calibration curve was obtained with a trolox (0–0.5 mg/g; y = 0.0001728x; R^2 = 0.9832). The results were expressed as trolox equivalent per gram dry weight (TE/g dw) [38].

2.5.3. DPPH Radical Scavenging Activity Assay

Here, 2.0 mL of DPPH solution (0.1 mM, in ethanol) was blended with 2.0 mL of the samples. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank using spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was obtained with a trolox (0–0.016 mg/g; y = 0.00623x; R^2 = 0.9923). The results were expressed as trolox equivalent per gram dry weight (TE/g dw) [39].

2.6. ABTS Post-Column Antioxidant Assay

The HPLC post-column method using ABTS reagent was carried out according to Marksa et al. [29].

The samples were introduced into the HPLC detection system, the mobile phase with test sample passes through the reaction loop into the mixing tee, where a 0.5 mL/min ABTS reagent solution is supplied simultaneously via a Gilson pump 305 (Middleton, WI, USA). The HPLC-ABTS system uses a 3 m (inner diameter 0.25 mm, outer diameter 1.58 mm) reaction loop, which was thermostated at 50 °C. Reaction of antioxidants with ABTS resulted in a color change of the reagent as determined using a Waters 2487 UV / VIS detector (Waters Corporation). Detection of test solutions was performed at a wavelength of 650 nm. Signal strength is expressed as peaks of negative active compounds. The antioxidant activity of the extract compounds was evaluated by the post-column method according to the equivalent of the trolox standard. Calibration curves were prepared using trolox ethanol solution. An ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase consisted of solvent A (acetic acid/methanol/deionized water) (1:10:89 $v/v/v$) and solvent B (acetic acid/methanol) (1:99 $v/v/v$). The linear gradient elution profile was as follows: 80% A/20% B at 0 min; 30% A/70% B at 30 min; 90% A/10% B at 39 to 40 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Calibration curve was prepared from a trolox ethanol solution at five dilutions in the range of 8.359–133.750 µg/mL, R^2 = 0.999565 (Figure 2).

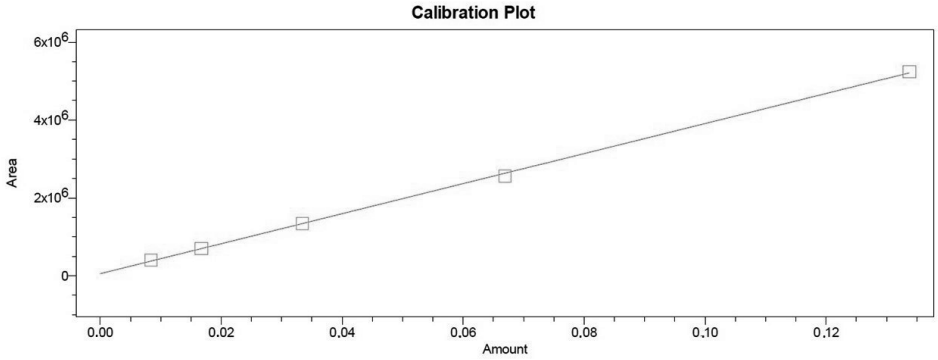

Figure 2. Trolox calibration curve.

2.7. LC-MS Qualitative Analysis

After performing reversed-phase liquid chromatographic (RP-LC) separation, the detailed qualitative profiling of red clover extract was carried out using electrospray (ESI) ionization (in negative and positive mode) followed by the mass spectrometric (MS) analysis. The LC/MS system was composed of a Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan) equipped with a LCMS-2020 mass spectrometer (Shimadzu, Tokyo, Japan).

The chromatographic separation was performed on a YMC-Triart C18 (YMC Karasuma-Gojo, Kyoto, Japan) (150 mm × 3.0 mm, 3 µm) analytical column, column temperature 40 °C. The mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. For each clover sample, 10 µL aliquots volumes were injected onto the chromatographic column. HPLC was run at 0.4 mL/min flow. The optimum ESI conditions were set as 350 °C for interface temperature, 250 °C for DL temperature, 400 °C for heat block temperature, 1.5 L/min for nebulizing gas flow, and 10 L/min for drying gas flow. Positive ion and negative ion measurements are performed while switching alternately between positive and negative ionization modes. The m/z ranges for positive and negative modes were 50–1000, scan speed 15,000 u/s, and 0.1 m/z steps.

Compounds present in the sample were identified by comparing the mass spectra obtained with both the literature data and mechanisms presented in freely available databases.

2.8. Statistical Data Analysis

Data were analysed using SSPS version 20.0 (IBM Corporation, Armonk, NY, USA). All experiments were performed in triplicate. Data are expressed as mean ± standard deviation (S.D.). The comparisons between three different measurements were made using Friedman and Wilcoxon tests. Comparisons between the two groups were made by the Mann–Whitney U test. Correlation and regression coefficients were performed using the Spearman test. The results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. The Influence of Excipients on the Total Amount of Phenolic Compounds and Flavonoids Content

There is very little information on the total phenolic and flavonoid compounds in the flower extracts of *Trifolium pratense* L. Extracts of red clover leaves have been studied the most, as they were thought to contain the highest amounts of isoflavones, which makes clover very valuable [40–42]. However, further research has shown that clover flowers also contain significant amounts of isoflavones [43–45]. In the previous research, the optimal extraction conditions were determined to conceive high isoflavones daidzein and genistein yields from red clover aerial parts [22]. In this study, these conditions were used to study the effects of CDs on total phenolic compounds, flavonoids content, and antioxidant activity in *Trifolium pratense* L. flowers extracts.

Antioxidants, including phenolic compounds (e.g., flavonoids, phenolic acids), have diverse biological effects. The antioxidant extracts were evaluated in terms of their total phenols and total flavonoids. Being plant secondary metabolites, polyphenols are very important judging from the virtue of their antioxidant activities by chelating redox-active metal ions, inactivating lipid free radical chains and avoiding the hydroperoxide conversions into reactive oxyradicals [2].

The total phenolic contents in samples of the extracts, expressed as gallic acid equivalents, varied from 44.78 ± 0.92 (BW1) to 32.31 ± 0.63 (BW3) mg GA/g dw when the solvent was water (Figure 3), and 54.12 ± 0.46 (GE3) to 34.38 ± 0.63 (AE2) mg GA/g dw when the solvent was 50% (*v*/*v*) ethanol (Figure 4). Most of the samples that were prepared with excipients, despite the solvent used, had illustrated higher total content of phenolic compounds compared with control samples prepared without CDs ($p < 0.05$).

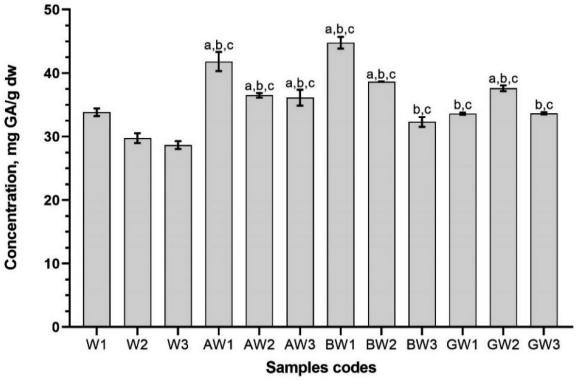

Figure 3. Total phenolic content in red clover flowers extracts with excipients using water as a solvent. Results are mean values (n = 3). [a] $p < 0.05$ vs. W1; [b] $p < 0.05$ vs. W2; [c] $p < 0.05$ vs. W3. Samples codes are provided in Table 1.

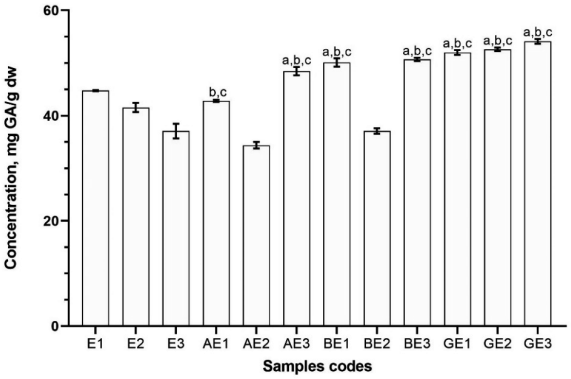

Figure 4. Total phenolic content in red clover extracts with 50% ethanol (v/v) as a solvent (n = 3). Results are mean values (n = 3). [a] $p < 0.05$ vs. E1; [b] $p < 0.05$ vs. E2; [c] $p < 0.05$ vs. E3. Samples codes are provided in Table 1.

Using water as a solvent the highest phenolic compound content was in sample BW1 (44.78 ± 0.92 mg GA/g dw), very similar amount was detected in sample AW1 (41.82 ± 1.51), that was prepared using same conditions as BW1 (Figure 3). The results showed that when using excipients in aqueous extractions, phenolic compound content increased significantly ($p < 0.05$) compared to that of control samples. Using α- or β-CDs, the most effective extraction method for phenolic compound extraction was reflux but combining it with ultrasound decreased phenolic compounds yield. Using γ-CD as an excipient and combining ultrasound processing with reflux was most effective, but the yield of total phenolic content obtained was lower than using α- or β-CDs (Figure 3). There were three samples (BW3; GW1; GW3) that were not significantly higher than control sample W1. This may have been since reflux alone was not sufficient with γ-CD, but the GW2 sample after sonication showed good results. Therefore, using sonication increases the phenolic compounds content, but by prolonging it from 10 to 30 min the amount of compounds decreases. The hypothesis can be made that using ultrasound for a longer period causes degradation of the phenolic compounds in the extract. This case was observed not only with γ- but also with β-CDs samples in water (BW3; GW3).

A lot of information can be found on the effect of solvent on the extraction of antioxidant phenolic compounds from different raw materials. The results of control samples (W1-3 and E1-3), that were prepared using the same conditions, but different solvents (water and 50% ethanol (v/v)) show that more phenolic compounds are recovered from red clover blossoms using ethanol as solvent. However, using water and CDs as excipients in the extraction increases the total amount of phenolic compounds. The use of CDs in an aqueous solution as extraction media is considered to be a green extraction since water is the main solvent and the existence of CD hydrophobic cavity boosts the extraction of phenolic compounds due to the formation of the inclusion complex [22].

Using 50% ethanol (v/v) as the solvent with excipients, it was determined that the highest content of phenolic compounds was found in the sample GE3 (54.11 ± 0.45 mg GA/g dw) (Figure 4). γ-CD was the most effective excipient used in ethanol compared to the results obtained from samples with α- and β-CDs. The best results for β-CD in ethanol were obtained using the same conditions as with water; the total amount of phenolic compounds was 50.11 ± 0,79 mg GA/g dw (BE1). Using ethanol with α-CD the best results was acquired employing ultrasound processing for 30 min and thermal hydrolysis—48.46 ± 0.80 mg GA/g dw (AE3).

All the results, except AE1, AE2, and BE2, were statistically significantly higher ($p < 0.05$) compared to the control samples. In ethanol, using α-CD and reflux or reflux combined with ultrasound processing for 10 min results decreased and had the opposite effect compared to α-CD samples prepared in water. However, increasing ultrasound-processing time to 30 min the total phenolic content increased. A similar case has been observed with β-CD as well. γ-CD in ethanol had the best extraction properties compared to other CDs.

Comparing total phenolic content results obtained from samples with different solvents (water and 50% ethanol (v/v)), it was observed that some of the aqueous test samples, prepared using an excipients-assisted method, showed higher total phenolic compounds content than ethanol samples with the same excipients. Aqueous samples obtained using α- or β-CDs excipients, sonicated for 30 min and then hydrolysed in high temperature, extracted more phenolic compounds than 50% ethanol. In addition, 6.17% more phenolic compounds were found in sample AW2 than in AE2 and in BW2—4.21% more than in BE2. The largest difference that was observed in this study was between samples GE3 and GW3—37.80%, but in this case ethanolic sample GE3 was higher than aqueous GW3.

The content of total flavonoids ranged from 21.74 ± 0.12 to 17.95 ± 0.08 mg RU/g dw using excipients and water as a solvent. Using excipients and ethanol as a solvent, the total flavonoids content ranged from 21.18 to 20.82 mg RU/g dw (Table 2). The total flavonoid content results were entirely synchronous with those of the total phenolic content. It was successfully shown that samples with a high level of phenolic content also contain flavonoids in a great amount. Plants rich in flavonoids can be a good source of antioxidants to help increase the body's overall antioxidant capacity and prevent it from lipid peroxidation [46].

Table 2. Flavonoid content (mg RU/g dw) of *Trifolium pratense* L. flowers.

Samples Codes *	Total Flavonoid Content (mg RU/g dw) **
W1	18.17 ± 0.67
W2	18.51 ± 0.67
W3	19.00 ± 0.39
AW1	20.35 ± 0.17 [a,b,c]
AW2	20.57 ± 0.18 [a,b,c]
AW3	20.57 ± 0.24 [a,b,c]
BW1	21.07 ± 0.20 [a,b,c]
BW2	21.74 ± 0.12 [a,b,c]
BW3	21.47 ± 0.23 [a,b,c]
GW1	19.56 ± 0.10 [a]
GW2	18.42 ± 0.38 [a]
GW3	17.95 ± 0.28
E1	20.51 ± 0.39
E2	21.16 ± 0.18
E3	19.81 ± 0.27
AE1	21.13 ± 0.13 [d,f]
AE2	21.18 ± 0.24 [d,f]
AE3	20.98 ± 0.28 [d,f]
BE1	21.11 ± 0.28 [d,f]
BE2	20.82 ± 0.16 [f]
BE3	20.84 ± 0.38 [f]
GE1	20.93 ± 0.23 [d,f]
GE2	20.91 ± 0.31 [d,f]
GE3	21.09 ± 0.19 [d,f]

* Samples codes are provided in Table 1. ** Results are mean values (n = 3); the ± values relative standard deviations SD. [a] $p < 0.05$ vs. W1; [b] $p < 0.05$ vs. W2; [c] $p < 0.05$ vs. W3; [d] $p < 0.05$ vs. E1; [f] $p < 0.05$ vs. E3. The control in water is compared only with samples prepared in water. Moreover, controls prepared in ethanol are compared only with ethanol samples.

Samples AW1-3 and BW1-3 were significantly higher ($p < 0.05$) compared to all control samples W1-3. GW1-2 was statistically significantly higher only compared to control sample W1. Highest total flavonoid content was found in aqueous samples BW1 and BW3, which were prepared using β-CD (21.74 $\pm$ 0.12 and 21.47 $\pm$ 0.23 mg RU/g dw, respectively). The lowest flavonoids yields were detected in samples prepared with γ-CD. Using ethanol as a solvent, the highest flavonoid content was found in AE2 (21.18 $\pm$ 0.24 mg RU/g dw). Similar quantities of flavonoids were observed in AE1 (21.13 $\pm$ 0.13 mg RU/g dw), BE1 (21.11 $\pm$ 0.28 mg RU/g dw), and GE3 (21.09 $\pm$ 0.19 mg RU/g dw) samples.

Biljana Kaurinovic et al. determined flavonoids content in red clover using water or ethanol was much lower comparing with the results obtained in this study [24]. However, as in our study, it was found that the use of water gives a similar yield of flavonoids as the use of ethanol.

Comparing samples in water and ethanol using CDs as excipients, reverse correlation was discovered between γ- and β-CDs depending on the solvent used.

Using γ-CD in water yielded lower amounts of flavonoids than under the same conditions in ethanol. The use of β-CD in water gave significantly better results than its use in ethanol. Thus, β-CD helps to extract flavonoids in water. Solvents impact to α-CD was hardly noticed, but more flavonoids were extracted in ethanol with this excipient. The different effects of CDs in complex formation in different solvents are shown in Figure 5.

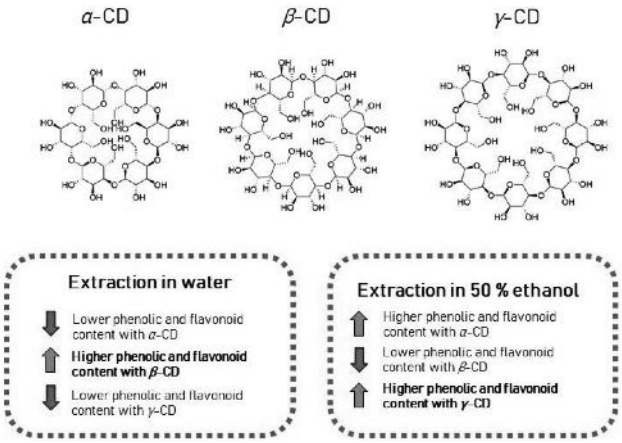

Figure 5. Comparison of the effect of excipients on the concentration of phenolic compounds using different solvents (water or 50% ethanol).

3.2. Antioxidant Activity

The antioxidant potential of red clover flowers extract with CDs as excipients in extraction media was investigated by using different antioxidant activity methods: the ABTS and DPPH methods as well as the ferric reducing antioxidant power (FRAP) test. It is strongly recommended to use a broad spectrum of methods, given that plant extracts contain many different classes and types of antioxidants. The phytochemical composition of extracts is affected by the extraction conditions, and, in our case excipients (CDs), which strongly influence the antioxidant effects [47–49].

3.2.1. DPPH Radical Scavenging Activity

In several studies, it has been reported that inclusion complex formation increases antioxidant capacity [49,50]. The presence of a guest molecule in a CD molecule results

in increased electron density, resulting in a greater likelihood that the guest molecule will release protons as radicals to quench the DPPH radical [50].

A DPPH scavenging activity was expressed as μg/g as trolox equivalent. DPPH discoloration, and a higher value signifies a higher antioxidant potential. In this research, significant differences ($p < 0.05$) between the antioxidant activities among the aqueous red clover samples with excipients and control samples were found (Figure 6). The DPPH radical scavenging in the tested aqueous samples varied from 9.75 ± 0.59 (GW1) to 11.52 ± 0.12 (BW1) μg TE/g dw.

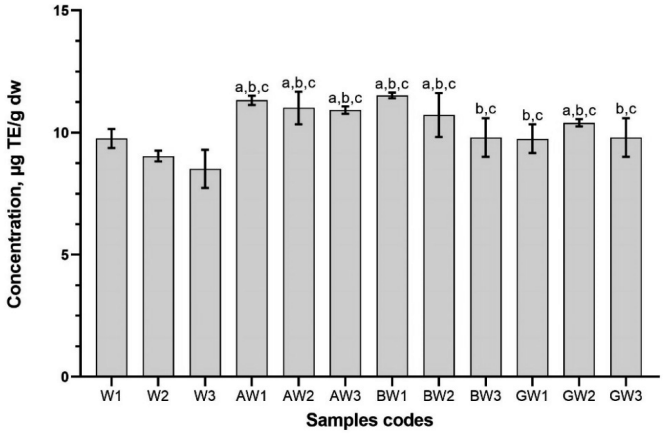

Figure 6. DPPH radical scavenging activity of aqueous red clover blossom extracts with excipients. Results are mean values (n = 3). [a] $p < 0.05$ vs. W1; [b] $p < 0.05$ vs. W2; [c] $p < 0.05$ vs. W3. Samples codes are provided in Table 1.

Only three samples (BW3, GW1 and GW3) did not have statistically higher antioxidant activity than control W1. The samples that contained highest total phenolic content had the strongest antioxidant activity using DPPH method. Therefore, CDs increase the quantity and/or concentration of phenolic compounds, which have strong antioxidant properties.

By using the DPPH assay on extracts prepared with 50% ethanol (v/v), CDs demonstrated the greatest radical scavenging activity—12.55 ± 0.59 μg TE/g dw in the sample GE2 and 12.45 ± 0.78 μg TE/g dw in the sample GE3 (Figure 7). These results are in good agreement with the phenolic and flavonoid contents in Figure 4 and Table 2. Using α-CD in the ethanolic red clover flowers extract antioxidant activity was weaker compared to extracts where β- or γ-CDs were used. Samples AE1 and AE2 were only significantly higher ($p < 0.05$) compared to the E3 control sample. Prolonging ultrasound from 10–30 min increased antioxidant activity in the AE3 sample that was statistically higher compered to E2 and E3 control samples.

Previous studies that used concentrated ethanol as a solvent reported that red clover extract in DPPH assay gave a value at 3.7 μg TE/g [51]. The results obtained in this study were significantly higher even in controls without excipients, compared to reported results in other articles, indicating that water or ethanol are suitable solvents for the extraction of compounds with strong antioxidant effects from red clover aerial parts.

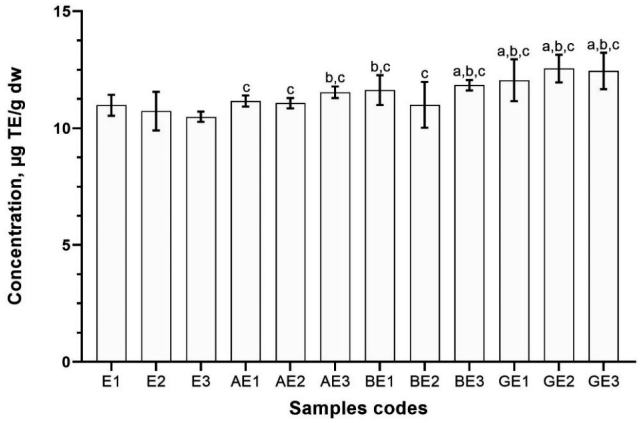

Figure 7. DPPH radical scavenging activity of red clover blossom extracts with 50% (*v*/*v*) of ethanol as a solvent with excipients. Results are mean values (n=3). [a] $p < 0.05$ vs. E1; [b] $p < 0.05$ vs. E2; [c] $p < 0.05$ vs. E3.

3.2.2. ABTS Radical Scavenging Activity

The ABTS radical cation decolorization test is one of the most widely used methods to evaluate antioxidant activity. Color reduction shows the decrease in the ABTS radical [2].

In this study, all the test samples prepared with CDs were significantly higher compared with control samples. The highest antioxidant activity (in samples where the solvent was water) after the reaction with ABTS assay was observed in sample BW1 (414.53 ± 2.09 µg TE/g) and GW3 (415.01 ± 1.45 µg TE/g) (Figure 8). From all the test samples (excluding control samples), the lowest antioxidant activity was observed in sample GW1 (386.768 ± 1.25 µg TE/g) (Figure 8). GW1 was the only sample that was statistically significantly smaller compared with all the test samples prepared with excipients. The ABTS radical scavenging capability using excipients of the test samples can be ranked, according to their average means, as follows: BW > AW > GW.

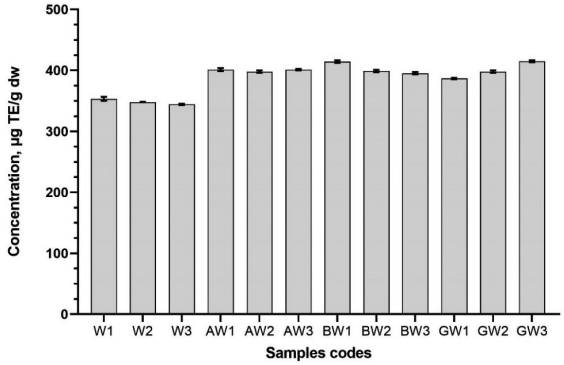

Figure 8. ABTS radical scavenging activity of aqueous red clover blossom extracts with excipients. Results are mean values (n = 3). All the test samples were statistically significant to controls, therefore, comparisons are not marked with letters in the graph. Samples codes are provided in Table 1.

The highest antioxidant activity (in samples where the solvent was 50% ethanol (v/v)) was detected in the sample GE1 433.122 ± 2.61 μg TE/g (Figure 9). Similar activity was noticed in the sample GE3 429.74 ± 2.54 μg TE/g). Lowest antioxidant activity was observed in sample AE2 (397.63 ± 0.72 μg TE/g) (Figure 9). All the samples prepared with ethanol, as in the case of aqueous extracts, were significantly higher compared to control samples ($p < 0.05$). The ABTS radical scavenging capability using excipients and 50% ethanol (v/v) as a solvent of the test samples can be ranked, according to their average means, as follows: GE > BE > AE.

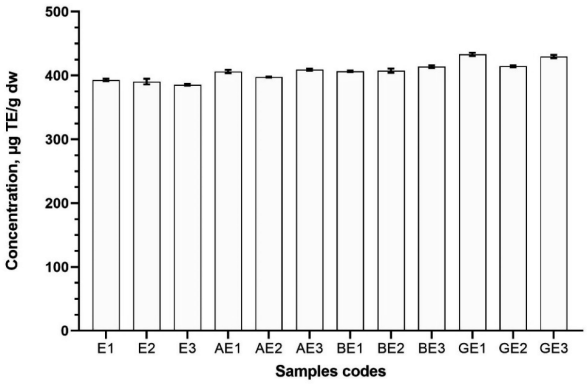

Figure 9. ABTS radical scavenging activity of ethanolic red clover blossom extracts with excipients. Results are mean values (n = 3). All the test samples were statistically significant to controls, therefore, comparisons are not marked with letters in the graph. Samples codes are provided in Table 1.

A trend between test samples was observed comparing the results obtained with the DPPH and ABTS methods. Samples that were prepared in water had higher radical scavenging activity when they were extracted using α- or β-CD. The results gained using these two excipients were similar in both antioxidant methods. Samples prepared using ethanol and CDs by both ABTS and DPPH methods showed identical results. The highest antioxidant activity was found in samples prepared with γ-CD and the lowest in extracts that contained α-CD.

3.2.3. Reducing Power Activity

The reducing capacity of a compound can be a significant indicator of its potential antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [52].

Total antioxidant activity, measured by the FRAP method, varied from 144.05 ± 0.73 (GE2) to 193.57 ± 1.94 (GE1) mg FE(II)/g dw in the test samples with excipients and 50% ethanol (v/v) as a solvent (Table 3). In aqueous samples extracted using different CDs as excipients, the highest antioxidant activity was observed in GW3 (143.37 ± 2.20 mg FE(II)/g dw) and the lowest—AW2 (106.45 ± 2.54 mg FE(II)/g dw). Examining the results obtained using FRAP assay, it was detected that only two samples—GE1 and AW2—had significant lower values compared with controls (Table 3).

Table 3. Ferric Reducing Antioxidant Power (FRAP) values in samples prepared with excipients.

Samples Codes *	FRAP, mg FE(II)/g dw **
E1	152.93 ± 1.93
E1	144.09 ± 1.47
E3	141.05 ± 1.94
AE1	167.78 ± 3.19 ^{d,e,f}
AE2	161.60 ± 1.94 ^{d,e,f}
AE3	161.35 ± 1.94 ^{d,e,f}
BE1	188.84 ± 5.13 ^{d,e,f}
BE2	162.07 ± 2.93 ^{d,e,f}
BE3	191.92 ± 0.73 ^{d,e,f}
GE1	193.57 ± 1.94 ^{d,e,f}
GE2	144.05 ± 0.73 ^{f}
GE3	179.62 ± 1.94 ^{d,e,f}
W1	109.92 ± 2.71
W2	106.58 ± 1.12
W3	102.60 ± 1.59
AW1	120.79 ± 1.27 ^{a,b,c}
AW2	106.45 ± 2.54 ^{c}
AW3	114.36 ± 0.73 ^{a,b,c}
BW1	142.10 ± 2.54 ^{a,b,c}
BW2	121.38 ± 1.47 ^{a,b,c}
BW3	116.43 ± 2.93 ^{a,b,c}
GW1	112.71 ± 0.73 ^{a,b,c}
GW2	136.44 ± 0.41 ^{a,b,c}
GW3	143.37 ± 2.20 ^{a,b,c}

* Samples codes are provided in Table 1. ** Results are mean values (n = 3); the ± values relative standard deviations SD. ^{a} $p < 0.05$ vs. W1; ^{b} $p < 0.05$ vs. W2; ^{c} $p < 0.05$ vs. W3; ^{d} $p < 0.05$ vs. E1; ^{e} $p < 0.05$ vs. E2; ^{f} $p < 0.05$ vs. E3. The control in water is compared only with samples prepared in water. Moreover, controls prepared in ethanol are compared only with ethanol samples.

In the literature, it was reported that red clover flowers have a low (0.0251 mg FE(II)/g dw) or even undetected reducing antioxidant power [53,54]. The test samples results obtained in this study were significantly higher than compared to controls. This means that it is very important to choose the right extraction conditions and solvent. However, the presence of phenolic compounds and their distribution in the red clover vary due to many factors—origin, cultivation influences, weather conditions, plant parts, growing stages, harvesting season, etc. [55]. Therefore, it would not be entirely appropriate to compare the results of different studies.

Some of the samples exhibited higher capacity in reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) than to scavenging free radicals. The use of β- or γ-CDs greatly increased the reducing power in both water and ethanol samples. Although the use of γ-CDs in ethanolic extracts gave a higher radical scavenging effect, aqueous extracts prepared with γ-CDs showed a higher reducing capacity. A similar trend was observed with β-CD. However, extracts prepared using β-CD and water showed stronger radical scavenging, but ethanolic samples extracted with β-CD had better reducing power. Even though aqueous extracts prepared using α-CD had a high radical scavenging activity, in both ethanol and water they had lower reducing properties compared to other test samples prepared using β- or γ-CDs. Once again, these results obtained with excipients could prove that using different CDs or/and solvent in the extraction media different compounds can be extracted, and the phenolic profile of the sample prepared with α-CD can be different from the samples prepared with β- or γ-CDs.

Comparing all antioxidant activity results obtained from three methods (FRAP, ABTS, DPPH), it was found that the CDs use increases antioxidant activity. This can be attributed not only to the higher yield of polyphenols and their improved diversity in the CD extracts, but also from the protection of the polyphenols from rapid oxidation by free radicals

(during all treatment steps) due to CD encapsulation [56]. In this study, CD whose extracts were least affected by the solvent used (water or ethanol) was β-CD.

3.2.4. Post-Column ABTS Antioxidant Assay and RP-LC/PDA/MS Qualitative Analysis

The post-column detection of the reduction of the ABTS radical in relation to antioxidant content is reflected by the negative chromatogram at 650 nm. HPLC separation is coupled with rapid identification of antioxidative active compounds. The major advantages of post-column reaction methods are that the antioxidant activity of an individual compound can be measured and its contribution to the total activity of a complex mixture can be estimated [29].

The highest antioxidant activity using the post-column ABTS method was determined in the ethanolic GE1 sample—30.16 $\pm$ 1.21 mg TE/g and the lowest in the BE1 10.73 $\pm$ 0.43 mg TE/g ethanolic sample (Table 4). All the samples had statistically higher values compared with controls.

Table 4. ABTS radical scavenging activity of red clover blossom extracts with 50% (v/v) of ethanol or water as a solvent with excipients.

Samples Codes *	ABTS Radical Scavenging, mg TE/g dw **
AW1	11.93 $\pm$ 0.48 ^{b}
AE1	32.68 $\pm$ 1.31 ^{a}
BW1	20.68 $\pm$ 0.83 ^{b}
BE1	10.73 $\pm$ 0.43 ^{a}
GW1	12.67 $\pm$ 0.51 ^{b}
GE1	30.16 $\pm$ 1.21 ^{a}
W1	8.35 $\pm$ 0.33
E1	9.38 $\pm$ 0.38

* Samples codes are provided in Table 1. ** ^{a} $p < 0.05$ vs. E1; ^{b} $p < 0.05$ vs. W1. The control in water is compared only with samples prepared in water. Moreover, controls prepared in ethanol are compared only with ethanol samples.

Results obtained from the post-column method show that using water the highest radical scavenging activity was in the BW1 sample (20.68 $\pm$ 0.83 mg TE/g) extracted using β-CD (Table 4). Much smaller results were obtained from the AW1 and GW1 samples. Ethanolic samples AE1 and GE1 had similar antioxidant properties, but BE1 antioxidant activity was almost three times lower. Therefore, samples obtained from aqueous β-CD assisted extraction had higher antioxidant activity than the same sample prepared in ethanol. Similar reverse correlation was observed in samples prepared with α- and γ-CDs.

Figure 10, part A, shows an example chromatogram of sample AW1. Antioxidant activity signal strength is expressed as peaks of negative active compounds. It shows that the extract is rich in many different compounds, but not all of them have antioxidant activity.

In chromatogram B (Figure 10), it is shown the results of concentrated control sample W1. It is widely reported in the literature [4,57,58] that red clover is rich in isoflavones, which have strong antioxidant properties. Therefore, it was decided to determine the antioxidant activity of isoflavones aglycones (daidzein (9) and genistein (10)) and glycosides (daidzin (7) and genistin (8)) in the extract using the ABTS post-column method.

In the study, we used known concentrations of isoflavones standards, and it was determined that isoflavones tested in the post-column method did not show antioxidant activity. The same isoflavones standard samples were investigated using the ABTS spectrophotometric method and the result was 134.64 $\pm$ 5.87 µg TE/g. This means that isoflavones need longer incubation time in the ABTS assay to reach equilibrium. It was reported in the literature that whenusing an ABTS assay not all the compounds react quickly, so longer incubation is needed [59,60]. Using spectrophotometric method incubation time was 30 min, but in column it was much shorter, which resulted in the absence of antioxidant activity.

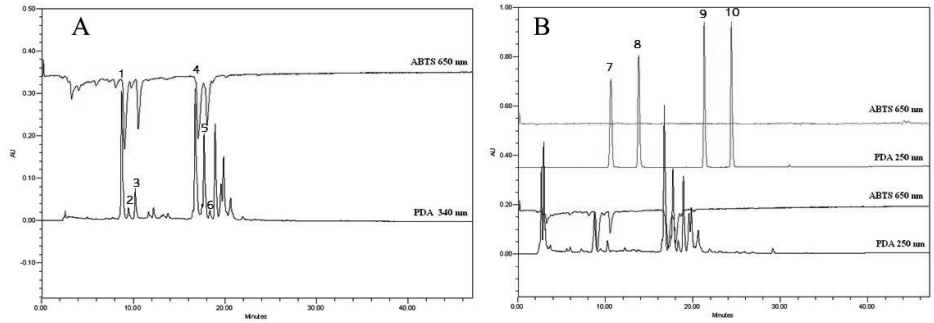

Figure 10. ABTS post-column chromatograms: (**A**) example profile of AW1 sample; (**B**) concentrated control sample profile with main isoflavones (7—daidzin; 8—genistin; 9—daidzein; 10—genistein) standards.

Using HPLC-DAD, only two compounds were identified in the red clover profile—caffeic acid (2) and hyperoside (4). To determine all the compounds that gave the highest antioxidant activity to the red clover extract (determined by the post-column method), it was decided to use LC-MS. It was determined that peak **1** (m/z 295.0 $[M\text{-}H]^-$) was caffeoylmalic acid, peak **3** (m/z 358.0 $[M\text{-}H]^-$) cis-clovamide, peak **5** (m/z 505.1 $[M\text{-}H]^-$) quercetin-O-hexoside-acetate, and peak **6** (m/z 447.0 $[M\text{-}H]^-$) kaempferol-O-hexoside, by comparing with the data found in the literature and databases [61].

According to ABTS post-column chromatogram (Figure 10A), caffeoylmalic acid gives the highest antioxidant respond compared to other compounds in the extract. Moreover, a quite high response was detected from hyperoside as well.

3.3. Correlation with Antioxidant Activities and Phytochemical Contents

In this study, four methods used to quantify the antioxidant activity of the samples prepared with CDs-assisted extraction. Furthermore, all the methods presented a significant correlation between them, with high values in the Spearman correlation coefficient. Numerous studies have also reported this correlation [43,55].

In the aqueous samples extracted, α-CD significant positive correlations were found between total phenolic content and DPPH and FRAP assays. Similar positive correlations were observed between total flavonoid content and DPPH and FRAP assays. ABTS assay correlations with total phenolic and flavonoid content were moderate (Table 5).

Table 5. Correlation between antioxidant activity and total phenolic and total flavonoid content of the red clover aerial extracts prepared with α-CD.

Assays	Correlations of Aqueous Extracts		Correlations of Ethanolic Extracts	
	Total Phenolic	**Total Flavonoid**	**Total Phenolic**	**Total Flavonoid**
DPPH radical scavenging activity	0.989 **	0.979 **	0.897 **	0.999 **
ABTS radical scavenging activity	0.447	0.500	0.986 **	0.830 **
Ferric Reducing Antioxidant Power	0.800 **	0.835 **	0.079	0.338

** Correlation is significant at the 0.01 level (2-tailed).

In ethanolic extracts, total phenolic and flavonoid content had significant positive correlations with DPPH and ABTS assays; in these samples, FRAP assay correlation was very weak (Table 5). The antioxidant ability of polyphenols, including mainly flavonoids,

seemed to be an important factor dictating the free radical scavenging capacity of red clover flower ethanolic extracts prepared with α-CD.

The most statistically significant correlations between the antioxidant assay and phenolic and flavonoids content were observed in aqueous test samples prepared with β-CD (Table 6). Even though flavonoid content presented nonsignificant moderate correlation with the DPPH assay, it demonstrated strong correlations with ABTS and FRAP assays.

Table 6. Correlation between antioxidant activity and total phenolic and total flavonoid content of the red clover aerial extracts prepared with β-CD.

Assays	Correlations of Aqueous Extracts		Correlations of Ethanolic Extracts	
	Total Phenolic	**Total Flavonoid**	**Total Phenolic**	**Total Flavonoid**
DPPH radical scavenging activity	0.999 **	0.558	0.979 **	0.344
ABTS radical scavenging activity	0.938 **	0.832 **	0.424	0.545
Ferric Reducing Antioxidant Power	0.939 **	0.830 **	0.998 **	0.480

** Correlation is significant at the 0.01 level (2-tailed).

In ethanolic extracts, correlations between flavonoid content and antioxidant assays were from weak (DPPH) to moderate (ABTS and FRAP). However, in ethanolic samples, the total phenolic content statistically significant correlated with DPPH and FRAP assays (Table 6).

Chelating power in aqueous samples prepared with γ-CD had a significant correlation between flavonoids, but nonsignificant with total phenolic content. Total phenolic content pointed to a significant correlation with DPPH (Table 7).

Table 7. Correlation between antioxidant activity and total phenolic and total flavonoid content of the red clover aerial extracts prepared with γ-CD.

Assays	Correlations of Aqueous Extracts		Correlations of Ethanolic Extracts	
	Total Phenolic	**Total Flavonoid**	**Total Phenolic**	**Total Flavonoid**
DPPH radical scavenging activity	0.999 **	0.293	0.836 **	0.570
ABTS radical scavenging activity	0.307	0.936 **	0.083	0.450
Ferric Reducing Antioxidant Power	0.316	0.998 **	0.024	0.353

** Correlation is significant at the 0.01 level (2-tailed).

In ethanolic extracts, the only statistically significant correlation was between total phenolic content and the DPPH assay.

All the samples prepared with excipients demonstrated a strong relationship between total phenolic content and DPPH assay. FRAP assay in all the aqueous samples (except samples prepared using γ-CD) had a significant correlation with total phenolic and total flavonoid contents.

4. Conclusions

The observed levels of phenolics, total flavonoid content and the antioxidant properties of red clover flowers extract indicate that it can be used as a natural source of biologically active components in the human nutrition, as well as in pharmaceutical industry.

Using α-, β-, and γ-CDs during aqueous or ethanolic red clover extraction enhanced antioxidant activity in samples. Either CD extracted different compounds in extraction media that had different activities. The total phenolic contents in samples of the extracts

varied from 54.12 ± 0.46 (GE3) to 34.38 ± 0.63 (AE2) mg GA/g dw when the solvent was 50% (*v*/*v*) ethanol and 44.78 ± 0.92 (BW1) to 32.31 ± 0.63 (BW3) mg GA/g dw when the solvent was water. All the samples that were prepared with excipients, despite the solvent used, had illustrated higher total content of phenolic compounds compared to control samples prepared without CDs. The total flavonoid content results were entirely synchronous with those of the total phenolic. It was successfully shown that samples with a high level of phenolic content also contain flavonoids in great amount.

Samples that were prepared in water had a higher radical scavenging activity than when they were extracted using *α*- or *β*-CD. The results gained using these two excipients were similar in both antioxidant methods. Samples prepared using ethanol and CDs by both ABTS and DPPH methods showed identical results. The highest antioxidant activity was found in samples prepared with *γ*-CD and the lowest in extracts that contained *α*-CD. Some of the samples exhibited higher capacity in reducing ferric ion to ferrous ion than to scavenging free radicals: samples with *β*- or *γ*-CDs greatly increased reducing power in both water and ethanol samples. Although the use of *γ*-CDs in ethanolic extracts gave a higher radical scavenging effect, aqueous extracts prepared with *γ*-CDs showed a higher reducing capacity. Using the post-column method, it is important to determine if active compounds react slowly with the ABTS assay, or quickly to optimize the method and obtain quality results. The most statistically significant correlations between the antioxidant assay and phenolic and flavonoids content were observed in aqueous test samples prepared with *β*-CD. Additionally, samples prepared using the *β*-CD assisted method in water showed the highest phenolic and flavonoid content as well as antioxidant activity. However, using *γ*-CD increased antioxidant activity and phenolic compounds in 50% ethanol samples. Thus, different CDs increase the amount of extracted phenolic compounds, which have strong antioxidant properties, in the desired safe solvent, making CDs a great and safe for food tool for obtaining rich extracts that can be used in the pharmaceutical industry for nutraceuticals production.

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Paper 4

Title: Application of Antiviral, Antioxidant and Antibacterial *Glycyrrhiza glabra* L., *Trifolium pratense* L. Extracts and *Myristica fragrans* Houtt. Essential Oil in Microcapsules

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Application of Antiviral, Antioxidant and Antibacterial *Glycyrrhiza glabra* L., *Trifolium pratense* L. Extracts and *Myristica fragrans* Houtt. Essential Oil in Microcapsules

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Abstract: Viruses and bacteria can disrupt normal human functions; therefore, ways to use the beneficial properties of plants to promote health are constantly being researched. Plant materials that accumulate biologically active compounds can be used to create a new pharmaceutical form. This study aimed to investigate the biological activity of selected plant extracts and essential oil and to produce microcapsules. The main compounds in extracts and essential oil were determined using chromatographic methods, antioxidant activity was evaluated spectrophotometrically, antimicrobial activity was assessed by monitoring the growth of nine pathogens, and the antiviral effect on infected bird cells with coronavirus was evaluated. *Trifolium pratense* L. extract had the highest antioxidant (26.27 ± 0.31 and 638.55 ± 9.14 µg TE/g dw by the DPPH and ABTS methods, respectively) and antiviral activity (56 times decreased titre of virus). Liquorice extract expressed antibacterial activity against Gram-positive pathogens and the highest antioxidant activity using the FRAP method (675.71 ± 4.61 mg FS/g dw). Emulsion stability depended on excipients and their amount. Microcapsules with extracts and essential oil were 1.87 mm in diameter, and their diameter after swelling was increased more than two times in intestinal media, while less than 0.5 times in gastric media.

Keywords: plant extract; essential oil; microcapsules; extrusion; antioxidant activity; antiviral activity; antimicrobial activity; *Glycyrrhiza glabra* L.; *Trifolium pratense* L.; *Myristica fragrans* Houtt. essential oil

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1. Introduction

Plant extracts and essential oils are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances, pharmaceutics, and cosmetics. Many plants have been recognized to have medicinal properties and beneficial impacts on health, e.g., antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects, and anticarcinogenic potential [1].

Phenolic compounds are one of the plants' most evident secondary metabolites, and their distribution is demonstrated throughout the entire metabolic process. The biological activity of plants is primarily attributed to these compounds. Polyphenols contain numerous varieties of compounds: simple flavonoids, phenolic acids, complex flavonoids, and coloured anthocyanins [2–4].

Botanical substances obtained from plants, such as *Trifolium pratense* L. flowers, *Glycyrrhiza glabra* L. roots, and *Myristica fragrans* Houtt. essential oil, which are generally

perceived as safe because of their natural origin, can be used for the prevention or treatment of menopausal health issues, various degenerative disorders, and possess many pharmacological activities, such as antiviral, antimicrobial, anti-inflammatory, antitumor, and other activities [5–11]. These plant substances could act synergistically and increase positive effects.

Trifolium pratense L. flower extracts are rich in isoflavones that possess estrogenic activity. However, it also has antioxidant and antibacterial activities, which may result from the presence of other flavonoids and phenolic compounds such as phenolic acids, clovamides, and saponins [12]. It is possible to obtain a preparation with strong antibacterial and antioxidant properties using *Trifolium pratense* L. extract with other plants.

Glycyrrhizin, a triterpenoid saponin glycoside, is considered the main bioactive component (4–10%) of *Glycyrrhiza glabra* L. roots (*Glycyrrhiza glabra* L.). It has been shown to possess glucocorticoid-like pharmacological effects and anti-inflammatory, antiviral, anti-tumour, and hepatoprotective activities [13]. A liquorice biological effect could be used in drugs and food supplements formulation, also it could be used in cosmetic products or in the food industry as a food and flavour additive [14]. Like *Trifolium pratense* L., the biological properties of this plant are due to phenolic compounds. Nevertheless, biological properties in plants can be provided not only by phenolic compounds in extracts but also by active compounds found in essential oils.

Essential oils contain important classes of compounds such as monoterpenes (C10 hydrocarbons based on two isoprene units), phenylpropanoids (C6 aromatic compounds with C3 side chains), sesquiterpenes (C15 hydrocarbons based on three isoprene units), diterpenes (C20), triterpenes (C30) and their oxygenated derivatives, and phenolic compounds (such as thymol and carvacrol) [15]. Several scientific researchers report that *Myristica fragrans* Houtt. essential oil has potential antioxidant, antimicrobial, anti-inflammatory, antiulcer, anticancer, aphrodisiac, and other activities caused by monoterpenes and phenolic compounds [10,16,17]. Not only can essential oils be used because of their biological properties but also because of the pleasant smell they can provide for the final product [18].

In order to protect volatile compounds found in essential oil to produce the final pharmaceutical, microencapsulation can be applied. Microencapsulation is a method that protects unstable compounds from environmental exposure. The excipients, which formulate a shell, could protect core substances.

Resistance to antibiotics has been on the rise in recent years. This is largely due to bacteria maintaining a genetic capacity to transmit and gain resistance against therapeutic compounds. In addition, many new multiresistant strains have arisen, which poses a serious threat both to individuals who are immunosuppressed as well as those who receive general hospital care [19,20]. Antibacterial agents derived from plants are a promising option for use in the treatment of infections. Plants lack some of the negative secondary effects and are often available and affordable.

Plant extracts that possess antiviral activity can be a valuable addition to an antiviral therapy. Antiviral drugs should target exactly the virus, but since viral particles live inside the host and gets integrated into the host cell, application of antiviral therapy through drugs is very difficult. Antiviral drugs may also kill the host cell [21]. Therefore, by using plant extracts that possess antiviral activity, the effective additional form or nutraceutical-like microcapsules can be created.

Sodium alginate is one of the most popular excipients used in extrusion [22–26]. It is a chemically stable polysaccharide and can form strong gel barriers from water [27,28]. Extrusion is a simple method that does not require expensive devices; for example, a medical syringe, syringe pump, and a crosslinker solution could be used to form particles [29,30]. Microcapsules prepared by extrusion could be used in wet and dry forms depending on the properties that are needed. This method's particle size usually ranges from 0.25 to 2.5 mm [31,32]. The size depends on the shell material, crosslinker solution and concentration, and the amount of excipient(s) [33].

Microcapsules could be used to create and supplement products for pharmacy, cosmetics, food, and other industries. Moreover, microcapsules increase the shelf-life of labile compounds [34,35]. This form was selected because it could be used as a final product or incorporated into many pharmaceutical forms in order to obtain different release effects. Using plant extracts and essential oils made by microencapsulation is an excellent opportunity to conduct research, evaluate the properties of active compounds, and use them in developing new products.

In this research, three plants were chosen: *Trifolium pratense* L. flower, *Glycyrrhiza glabra* L. roots, and *Myristica fragrans* Houtt seeds. Their extracts and essential oil were used to formulate an emulsion and microcapsules by extrusion, and then their physical properties were evaluated. The main active compounds were determined and antioxidant, antimicrobial, and antiviral effects were assessed from *Trifolium pratense* L. and *Glycyrrhiza glabra* L. extracts and *Myristica fragrans* Houtt. essential oil.

This research could help create microcapsules with high antibacterial and antiviral effects and could be incorporated into a pharmaceutical form.

2. Materials and Methods

2.1. Plant Material and Reagents

Trifolium pratense L. samples were collected in *Trifolium pratense* L. fields in Laičiai, Kupiškis district, Lithuania (latitude 55°53024.2″ N; longitude 25°19036.0″ E). The collections of *Trifolium pratense* L. flower buds were made on the 31 July 2021. *Glycyrrhiza glabra* L. roots (the country of origin is China) were bought from LSMU pharmacy (Kaunas, Lithuania). Myristica fragrans seeds' country of origin was Grenada (supplier Spaisvilė, Pašaltuonys, Lithuania). Voucher specimens (*Trifolium pratense* L.—J21731; *Myristica fragrans* Houtt.—I18922; and Glycyrrhiza glabra L.—K20911) were placed for storage at the Herbarium of the Department of Drug Technology and Social Pharmacy, Lithuanian University of Health Sciences, Lithuania.

In this experiment, purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionised water was prepared with Millipore, SimPak 1 (Merck, Darmstadt, Germany). The following reagents were used: standards genistein, daidzein, and glycyrrhizin acid (Sigma Aldrich, Steinheim, Germany). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and β-CDs purchased from Sigma Aldrich (Hamburg, Germany); aluminium chloride, hexaethylenetetraamine, dimethyl sulfoxide (DMSO), acetic acid, and Sabouraud dextrose agar (dehydrated) obtained from Sigma-Aldrich (Buchs, Switzerland); potassium persulfate obtained from Alfa Aesar (Karlsruhe, Germany); ethanol (96%) obtained from Vilniaus Degtinė (Vilniaus, Lithuania); Folin–Ciocalteu's phenol reagent (Merck, Darmstadt, Germany); monosodium phosphate, ferrous sulfate heptahydrate, saline phosphate buffer, and hydrogen peroxide obtained from Sigma Aldrich (Schnelldorf, Germany); disodium hydrogen phosphate obtained from Merck (Darmstadt, Germany); Mueller–Hinton Agar obtained from BBL (Baltimore, MD, USA); foetal bovine serum obtained from FBS (Gibco, TX, USA); and as the shell material, alginic acid sodium salt from brown algae obtained from Sigma-Aldrich (Shanghai, China) was used. Calcium chloride (Farmalabor, Pozzillo, Italy) salt was used to formulate microcapsules as a crosslinker, which linked sodium alginate chains and formed a solid gel.

2.2. Extracts and Essential Oil Preparation

2.2.1. Preparation of *Glycyrrhiza glabra* L. Extract

Glycyrrhiza glabra L. extract was prepared by using dried and milled plant root powder. The extraction was done using 1 g $\pm$ 0.001 g of powder in 10 mL of purified water. First, the plant material was macerated in water for 4 h. After that, ultrasound-assisted extraction was performed using an ultrasound bath (frequency 38 kHz) (Grant Instruments™, XUB12 Digital, Cambridge, England). The extraction time was 30 min; the processing temperature was 40 $\pm$ 2 °C. After extraction, samples were centrifuged for 10 min at 3382 g (5500 rpm)

followed by the decantation of the supernatant. The extract was filtered through the paper filter and used in the following research.

2.2.2. Preparation of *Trifolium pratense* L. Extract

Before use, clover flowers were ground to a fine powder using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Grinding was performed at 4025 g (6000 rpm) using a 0.5 mm trapezoid hole sieve. The extraction was performed as in the previous study using excipient β-cyclodextrin (β-CD) [36].

Ultrasound-assisted extraction was performed using an ultrasound bath (38 kHz) (Grant Instruments™ XUB12 Digital, Cambridge, England). A sample of 0.3 ± 0.001 g of dried and milled flower heads was macerated in 10 mL of 50% ethanol. Additionally, 0.1 ± 0.001 g of β-CDs was added to the extraction mixture samples (10 mL) to prepare samples with CD concentrations of 1% (*w*/*v*). Ultrasound extraction time was 10 min, and the processing temperature was 40 ± 2 °C. After ultrasound processing, the samples were put in a round bottom flask and refluxed in a sand bath at 100 °C for 1 h. After that, the mixture was left to cool at a temperature of 25 ± 2 °C. The samples were centrifuged for 10 min at 3382 g (5500 rpm), followed by the decantation of the supernatant. The extract was filtered through the paper filter and used in the following research.

2.2.3. Essential Oil Preparation

Myristica fragrans Houtt. essential oil was prepared by modified hydrodistillation. *Myristica fragrans* Houtt. seed powder was mixed with magnesium aluminometasilicate, and then distilled water was added to the mixture (5:1:300). Hydrodistillation with Clevenger-type apparatus (manufacturer of apparatus Winzer Corporation, bought from Carl Roth, Drogen, Germany) and heating mantle (Pilz®, Winkler, Germany) was carried out for 2 h. A colourless essential oil was obtained and collected in an airtight bottle. The essential oil was stored in a refrigerator at 4 °C until needed.

2.3. Chromatografical Analysis of Extracts

2.3.1. HPLC–PDA Conditions

High-performance liquid chromatography with diode array detectors (HPLC-PDA) was performed as described in previous work by Kazlauskaite et al. [36]. It was carried out using the Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan), equipped with an SPD-M20A diode array detector (DAD).

For the determination of polyphenols in plant extracts, an ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase consisted of solvent A (acetic acid/methanol/deionised water) (1:10:89 *v*/*v*/*v*) and solvent B (acetic acid/methanol) (1:99 *v*/*v*). The linear gradient elution profile was as follows: 80% A/20% B at 0 min, 30% A/70% B at 30 min, and 90% A/10% B at 39 to 40 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. Absorption was measured at 260 nm. Quantification of compounds was performed using reference standards.

2.3.2. GC-MS Conditions

Gas chromatography-mass spectrometry (GC-MS) analysis was performed as described in Matulyte et al. [37]. GC-MS was carried out using the GCMS-QP2010 system (Shimadzu, Tokyo, Japan). The column used in the process was RTX-5MS, 30 m × 0.25 mm i.d. ×0.25 µL film thickness. The flow rate of helium (99.999%, AGA Lithuania) carrier gas was set at 1.23 mL/min.

A total of 20 µL of the sample (extract or essential oil) was diluted to 1 mL with hexane (≥99%, Sigma-Aldrich, Germany). The oven temperature was maintained at 40 °C for 2 min after injection and then programmed at 3 °C/min to 210 °C, at which the column was maintained for 10 min. The split ratio was 1:10. The mass detector electron ionisation

was 70 eV. Identification of volatile compounds was carried out using mass spectra library search (NIST 14).

2.4. Total Phenolic and Flavonoid Content and Antioxidant Activity

Total phenolic and flavonoid content and antioxidant activity methods ABTS, DPPH, and FRAP were carried out as described in Kazlauskaite et al. [36].

2.4.1. Determination of Total Phenolic Content

A total of 0.5 mL of extract was mixed with 2.5 mL of 1:9 diluted Folin–Ciocalteu's phenol reagent and 2.0 mL of 7% (*w*/*v*) sodium carbonate. Absorbance was measured at 765 nm after 1 h using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was prepared using gallic acid (0–0.1 mg/g; y = 11.108; R^2 = 0.9981). The results were expressed as gallic acid equivalent per gram dry weight (mg GA/g dw).

2.4.2. Determination of Total Flavonoid Content

A total of 0.1 mL of extract was added to 1.0 mL 96% (*v*/*v*) of ethanol, 0.05 mL of 33% acetic acid, 0.15 mL 10% aluminium chloride, and 2.0 mL 5% hexamethylenetetramine solutions. Spectrophotometric analysis was performed after 30 min at 475 nm wavelength using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The results were expressed as rutin equivalent per gram dry weight (RE/g dw) and calculated by the formula TFC = C · V_e · F/M, where TFC is the total flavonoid content (mg RE/g dw); C is the concentration of the used standard (mg/L); V_e is the volume of the used solvent (L); F is the dilution coefficient of the sample; and M is the mass of the sample (g). The calibration curve was obtained with a rutin (0–0.5 mg/g; y = 5.0867; R^2 = 0.9985). The results were expressed as rutin equivalent per gram dry weight (RU/g dw).

2.4.3. ABTS Radical Scavenging Activity Assay

Aqueous ABTS solution (7 mM) was mixed with potassium persulfate (2.45 mM) solution and stored in the dark for 12–16 h to produce a dark-coloured solution containing ABTS radical cation. ABTS working solution was prepared using ABTS radical solution and diluting it with water. Working solution absorbance should be regulated to 0.90 (±0.02) at 734 nm using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan).

Free radical scavenging activity was evaluated by mixing 2.0 mL of ABTS working standard with 200 µL of the test sample in the cuvette. The samples were incubated in the dark at room temperature for 30 min. The calibration curve was obtained with a Trolox (0–0.5 mg/g; y = 0.0001728x; R^2 = 0.9832). The results were expressed as Trolox equivalent per gram dry weight (TE/g dw).

2.4.4. DPPH Radical Scavenging Activity Assay

A total of 2.0 mL of DPPH solution (0.1 mM in ethanol) was mixed with 2.0 mL of the samples. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was obtained with a Trolox (0–0.016 mg/g; y = 0.00623x; R^2 = 0.9923). The results were expressed as Trolox equivalent per gram dry weight (TE/g dw).

2.4.5. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was prepared by mixing 0.3 M acetate buffer, 10 mM TPTZ solution with 40 mM HCl, and 20 mM ferric chloride solution. A total of 10 µL of the sample was combined with 200 µL of FRAP reagent; the contents were mixed vigorously. The absorption was measured at 593 nm using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was obtained with ferrous sulfate (0–1 mg/g; y = 2.6272; R^2 = 0.9985). The results were expressed as ferrous sulfate equivalent per gram dry weight (FS/g dw).

2.5. Antimicrobial Activity

Antimicrobial activity was determined with the diffusion method in a solid nutrient media of agar. Mueller–Hinton Agar (Mueller–Hinton II Agar, BBL, Cockeysville, MD, USA) was used.

Standard cultures of nonspore bacteria (all bacteria were obtained from American Type Culture Collection (ATCC))—*Staphylococcus aureus* (ATCC 25923; human nasal microbiota), *Staphylococcus epidermidis* (ATCC 12228; human skin microbiota), *Enterococcus faecalis* (ATCC 29212; human colonic microbiota), *Escherichia coli* (ATCC 25922; human colonic microbiota), *Klebsiella pneumoniae* (ATCC 13883; human microbiota), *Pseudomonas aeruginosa* (ATCC 27853; human microbiota), and *Proteus vulgaris* (ATCC8427; human microbiota). Bacteria were grown for 20–24 h at 35–37 °C on Mueller–Hinton Agar. The bacterial suspension was prepared from cultures of cultivated bacteria in sterile physiological sodium chloride (0.9%) solution, standardised with a McFarland standard indicator. The bacterial suspension was considered standardised when the indicator value was 0.5 (1 mL of bacterial suspension contains 1.5×10^8 cells of the micro-organism).

Standard spore bacteria cultures of *Bacillus cereus* (ATCC 6633; soil microbiota) were grown for 7 days at 35–37 °C on Mueller–Hinton Agar. After growing the culture of spore bacteria, it was washed off the surface of the medium with a sterile physiologic solution. The prepared suspension was heated for 30 min at 70 °C and diluted with physiological saline until the spore concentration in 1 mL was between 10×10^6 and 100×10^6.

The standard culture of the fungus *Candida albicans* (ATCC 10231; human microbiota) was grown for 20 to 24 h at 30 °C for 72 h on Sabouraud agar. The fungal suspension was prepared from cultivated fungal cultures in physiological saline and standardised with a McFarland standard indicator.

A 0.5 McFarland turbidity suspension of the standard bacteria was prepared. The bottom of the Petri dishes was divided into 9 segments. The technology of reference microorganisms to Mueller–Hinton agar was used to determine the antimicrobial activity of *Glycyrrhiza glabra* L. and *Trifolium pratense* L. extracts. The disk method was used to determine the antimicrobial activity of *Myristica fragrans* Houtt. essential oil.

2.5.1. Antibacterial Activity of Extracts

The chosen amount of extract was poured into a sterile Petri dish (*Glycyrrhiza glabra* L. extract from 1 mL to 0.0075 mL; clover from 1 mL to 0.1 mL). Then, 5 mL of Mueller–Hinton agar was added. After the solidification of the agar, suspensions of reference microorganisms were inoculated. Samples were kept in a thermostat for 20–24 h at 35 °C, then stored for 24 h at room temperature. Antimicrobial activity was evaluated. If the cultures had grown—the sample did not inhibit the growth of bacteria. If the reference culture did not grow, the sample has an antimicrobial effect against the microorganism.

2.5.2. Antibacterial Activity of Essential Oil

A total of 0.5 mL of bacterial suspensions were separately poured into sterile Petri dishes. After that, 5 mL of Mueller–Hinton agar was poured into the plates. After the agar solidified, an 8 mm disk was placed on top, and 30 µm of *Myristica fragrans* Houtt. essential oil was added. Samples were kept in a thermostat for 20–24 h at 35 °C, then stored for 24 h at room temperature. Antimicrobial activity was then evaluated: if the cultures grew around and under the disk—the sample did not inhibit the growth of bacteria. If the reference culture did not grow, the sample has an antimicrobial effect against the microorganism.

2.6. Antiviral Activity

Dr. I. Jacevičienė provided a Vero cell line (ATCC CCL-81) from the Department of Virus Research at the National Food and Veterinary Risk Assessment Institute in Lithuania. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, TX, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, TX, USA) at 37 °C in a 5% CO_2

incubator. Gentamycin (50 µg/mL, Gibco, TX, USA) and nystatin (100 units/mL, Gibco, TX, USA) were used to prevent microbial contamination.

The Vero-adapted Beaudette infectious bronchitis virus (IBV) strain was used. Dr. M.H. Verheije of Utrecht University in The Netherlands provided the virus. The virus stocks were prepared and stored at −80 °C in aliquots.

Determination of TCID50 of the control and the treated IBV were performed in 96-well plates (TPP, Switzerland) with Vero cells. Serial dilutions of IBV in tenfold steps were prepared. Each sample was tested in octuplicates, and the experiments were repeated twice. CPE was evaluated after 72 h. Virus titres and standard deviations were calculated using the Kärber method (Kärber 1931), and the virus reduction capacity was assessed [38].

The cytotoxicity assay was performed to determine the 50% cytotoxic concentration (CC_{50}). CC_{50} was determined for each extract on Vero cells using an MTT assay [39]. First, cells were seeded at a concentration of 1×10^4 cells/well in a 96-well plate and grown at 37 °C for 1 day. The assay was performed in octuplicate for each extract. After 72 h, the MTT reagent (10 µL, 5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 4 h at 37 °C. A total of 100 µL dimethyl sulfoxide was added to each well, and the plates were placed on the shaker for 5 min. The absorbance of each well was measured at 620 nm in a microplate reader (Thermo Scientific™, Multiskan™ FC Microplate Photometer, Tokyo, Japan), and the percentage of cell survival was calculated. The relative viability in untreated cells was set as 100%. Finally, dose–response curves were plotted to calculate CC_{50} that caused lysis and death of 50% of cells (Chang, 2016). The dilutions of plant extracts were prepared for screening and determination of antiviral and virucidal activity.

Testing solutions were prepared by mixing IBV ($10^{5.25}$ $TCID_{50/mL}$) suspensions in DMEM with previously established noncytotoxic extract volumes (dilutions 1:15 and 1:30). The controls of diluents of plant preparations were used. The virus and extract mixtures were incubated at 20 °C for 1 h and then titrated as described above. Controls of cells, the virus, and extracts were included. After 72 h of incubation, the plates were examined using an inverted microscope (Leica, Germany) to detect CPE. The reduction factor was used to describe plant preparations' viral reduction potential [38]. The reduction factor ≤ 1 $\log_{10}$—regarded as insignificant; 1–2 $\log_{10}$—indicative/contributable; 2–4 $\log_{10}$—moderate; and >4 $\log_{10}$—high.

Antiviral activity was evaluated by infection of cells after virus treatment, during virus treatment, and the treatment of cells before infection and after infection. A total of 0.05 IBV particles per cell multiplicity of infection (MOI) was used. Prepared extracts were serially diluted twofold per seven wells in DMEM and assessed for their ability to inhibit IBV replication using four mechanisms.

In the first method, IBV was treated with the diluted extract for 1 h in a separate 96-well plate and then poured onto the cells. After incubating for 1 h at 37 °C in 5% CO_2, the mixtures were discarded, and then the cells were washed twice with PBS. After washing, 200 µL of DMEM containing 2% FBS was added. Observation by microscopy for inhibition of CPE was performed after incubation for 72 h at 37 °C in 5% CO_2.

In the second method, the diluted extract and IBV mixtures were poured onto the cells immediately. After incubating for 1 h at 37 °C in 5% CO_2, the mixtures were discarded, and then the cells were washed twice with PBS. After washing, 200 µL of DMEM containing 2% FBS was added. Observation by microscopy for inhibition of CPE was performed after incubation for 72 h at 37 °C in 5% CO_2.

In the third method, the cells were inoculated with the IBV and then treated with the extract. First, the cells were inoculated with the virus and incubated for 1 h at 37 °C in 5% CO_2. Then the unadsorbed IBV was discarded, and the cells were washed twice with PBS. After washing, the cells were treated with the diluted extracts for 1 h at 37 °C in 5% CO_2. After washing the cells twice with PBS, 200 µL of DMEM containing 2% FBS was added. Observation by microscopy for inhibition of CPE was performed after incubation for 72 h at 37 °C in 5% CO_2.

In the fourth method, the cells were treated with extract before inoculation. First, the cells were treated with the diluted extracts for 1 h at 37 °C in 5% CO_2. Then the cells were washed twice with PBS and inoculated with IBV. After incubation for 1 h at 37 °C in 5% CO_2, the cells were washed twice with PBS, and 200 µL of DMEM containing 2% FBS was added. Observation by microscopy for inhibition of CPE was performed after incubation for 72 h at 37 °C in 5% CO_2.

Every sample of the extract was tested twice in quadruplicate.

The cytopathic effect (CPE) of IBV was evaluated by optical microscopy. The endpoint was the extract dilution that inhibited 100% of the CPE at two noncytotoxic concentrations.

2.7. Emulsion Preparation

First, a 4% sodium alginate solution was prepared from distilled water and alginic acid sodium salt. It was used throughout the experiment for emulsion preparation as the shell material. Emulsion with *Trifolium pratense* L. and *Glycyrrhiza glabra* L. extracts, and *Myristica fragrans* Houtt. essential oil were prepared as follows: solution with excipients (maltodextrin, inulin, and/ or gum Arabic) was mixed with sodium alginate solution (stirred for 15 min with a magnetic stirrer MSH-20A (Witeg, Wertheim, Germany)) and then extracts with essential oil were added. The solution was homogenised for 15 min at 5000 rpm using an IKA T18 homogeniser (IKA-Werke GmbH & Co., KG, Staufen, Germany).

The emulsion's stability was tested using a centrifuge Sigma 3-18KS (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The test was repeated three times using 23 °C temperature, 3000 rpm, and the duration was 5 min. The centrifugation index (CI) was calculated to evaluate emulsion stability.

$$CI(\%) = \frac{V_e}{V_i} \cdot 100 \quad (1)$$

where V_e is the volume of the remaining emulsion after centrifugation and V_i is the volume of the initial emulsion.

2.8. Particle Size and Distribution Measurements

Myristica fragrans Houtt. essential oil particles' size and distribution were assessed using Mastersizer 3000 with a Hydro EV unit (Malvern analytical Ltd., Malvern, UK). The emulsion was added dropwise in the dispersant (water) to obtain laser obscuration between 9.5% and 10.5%. The pump speed was kept constant at 2400 rpm. The refractive index used for dispersant and dispersing material was 1.330 and 1.478, respectively. The average was calculated for particle size distribution in five runs. The percentile (D10, D50, and D90) values described the formulations.

2.9. Microcapsule Preparation Using the Extrusion Method

Microcapsules were prepared by extrusion method. The medical syringe (Jiangsu Zhengkang Medical Apparatus, Yangzhou, China) and NE-1000 Programmable Single Syringe Pump (KF T technology SRL, Rozzano, Italy) were used to prepare droplets for microcapsules. The drops of emulsion were ejected from the needle into the crosslinker solution. The height from the needle to the solution surface was 10–15 cm, and the pumping speed was 3 mL/min. A solution of 5% calcium chloride was used as a crosslinker. Microcapsules were prepared by using a magnetic stirrer. Particles in the crosslinker solution were stirred for 15 min, and then the microcapsules were filtered using filter paper and washed with distilled water. Manufactured capsules were left to dry at room temperature (20 ± 2 °C) for 24 h. Dried and wet microcapsules were stored in sealed tubes until further tests.

2.10. Physical Parameters of Microcapsules

2.10.1. Size of Dry and Wet Microcapsules

The microcapsules' size was measured using a Digital Calipiper micrometre (BGS technic, Wermelskirchen, Germany). The diameter of 30 units of dried and freshly made capsules was measured, and the average was calculated.

2.10.2. Firmness of Microcapsules

The force of firmness was measured on freshly made microcapsules by texture analyser TA.TX.plus (Texture Technologies, Hamilton, MA, USA). For one sample, 5 units of microcapsules were taken, and the force which was required to compress 2 mm was measured using a P/100 probe.

2.10.3. Swelling Characteristic of Microcapsules

Dried microcapsules were swelled in gastric and intestinal media [33]. The microcapsules were weighed at successive time intervals of 0, 0.5, 1, 2, 4, and 24 h. The swollen microcapsules were removed and filtered using metal mesh and dried with a paper towel to eliminate the excess fluid. To determine the swelling index (SI), the following formula for calculation was used:

$$SI(\%) = \frac{W_s - W_i}{W_i} \cdot 100 \quad (2)$$

where W_s is the weight of swollen microcapsules at the time and W_i is the dried microcapsules' weight.

2.11. Statistical Data Analysis

Data were analysed using SSPS version 20.0 (IBM Corporation, Armonk, NY, USA). All experiments were performed in triplicate. Data are expressed as mean ± standard deviation (SD). The comparisons between three different measurements were made using Friedman and Wilcoxon tests. The results were considered statistically significant at $p < 0.05$. For antiviral experiments, the differences between the methods and extracts were evaluated by Fisher's criteria and the Student's t-test. The data were regarded as significant when $p < 0.05$.

3. Results and Discussion

3.1. Quantification of Main Compounds in Plant Extracts and Essential Oil

The main compound with higher biological activity in *Glycyrrhiza glabra* L. extract is glycyrrhizic acid [40]. *Glycyrrhiza glabra* L. extract was made by maceration combined with ultrasound processing. The extracts yielded 349.29 ± 14.55 μg/g glycyrrhizic acid. The extract was concentrated by evaporation of water (half the weight of the extract), and the glycyrrhizin content determined after concentration was 2.07-fold higher. In the literature, there are other extraction methods to obtain glycyrrhizin. Using sonification and pressurised hot water extraction, the amount of glycyrrhizin acid was 17.21 ± 1.27 mg/g and 13.20 ± 2.37 mg/g, respectively [41]. A total of 36.4 μg/g of glycyrrhizic acid was obtained by ultrasound-assisted extraction (30:1 solvent to solute ratio, 40 °C temperature) [42]. Ultrasound alone yields lower concentrations of glycyrrhizic acid; therefore, combining it with maceration gives better results.

Trifolium pratense L. flower extract (extraction with excipient β-CD) contained 171.57 ± 12.36 μg/g of genistein and 393.23 ± 23.71 μg/g of daidzein. This amount of isoflavones is higher than the average reported in the literature. It was reported that petioles contained 0.028 mg/g of daidzein and 0.054 mg/g of genistein, and flowers contained 0.44 μg/g of genistein and daidzein, except for minimal traces were not detected [43]. Using reflux (85 °C) extraction, 0.11 ± 00.28 mg/g of daidzein and 0.10 ± 0.0053 mg/g of genistein were obtained (extrahent was methanol). These concentrations were a lot lower than those obtained in this study. Adding excipient β-CD to extraction media helps extract target compounds. β-CDs form host–guest inclusion

complexes with various drugs in a solution or a solid state. The existence of a β-CD hydrophobic cavity boosts the extraction of phenolic compounds, including isoflavones [44]. Clover extract was also concentrated by evaporating water (50%), and the isoflavones concentration found in the sample were genistein 265.46 μg/g and daidzein 598.16 μg/g.

Myristica fragrans Houtt. essential oil (prepared by modified hydrodistillation; origin country: Grenada) had the highest concentration of sabinene 42.55%, α-pinene 13.58%, β-terpinene 8.17%, and β-myrcene 3.47%. The essential oil yield was 10.88 ± 0.75%. Compared with other studies, it was found that *Myristica fragrans* Houtt. essential oil yield prepared using hydrodistillation was from 3.2% to 10.3% and when prepared by steam distillation was 0.3–12.5% [10]. The essential oil isolated from the seeds of *Myristica fragrans* Houtt. (origin country Nigeria) was found to contain 49.09% sabinene, 13.19% α-pinene, 6.72% α-phellandrene, and 6.43% terpinen-4-ol as major constituents [45]. Therefore, the monoterpenes identified from *Myristica fragrans* Houtt. essential oil in our sample contained higher concentrations. *Myristica fragrans* Houtt. Essential oil from Brasilia had an even lower amount of sabinene (25.0%) but a higher amount of myrcene (10.9%) compared with this study's results [46].

3.2. Phenols and Antioxidant Activity of Extracts and Essential Oil

Many components in extracts and essential oil, i.e., carotenoids, vitamin C, vitamin E, and phenolic compounds, contribute to the overall antioxidant activity; it is difficult to measure the total antioxidant activity based on individual active components. This is because compounds can interact with each other to produce antagonistic or synergistic effects [47]. As a result, the extracts were tested for total phenolic compounds, flavonoid content, and antioxidant activity using three different methods—ABTS, DPPH, and FRAP. The research was done using concentrated *Glycyrrhiza glabra* L. and *Trifolium pratense* L. extracts.

3.2.1. Total Phenolic and Flavonoid Content

The total phenolic content found in *Glycyrrhiza glabra* L. extract was 43.14 ± 0.06 mg GA/g dw. Higher results were obtained from the *Trifolium pratense* L. flowers extract—74.00 ± 0.15 mg GA/g dw (Table 1). Ethanolic *Trifolium pratense* L. extract had a significantly higher total phenol content compared to aqueous *Glycyrrhiza glabra* L. extract ($p < 0.05$), and the exact correlation was observed comparing total flavonoids. Varying solubility of the phenolic compounds due to different solvents could be explained by the solvent polarity, and ethanol is more efficient in extracting lower molecular weight polyphenols, especially glycosides [48,49].

Table 1. The results of samples total phenol and flavonoid content.

Samples	Total Phenols (mg GA/g dw)	Total Flavonoids (mg RU/g dw)
Glycyrrhiza glabra L. extract	43.14 ± 0.06	16.89 ± 0.02
Trifolium pratense L. extract	74.00 ± 0.15	19.50 ± 0.04
Myristica fragrans Houtt. Essential oil (1%)	7.49 ± 0.04	6.84 ± 0.05

The essential oil of *Myristica fragrans* Houtt., because of its preparation method, contained only a small amount of phenols (7.49 ± 0.04 mg GA/g dw). The main compounds found in our sample were monoterpenes (identified as 67.77%). This correlates with the results published by other researchers [10,15]. The total flavonoids determined in the essential oil was 6.84 ± 0.05 mg RU/g dw.

3.2.2. Antioxidant Activity of Extracts and Essential Oil

In the literature, a few antioxidant assays have been developed based on methodological differences to detect antioxidant activity in samples taken from natural sources, including extracts and essential oils. In this study, DPPH, ABTS, and FRAP methods were used to determine the antioxidant potentials of *Glycyrrhiza glabra* L. and *Trifolium pratense*

L. extract, also *Myristica fragrans* Houtt. essential oil. Radical scavenging antioxidants are necessary for the antioxidative defence to protect cells from the injurious effects of free radicals [50]. Antioxidants can delay the progress of many chronic diseases as well as lipid peroxidation [51].

The antioxidant activity of *Glycyrrhiza glabra* L. and *Trifolium pratense* L., using the DPPH method, was similar (Table 2). *Trifolium pratense* L. extract showed better antioxidant activity using the ABTS method (638.55 ± 9.14 μg TE/g dw), but *Glycyrrhiza glabra* L. extract had higher ferric reducing power (675.71 ± 4.61 mg FS/g dw). Studies in the literature have shown that both *Trifolium pratense* L. and *Glycyrrhiza glabra* L. are good antioxidants. *Trifolium pratense* L. is known to contain isoflavones (daidzein, genistein, formononetin, and biochanin A), which possess estrogenic effects, but antioxidant properties of extract can be related to quercetin, hyperoside, clovamides, and other phenolic compounds [52]. The reported main phenolic compounds in *Glycyrrhiza glabra* L. extract responsible for its antioxidant activity are also isoflavones, such as glabridin, hispaglabridin A, and 30-hydroxy-4-O-methylglabridin [53].

Table 2. Antioxidant capacity of plant extracts and essential oil.

Samples	DPPH (μg TE/g dw)	ABTS (μg TE/g dw)	FRAP (mg FS/g dw)
Glycyrrhiza glabra L. extract	26.22 ± 0.27	524.67 ± 6.32	675.71 ± 4.61
Trifolium pratense L. extract	26.27 ± 0.31	638.55 ± 9.14	526.86 ± 3.21
Myristica fragrans Houtt. Essential oil (1%)	8.69 ± 0.01	92.14 ± 1.26	176.05 ± 0.12

Myristica fragrans Houtt. essential oil reported possessing antioxidant activity as well as *Trifolium pratense* L. or *Glycyrrhiza glabra* L. extracts. Nevertheless, the concentration of the essential oil used in this study was 1%. The isomers α-pinene and β-pinene possess antioxidant and antimicrobial activity, but the antimicrobial activity of β-pinene is more robust [17].

3.3. Antimicrobial and Antiviral Activity

The antimicrobial activity of *Trifolium pratense* L. and *Glycyrrhiza glabra* L. extract was determined using nonconcentrated extracts. *Glycyrrhiza glabra* L. extract had expressed antimicrobial activity against Gram-positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Bacillus cereus*. Additionally, it suppressed the growth of the fungus *Candida albicans*.

A concentration of 0.2% extract inhibited these pathogens, whereas *Bacillus aureus* was inhibited by a 2% *Glycyrrhiza glabra* L. extract solution. *Glycyrrhiza glabra* L. extract had no antimicrobial activity against Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*. The results are presented in Table 3.

The antimicrobial activity of *Trifolium pratense* L. extract was not expressed, but the obtained results showed that this extract inhibits gram-positive bacteria more. 0.75 mL of extract in 5 mL of agar medium (13.04% concentration of extract) inhibited the growth of *Staphylococcus epidermidis* and inhibited *Staphylococcus aureus* and *Bacillus cereus* weakly. The growth of other pathogens was not inhibited (Table 4).

According to the literature, the extraction solvent capability to extract active substances of the active principles has a major importance on the antibacterial and antifungal activity of the *Trifolium* species. Studies on the antimicrobial properties of *Trifolium pratense* L. included a comparison of the actions of different extracts solvents, concentration of the extract and drying conditions [19,54–56]. In our study case, the concentration of isoflavones, which are responsible for antibacterial effect in *Trifolium pratense* L. was too low to have strong antibacterial response.

Essential oils are volatile liquids, so it is difficult to determine their effect due to evaporation. One essential oil concentration was chosen in this study, and antimicrobial activity against 9 pathogens was evaluated. Results (Table 4) showed that Myristica fragrans

Houtt. Essential oil suppressed the growth of half of referenced gram-positive and half of referenced gram-negative pathogens (*Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*, *Klebsiella pneumoniae,* respectively), and the other pathogens were not suppressed (two of gram-positive and two of gram-negative).

Observing the results obtained by other scientists and comparing it with this study, it was found that a two times higher amount of nutmeg essential oil suppressed *Candida albicans*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus luteus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [17]. In the other study, it was found that *Myristica fragrans* Houtt. essential oil was more effective in inhibiting the growth of *S. aureus* than gentamicin (sample and gentamicin (as control) concentrations were the same, and the zone of inhibition of *Myristica fragrans* Houtt. was 1.22% greater than the antibiotic) [57].

In the literature, it was reported that *Trifolium pratense* L. extract suppressed *Bacillus subtilis* growth. The diameter of the nongrowth zone was 20 mm, while the essential oil nongrowth zone was 12 mm. The extract had 40% higher activity against *B. subtilis* compared to essential oil [58]. The antibacterial activity of the essential oil against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus cereus* was also tested, and it was found that clover extract did not inhibit the growth of either pathogen [59]. Thus, it can be concluded that to increase the antibacterial activity in products, clover extract should be used instead of essential oil.

In publications about *Glycyrrhiza glabra* L. roots, it was revealed that an ethanolic extract of *Glycyrrhiza glabra* L. had a positive effect on suppressing *B. cereus*, *B, subtilis*, *K. pneumoniae*, and *S. aureus*; however, the ethanolic extract did not suppress *E. faecalis* growth [60]. In our study it was found that 0.01 mL of the *Glycyrrhiza glabra* L. extract in a 5 mL agar medium effectively suppressed *E. faecalis*.

Table 3. Antimicrobial effect of *Glycyrrhiza glabra* L. extract on the growth of reference microorganism cultures.

Reference Cultures of Microorganisms	**Amount of *Glycyrrhiza glabra* L. Extract (mL)**									
	1.1	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**1.7**	**1.8**	**1.9**	**1.10**
	1.0	**0.75**	**0.5**	**0.25**	**0.1**	**0.075**	**0.05**	**0.025**	**0.01**	**0.0075**
Staphylococcus aureus ATCC 25923	S	S	S	S	S	S	S	S	S	N
Staphylococcus epidermidis ATCC 12228	S	S	S	S	S	S	S	S	S	N
Enterococcus faecalis ATCC 29212	S	S	S	S	S	S	S	S	S	N
Escherichia coli ATCC 25922	N	N	N	N	N	N	N	N	N	N
Klebsiella pneumoniae ATCC 13883	N	N	N	N	N	N	N	N	N	N
Pseudomonas aeruginosa ATCC 27853	N	N	N	N	N	N	N	N	N	N
Bacillus cereus ATCC 11778	S	S	S	S	S	N	N	N	N	N
Candida albicans ATCC 10231	S	S	S	S	S	S	S	S	±	N
Proteus vulgaris ATCC 8427	N	N	N	N	N	N	N	N	N	N

S—inhibits the growth of reference culture of microorganisms (the antimicrobial effect was present); N—does not inhibit the growth of reference culture of microorganisms (no antimicrobial effect); ±—very weak growth.

Table 4. Antimicrobial effect of *Trifolium pratense* L. extract and *Myristica fragrans* Houtt. Essential oil on the growth of reference microorganism cultures.

Reference Cultures of Microorganisms	Amount of Clover Extract (mL)					Amount of *Myristica fragrans* Houtt. Essential Oil (μg)
	2.1	**2.2**	**2.3**	**2.4**	**2.5**	**4**
	1.0	**0.75**	**0.5**	**0.25**	**0.1**	**30.0**
Staphylococcus aureus ATCC 25923	S	±	N	N	N	S
Staphylococcus epidermidis ATCC 12228	S	S	N	N	N	N
Enterococcus faecalis ATCC 29212	N	N	N	N	N	N
Escherichia coli ATCC 25922	N	N	N	N	N	S
Klebsiella pneumoniae ATCC 13883	N	N	N	N	N	S
Pseudomonas aeruginosa ATCC 27853	N	N	N	N	N	N
Bacillus cereus ATCC 11778	S	±	N	N	N	N
Candida albicans ATCC 10231	±	N	N	N	N	S
Proteus vulgaris ATCC 8427	N	N	N	N	N	N

S—inhibits the growth of reference culture of microorganisms (the antimicrobial effect was present); N—does not inhibit the growth of reference culture of microorganisms (no antimicrobial effect); ±—very weak growth.

3.4. Antiviral Activity of Used Plant Extracts and Essential Oil

It was determined that the CC_{50} of *Trifolium pratense* L. was 4.8 mg/mL and the CC_{50} *Glycyrrhiza glabra* L. was 10.0 mg/mL for Vero cells. The diluent of *Glycyrrhiza glabra* L. had no virucidal effect upon IBV, whereas the diluent, ethanol, used for *Trifolium pratense* L. at concentrations of 8.0% ethanol (*Trifolium pratense* L. with 4.8 mg/mL) and 6.67% ethanol (*Trifolium pratense* L. with 4.0 mg/mL) had a virucidal effect (Table 5). The diluent of the almond EO did have a positive impact on virucidal activity ($p < 0.05$).

Based upon cytotoxicity and control results, dilutions of 1:15 and 1:30 were chosen for comparison of the virucidal activity of extracts.

All plant preparations (1:15 and 1:30) showed a ≥90% reduction of the viral titre and had moderate or contributable virucidal activity. At 1:15 dilution (2 mg/mL), *Trifolium pratense* L. showed moderate virucidal activity (virus reduction 2.75 $\log_{10}$), while at a 1:30 dilution (1 mg/mL), virus reductions were between 1.125 $\log_{10}$ and 1.75 $\log_{10}$. Other plant preparations showed contributable virucidal activity at both dilutions. The virucidal activity of *Trifolium pratense* L. and *Glycyrrhiza glabra* L. was dose-dependent ($p < 0.05$), while the concentration of almond had no effect ($p > 0.05$).

Antiviral activity was tested for the extracts of *Trifolium pratense* L. and *Glycyrrhiza glabra* L. (Table 6). It was shown that the extracts were effective against IBV and could protect the Vero cells prior to or during infection. IBV pretreatment with extracts after infection and cell pretreatment prior to infection could not inhibit 100% of the CPE. Combining these extracts together can result in a promising preparation that will have high antioxidant, antibacterial, and antiviral properties.

Table 5. Comparison of the virucidal effect of plant extracts on IBV.

Material, Dilution and Concentration			Virus Titre, $TCID_{50}$	Virus Reduction ($TCID_{50}$)		
Name	Dilution	Concentration		log_{10}	%	Reduction Factor
Trifolium pratense L. extract	1:7.5	4 mg/mL	NA	NA	NA	NA
	1:15	2 mg/mL *	2.50 ± 0.26	2.72	99.82	moderate
	1:30	1 mg/mL *	3.50 ± 0.00	1.74	98.23	contributable
50% ethanol (control)	1:15	3.33%	5.22 ± 0.16	-	-	-
	1:30	1.67%	5.24 ± 0.16	-	-	-
Glycyrrhiza glabra L. extract	1:15	13.3 mg/mL *	3.50 ± 0.18	1.72	98.23	contributable
	1:30	6.6 mg/mL *	4.00 ± 0.19	1.24	94.38	contributable
Water (control)	1:15	6.66%	5.22 ± 0.16	-	-	-
	1:30	3.33%	5.24 ± 0.16	-	-	-
Myristica fragrans Houtt. essential oil **	1:15	13.3 µL/mL	4.13 ± 0.18	1.10	92.50	contributable
	1:30	6.6 µL/mL	4.00 ± 0.18	1.24	94.38	contributable
Almond oil (control)	1:15	13.3 µL/mL	3.88 ± 0.18	1.34	95.80	contributable
	1:30	6.6 µL/mL	3.75 ± 0.16	1.49	96.84	contributable
IBV control	undiluted	100%	5.25 ± 0.16	-	-	-

* Dose-dependent effect ($p < 0.05$). ** *Myristica fragrans* Houtt. essential oil was diluted in almond oil; the essential oil concentration was 1%. NA—nonapplicable because of ethanol virucidal effect.

Table 6. Antiviral activity of plant extracts.

Plant Extract	Antiviral Effect Evaluated by CIA100			
	Virus Pre-Treatment with Extract			Cell Pre-Treatment Prior to Infection
	Prior to Infection	During Infection	After Infection	
Trifolium pratense L.	+	+	-	-
Glycyrrhiza glabra L.	+	+	-	-

+: had an effect; -: no effect.

3.5. Emulsion Physical Stability

Six emulsion samples were prepared, and their stability was evaluated (the emulsion's composition is shown in Table 7). Sample E3 became an inhomogeneous texture after all ingredients were mixed, and its centrifugation index was the lowest (Figure 1).

When comparing the stability of the emulsion, it was established that one excipient (maltodextrin/inulin/gum Arabic) was unable to keep the emulsions stable (CI was lower than 50%). Then, two different excipients were added to the composition, and the stability of the emulsion was increased by approximately 46% compared with E1. The highest stability of the emulsion was evaluated, and then three excipients were added. Emulsion E6 was the most stable, and its CI was 100%.

In an emulsion, particle size, distribution, and essential oil drop diameter were determined using the Mastersizer 3000 (Figure 2). *Myristica fragrans* Houtt. essential oil drop diameter in the emulsion was 0.571 µm. Evaluated percentiles were measured: D10 was 0.328 ± 0.051 µm, D50 was 0.46 ± 0.03 µm, and D90 was 0.89 ± 0.094 µm. The percentiles D10, D50, and D90, indicate the size for which 10%, 50%, and 90% of the particles are equal to or less, respectively [61]. The emulsion particle size with different essential oils was 16.70–55.56 µm. The base was made from soy protein isolate and gum Arabic [62]. Studies similar to this, with *Myristica fragrans* Houtt. essential oil, extracts, and gum Arabic,

maltodextrin, and inulin, were not found. This stable emulsion (E6) was used to prepare microcapsules by extrusion.

Table 7. Emulsions' composition.

Ingredients	Samples and Ingredients Quantities					
	E1	**E2**	**E3**	**E4**	**E5**	**E6**
4% sodium alginate solution (g)	47.0	47.0	47.0	47.0	47.0	47.0
Myristica fragrans Houtt. essential oil (mL)	0.3	0.3	0.3	0.3	0.3	0.3
Trifolium pratense L. extract (mL)	4.2	4.2	4.2	4.2	4.2	4.2
Glycyrrhiza glabra L. extract (mL)	16.5	16.5	16.5	16.5	16.5	16.5
Maltodextrin (g)	12.0	-	-	8.6	10.3	6.2
Inulin (g)	-	12.0	-	3.4	-	4.3
Gum Arabic (g)	-	-	12.0	-	1.7	1.5
Purified water (mL)	20.0	20.0	20.0	20.0	20.0	20.0

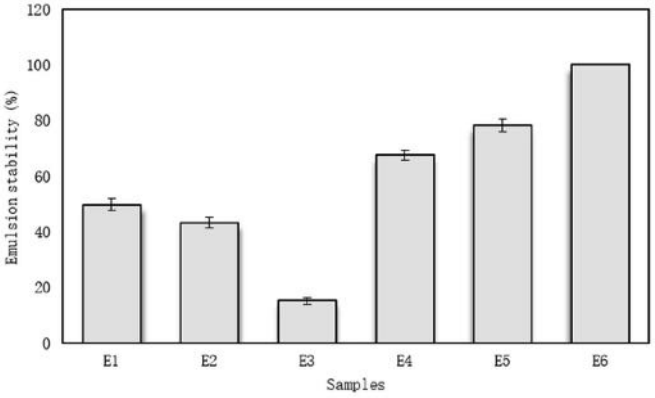

Figure 1. The emulsion stability (centrifugation index). The code of emulsion is given in Table 7.

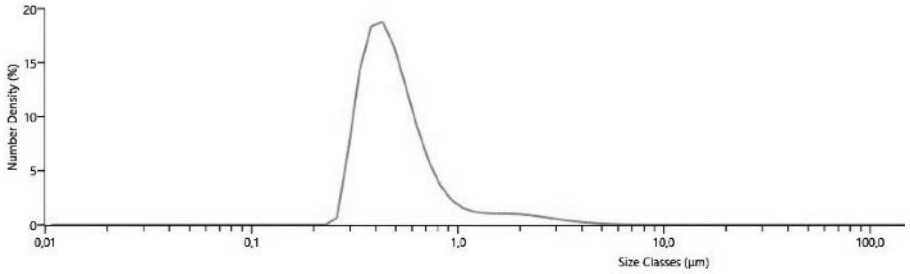

Figure 2. Particle size distribution.

3.6. Microcapsules' Physical Parameters

In a 5% crosslinker solution, microcapsules were formulated (Figure 3), and all microcapsules were spherical and of light brown colour. The yield of microcapsules was 68.67 ± 3.08%. The freshly prepared microcapsules' diameter was 1.87 ± 0.35 mm and soft (easily crushed between the fingers). The force of crushing (measured with a Texture

analyser) was 3758.52–4135.87 g. The dried microcapsules' diameter was 0.76 ± 0.11 mm, and the device did not measure the force because the maximum force value is 6500 g; dried microcapsules were harder than this value.

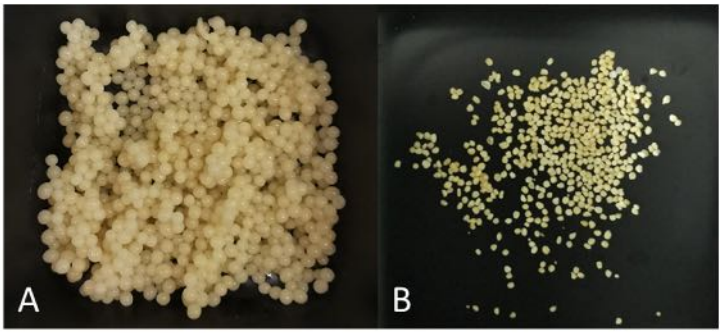

Figure 3. Extruded microcapsules (**A**) immediately after extrusion and (**B**) after drying the microcapsules.

Extrusion technology to produce microcapsules with essential oils and extracts is rarely used, but similar research results have been found. In a study with *Myristica fragrans* Houtt. essential oil, when polysorbate 80 is used as an emulsifier, microcapsules of size 2.120–2.280 mm with a strength of 4333.46–5116.70 g were formed in a 2% crosslinker solution [33]. Microcapsules with 1% of rosemary essential oil and without other excipients had a diameter between 0.950 mm and 0.756 mm (non-dry and dry, respectively; only sodium alginate 4% solution was used) [25].

Microcapsules' swelling index in gastric media was from 19.74% to 13.96%, and alginate microcapsules did not swell in gastric fluid. At first, the microcapsules swelled a little, but after 30 min, the swelling index decreased. Conversely, microcapsules swelled from 25.22% to 121.59% in the intestinal medium (Figure 4). After a day, the microcapsules had lost their shape and softened. Another study found that alginate microcapsules lost weight in the gastric media (alginate, soy protein microcapsules with thyme essential oil) [26]. The maximum swelling index was reached in 40–60 min, and then the amount of microcapsules in the gastric medium decreased [63]. Another study confirmed that alginate microcapsules swell in the intestinal medium, and the swelling index could be higher than 20 times [33]. In contrast, microcapsules almost did not swell, or their amount decreased in gastric media (pH = 1–2.5) [64]. The results of other studies are in agreement with the results obtained in this study and show that sodium alginate microcapsules swell in the intestinal medium. Sodium alginate is a polymer and forms hydrogels. It is well known that a hydrogel can respond to surrounding conditions such as pH, ionic strength, temperature, and electric current. The pH sensitivity is an important factor for controlled drug release in the gastrointestinal tract, which has a variation of pH from the stomach to the intestine. Many studies support the results that sodium alginate microcapsules or gels do not swell in the intestinal medium. The pH controls the degree of dissociation of the guluronic and mannuronic acid groups (alginate molecule parts); therefore, alginate does not swell in an acidic medium [65,66]. Alginate microcapsules can be used to develop gastric insoluble pharmaceutical formulations.

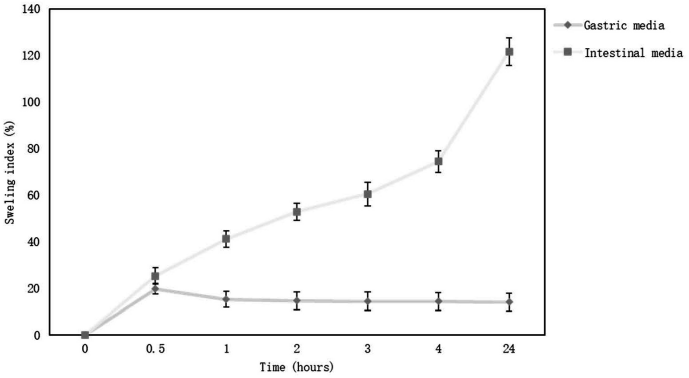

Figure 4. Microcapsules swelling graph in simulated intestinal and gastric media.

4. Conclusions

This study determined that the chosen plant extracts and essential oil had a significant concentration of active substances. The main component in liquorice extract was glycyrrhizin acid (349.29 ± 14.55 μg/g); the red clover extract contained 171.57 ± 12.36 μg/g of genistein and 393.23 ± 23.71 μg/g of daidzein. Nutmeg essential oil had four main chemical compounds: sabinene, α-pinene, β-terpinene, and β-myrcene.

All the plant extracts possess antioxidant effects. The highest effect was determined in the red clover extract, which also was the most effective against viruses. The highest antibacterial effect was found in the liquorice extract, which was most effective against Gram-positive pathogens. Therefore, plant-derived products can be considered promising when being used as antimicrobial agents. Extruded microcapsules with essential oil and extracts could be an additional form to incorporate in other pharmaceutical forms for therapeutic benefits. Considering the obtained research results, it is planned to evaluate the release of active compounds from microcapsules in the future.

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Paper 5

Title: Nutmeg Essential Oil, Red Clover, and Liquorice Extracts Microencapsulation Method Selection for the Release of Active Compounds from Gel Tablets of Different Bases

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Article

Nutmeg Essential Oil, Red Clover, and Liquorice Extracts Microencapsulation Method Selection for the Release of Active Compounds from Gel Tablets of Different Bases

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Abstract: The current study presents the most suitable method for encapsulating nutmeg essential oil with liquorice and red clover. Two widely used methods, spray-drying and freeze-drying, were employed to find the most suitable for essential oil volatile compounds' protection. Results showed that freeze-dried capsules (LM) had a higher yield (85.34%) compared to the exact formulation of spray-dried microcapsules (SDM)—45.12%. All the antioxidant and total phenolic compounds' results obtained with the LM sample were significantly higher compared with SDM. LM microcapsules were incorporated in two different bases with no additional sugar (gelatin and pectin) for targeted release. Pectin tablets had firmer and harder texture properties, while gelatin tablets had a more elastic texture. There was a significant impact on texture changes caused by microcapsules. Microencapsulated essential oil with extracts can be used alone or in a gel base (pectin or gelatin, depending on user preferences). It could be an effective product to protect the active volatile compounds and regulate the release of active compounds and give a pleasant taste.

Keywords: essential oil; nutmeg; red clover; polyphenols; liquorice; spray-drying; freeze-drying; gel tablets; gelatin; pectin

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1. Introduction

Microencapsulation has many applications in the food, cosmetic and pharmaceutical industries, such as protecting, isolating, or controlling a given substance's release. Microcapsules as delivery systems offer numerous advantages over conventional dosage forms, including improved efficacy, patient compliance, and convenience. Changing a liquid into a powder enables a variety of different applications for substances [1,2]. Microcapsules are constructed from an encasing substance that surrounds a core containing the active ingredient. The final particle size can range from 0.2 to 5000 m in diameter depending on a number of variables, including processing and material selection [3]. For microcapsules' preparation, various techniques can be employed, including spray drying, spray chilling or spray cooling, extrusion coating, fluidized-bed coating, liposomal entrapment, lyophilization, coacervation, centrifugal suspension separation, co-crystallization, and inclusion complexation [4].

Essential oil microencapsulation is needed to protect active compounds' evaporation and oxidative decomposition, to mask strong odors, and in pharmaceuticals, to control release [2]. Additionally, plant extracts that contain active polyphenols can be encapsulated. These compounds sometimes needs to be microencapsulated because they have an unpleasant bitter taste or limited concentration that remains available following oral administration

due to an insufficient gastric residence time, low permeability and/or solubility within the gut, and instability [4,5].

Emulsification, which affects encapsulation efficiency, powder characteristics, and storage stability, is a crucial step that should be performed before drying, particularly in the microencapsulation of essential oils, which requires the use of various wall materials. Different wall materials, including some carbohydrates, protect active substances against adverse temperatures, pH, humidity, oxygen, and other components that can react with the protected material [6].

One of the most used microencapsulation methods is spray-drying. This process is affordable, simple to alter and use, and results in a high-quality product [7]. The feed emulsion's composition and drying parameters have an impact on the quality of spray-dried microcapsules. The coating substance can be altered and customized to deliver ideal features. Coverage of the active substance gives oxidation protection and wall materials with high solubility, good emulsification, film-forming, and drying capabilities in the finished microcapsules [8]. The main concern when spraying emulsions containing essential oils is the evaporation of volatile compounds due to the high spraying temperature. The researchers proved that it is possible to produce powders of heat-labile compounds even using 200 °C temperature; therefore, it should be safe to use a spray-drying process in preparation for essential oil microcapsules [9].

Freeze-drying (lyophilization) is a process that can protect product quality by limiting chemical reactions, and biological and microbiological deterioration [10]. It is particularly appealing for drying heat-sensitive and biologically active components because drying is carried out in a vacuum and at temperatures lower than the materials' ambient temperature. Nevertheless, it has downsides like high energy consumption and long processing times [11–13].

Choosing the right inclusion material is a crucial step since encapsulating shields active volatile molecules from the environment, as was previously discussed. The increase in the stability of essential oil volatile compounds and the controlled delivery of encapsulated substances depends on the wall material. However, microcapsules after production often lack the desired effect, therefore incorporating microcapsules into pharmaceutical form can solve several problems.

Oral administration forms come in a variety of formats, such as tablets, soft and hard gel capsules, elixirs, suspensions, chewable tablets, etc. [14]. The latter form is becoming increasingly popular due to its convenient consumption in people with dysphagia, colorful appearance, smell, and because of a high sugar content pleasant taste. Most often, this form is found in food supplements [15]. Various polymeric materials are used for the formation of gels, such as gelatin, pectin, agar, and sugar is needed for the stabilization of their structure [16]. Nevertheless, natural substitutes can be used to form these gels, thus avoiding added sugar.

This study aims to compare freeze drying with spray drying technology to preserve extracts and nutmeg essential oil and to choose the best microcapsules to incorporate in different base, sugar-free chewable tablets on a laboratory scale.

2. Materials and Methods

2.1. Plant Material and Reagents

Myristica fragrans seeds' country of origin was Grenada (supplier Spaisvilė, Pašaltuonys, Lithuania).

Trifolium pratense L. samples were collected in *Trifolium pratense* L. fields in Laičiai, Kupiškis district, Lithuania (latitude 55°53,024.2″ N; longitude 25°19,036.0″ E). *Glycyrrhiza glabra* L. roots (the country of origin is China) were bought from LSMU pharmacy (Kaunas, Lithuania). Dried herbs and seeds (blower buds and roots) were ground separately using an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany).

Gum Arabic and maltodextrin were purchased from Sigma-Aldrich, (Steinheim, Germany). Ethanol (96%) used for extraction was purchased from Vilniaus degtinė

(Vilnius, Lithuania). In this experiment, purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionized water was prepared with Millipore, SimPak 1 (Merck, Darmstadt, Germany). The following reagents were used as standards, genistein, daidzein, and glycyrrhizin acid (Sigma-Aldrich, Steinheim, Germany). The 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and β-CDs were purchased from Sigma-Aldrich (Hamburg, Germany); aluminum chloride, hexaethylenetetraamine, dimethyl sulfoxide (DMSO), acetic acid obtained from Sigma-Aldrich (Buchs, Switzerland); potassium persulfate obtained from Alfa Aesar (Karlsruhe, Germany); Folin–Ciocalteu's phenol reagent (Merck, Darmstadt, Germany); monosodium phosphate, ferrous sulfate heptahydrate, saline phosphate buffer, and hydrogen peroxide obtained from Sigma-Aldrich (Schnelldorf, Germany); disodium hydrogen phosphate obtained from Merck (Darmstadt, Germany); alginic acid sodium salt from brown algae obtained from Sigma-Aldrich (Shanghai, China). Calcium chloride (Farmalabor, Pozzillo, Italy).

2.2. Microcapsules' Formulation and Preparation

Ethanolic red clover extract, aqueous liquorice extract and nutmeg essential oil were prepared as described in a previous study [17].

The emulsion for lyophilization/spray-drying was prepared using maltodextrin, gum Arabic and inulin as excipients. The composition of the emulsion is given in Table 1.

Table 1. Emulsion composition.

Ingredient	Amount (%)
Red clover extract	12.4
Liquorice extract	49.59
Nutmeg essential oil	0.83
Gum Arabic	5.58
Maltodextrin	18.6
Inulin	13.2

Emulsion preparation: excipients were dissolved in liquorice extract and mixed with red clover extract then the essential oil was added. The liquid was homogenized with IKA® ULTRA-TURRAX® T18 homogenizer (IKA-Werke GmbH & Co., KG, Staufen, Germany) for 15 min, 3000 rpm.

2.2.1. Lyophilization Method Conditions

The emulsion (Table 1) required storage in the refrigerator for 48 h at 18 °C prior to lyophilization. For freeze-drying Beta 1-8 LSC plus lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) was equipped. Prior to the lyophilization process, the apparatus' condenser was turned on for roughly 30 min to attain the necessary temperature (−53 °C). The product was lyophilized for 48 h. Throughout the article, microcapsules prepared using lyophilization will be named LM.

2.2.2. Spray-Drying Method Conditions

Spray drying was done using a Buchi B-291 Mini Spray-Dryer (BÜCHI Labortechnik AG, Flawil, Switzerland) under the following conditions: according to previous pilot studies inlet temperature was 160 °C, outlet temperature was 80–90 °C, spray flow feed rate—30 mL/min, air pressure—6 bar, aspirator—100%. Throughout the article, microcapsules prepared using spray-drying will be named SDM.

2.3. Evaluation of Physical Parameters of Microcapsules

The microcapsules obtained by spray-drying and lyophilization were analyzed for their product yield, moisture content, bulk and tapped volumes, solubility, and size distribution.

2.3.1. Microcapsule Yields

The powder yield after spray-drying/lyophilization is given by the percentage ratio between the total mass of the final product by the non-solvent mass of the emulsion.

2.3.2. Moisture Content

Using the Moisture Analyzer DAB (KERN & SOHN Gmb, Balingen, Germany), moisture content was determined. Microcapsules were weighed (approximately 0.2 ± 0.05 g) and heated at 105 °C until all the moisture was evaporated, and the product weight was constant. The device's screen displayed the moisture content (%) after the procedure.

2.3.3. Bulk and Tapped Volumes

The microcapsules' compressibility index and Hausner ratio were evaluated using the TD 1 Tap Density Tester (SOTAX, Hopkinton, United States). The tapped density was obtained mechanically tapping a graduated measuring cylinder containing the powder sample. The Hausner ratio with compressibility index was calculated (values were shown on device's screen). All measurements were carried out three times.

2.3.4. Shape of the Microcapsules

The morphological characteristics of the microcapsules produced with different technological methods were evaluated by optical microscopy, Eclipse 50i (Nikon, China). Small amounts of powders were placed on the surface of double-sided tape and 100× magnification was used.

2.3.5. Solubility

A total of 5.0 ± 0.05 g of the microcapsules were mixed with 30 mL of purified water for 30 min using a vortex mixer (IKA, Staufen, Germany). The solution was centrifuged at 1789× *g* for 20 min at 25 °C. After centrifugation, the sediment was transferred to preweighed Petri dishes and dried at 105 °C to constant weight. The solubility (%) of the microcapsules' powder was calculated as the percentage of weight of sediment divided by the weight of the sample and multiplied by 100%.

2.3.6. Size Distribution of Microcapsules

By using a laser, the volume weighted mean size (D 4,3) of the essential oil emulsions were calculated. Using the Mastersizer 3000 with the Hydro EV unit (Malvern Panalytical Ltd., Malvern, UK), diffraction size and distribution were evaluated. To achieve laser obscuration, samples were introduced drop-by-drop to the dispersant (water). The D10, D50, and D90 percentile values were used to describe the formulations.

2.4. Total Content of Active Compounds and In Vitro Release and Analysis of Microcapsules and Gel Tablets

2.4.1. Total Phenolic and Flavonoid Content

Total phenolic content was determined using the modified Slinkard et al. method [18]. A 0.5 mL volume of sample was mixed with 2.5 mL Folin–Ciocalteu's phenol reagent (1:9 diluted in distilled water) and 2.0 mL of 7% (*w*/*v*) sodium carbonate. Absorbance was measured at 765 nm after 1 h using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The calibration curve was prepared using gallic acid. The results were expressed as gallic acid equivalents per gram dry weight (mg GA/g dw).

Flavonoid content was also determined using a slightly modified spectrophotometric method by Zhishen et al. [19]. Briefly, 0.1 mL of sample was added to 1.0 mL 96% (*v*/*v*) ethanol, 0.05 mL 33% acetic acid, 0.15 mL 10% aluminum chloride, and 2.0 mL 5% hexamethylenetetramine solutions. The samples were kept in the dark for 30 min and the absorbance was measured at a wavelength of 475 nm. The results were expressed as rutin equivalents per gram dry weight (RE/g dw).

2.4.2. In Vitro Release of Active Compounds

In vitro release was performed in gastric and intestinal media as previously performed in the study of Matulyte et al. [20]. The research was achieved using the Sotax AT7 Smart Dissolution System (SOTAX AG, Aesch, Switzerland). Gastric medium was prepared according to the European pharmacopeia [21] using: 2.0 g of NaCl, 80 mL of 1 M HCl solution, 3.2 g of pepsin, and distilled water up to 1000 mL (pH = 1.2).

Simulated intestinal juice was prepared using 6.8 g of KH_2PO_4, 77.0 mL of 0.2 M NaOH solution, 10 g of pancreas powder, and distilled water up to 1000 mL. The samples in gastric medium were incubated for 30–90 min and then moved to intestinal medium for 30–90 min. Samples for HPLC analysis were taken every half hour. The total in vitro release time interval was 0–180 min. The samples were filtered and prepared for analysis using headspace-gas chromatography with mass spectroscopy (HS-GC–MS) for volatile compounds from essential oil and high-performance liquid chromatography (HPLC) for isoflavones daidzein and genistein, and glycyrrhizin.

2.4.3. Chromatographic Analysis of Microcapsules

Nutmeg essential oil's chemical compounds were determined using HS-GC–MS. Conditions and equipment used are described in a previous study by Matulyte et al. [22]. Quantitative analysis of volatile compounds was determined in microcapsules and chewable gel tablets.

The release of daidzein, genistein and glycyrrhizin were determined using high-performance liquid chromatography with diode array detectors (HPLC–PDA). The analysis was conducted using the Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan), equipped with an SPD-M20A diode array detector (DAD). The isoflavones' research conditions are described in the previous work of Kazlauskaite et al. [17].

The mobile phase for glycyrrhizin detection consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (acetonitrile). The linear gradient elution profile was as follows: 95% A/5% B at 0 min, 5% A/95% B at 30 min, 95% A/5% B at 36 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. The chromatographic column was ACE C18 (250 × 4.6 mm) 5 µm, column storage temperature was 30 °C, UV/Vis range was 200 to 400 nm. The range of linearity of glycyrrhizin was 0.480 to 492 µg/mL. The contents were expressed as µg/g dry weight (dw). The calibration equation was y = 7210x + 9660, coefficient of determination (R^2) was 0.9997. The limit of detection (LOD) was 0.095; limit of quantification (LOQ) was 0.42.

2.5. Antioxidant Activity of Microcapsules

The antioxidant activity of microcapsules and gel tablets were analyzed by spectrophotometric ABTS, DPPH and FRAP assays. ABTS, DPPH and FRAP methods were performed in the same as described in our previous study [17].

The ABTS method was initially reported by Miller and colleagues. It is based on the ability of an antioxidant to stabilize the ABTS colored cation radical, which can be previously formed by the oxidation of ABTS by methemoglobin and hydrogen peroxide [23]. Briefly, the test solution was prepared by mixing 2 mL of ABTS working solution with 200 µL of each test sample. The mixture was stored in a dark at room temperature for 30 min. The change in absorbance of the mixture was measured with a spectrophotometer at 734 nm. The calibration curve was obtained with a Trolox. The results were expressed as Trolox equivalents per gram dry weight (TE/g dw).

The DPPH method was first reported by M. Blois [24]. DPPH free radical scavenging is an accepted mechanism for screening antioxidant activity. The method used 2.0 mL of prepared DPPH solution mixed with 2.0 mL of the test samples. The reaction mixture was mixed and then incubated in the dark for 30 min. The absorbance was read at 517 nm. The calibration curve was obtained with a Trolox. The results were expressed as Trolox equivalents per gram dry weight (TE/g dw).

The FRAP assay was established by Benzie and Strain (1996) [25] and was used to determine the reducing activity in the plant raw material. The FRAP working reagent was prepared using 0.3 M acetate buffer, 10 mM TPTZ solution with 40 mM HCl, and 20 mM ferric chloride solution. A 10 µL amount of test sample was mixed with 200 µL of FRAP reagent and left in the dark. After 1 h, the absorbance of the mixture was measured at 593 nm. The calibration curve was obtained with ferrous sulphate. The results were expressed as ferrous sulphate equivalents per gram dry weight (FS/g dw).

2.6. Gel Tablets Preparation

2.6.1. Gelatin Gel Tablets' Preparation

Gelatin was poured with water and glycerol solution and left for 20 min to swell. In the porcelain dish apple juice, apple puree, and fruit powder were mixed. Gelatin was dissolved using a water bath (the temperature of gelatin mass was 50–60 °C) and the mass of the fruit was added to it. Microcapsules (5% *w*/*w*) were added in the gelatin gel mass, then poured into silicone molds and kept for 4 h in the refrigerator. After that gel tablets were taken out and kept in a plastic container at room temperature. The composition of gelatin gel tablets is given in Table 2. Although apple juice and apple puree were prepared in the laboratory, the products had no added sugar.

Table 2. Gelatin gel tablets composition.

Ingredient	Amount (%)
Apple juice	38.8
Apple puree	14.9
Banana powder	20.7
Gelatin	7.3
Water	10.3
Glycerol	8.0

2.6.2. Pectin Gel Tablets Preparation

Five different compositions of pectin gel tablets were prepared (Table 3). First, apple juice, apple puree and other ingredients (except citric acid and pectin) were mixed and heated up to 40 °C. Pectin was added to the warm mass and dissolved. Next, mass was heated up to 100 °C and citric acid was added. Microcapsules (5% *w*/*w*) were added to the pectin gel mass, then poured into silicone molds and kept for 24 h at room temperature. After that pectin gel tablets were taken out and kept in a plastic container at room temperature.

Table 3. Pectin gel tablets' composition.

Ingredient	Sample				
	A	B	C	D	E
	Amount (%)				
Apple juice *	76.2	65.2	46.1	40.4	-
Apple puree *	20.3	25.3	41.4	23.2	55.7
Blackcurrant puree *	-	-	9.5	-	-
Buckthorn puree *	-	6.3	-	-	-
Banana powder	-	-	-	-	29.3
Lingonberry powder	-	-	-	15.4	-
Pectin	2.0	1.7	1.5	2.0	2.0
Citric acid 50% solution	1.5	1.5	1.5	1.5	2.0
Water	-	-	-	17.5	11.5

* Ingredients were prepared in the laboratory, the products had no added sugar.

2.7. Gel Tablets' Physical Parameters Determination

Firmness, springiness, hardness, and stickiness were measured (n = 3) by a texture analyzer, Ta.XT.plus (Texture Technologies, New York, NY, USA).

The parameters of texture were: return speed 10 mm/s; force 1 g; strain 50%; pre-test speed 1.00 mm/s; test speed 1.00 mm/s; post-test speed 10 mm/s; hold time 60 s; and trigger force 5.0 g.

2.8. Statistical Analysis

Data were analyzed using SSPS version 20.0 (IBM Corporation, Armonk, NY, USA). Physical parameters, antioxidant activity, total phenolic and flavonoid content experiments were performed three times. Data are expressed as mean ± standard deviation (S.D.). Comparisons between three different measurements were made using Friedman and Wilcoxon tests. In addition, comparisons between the two groups were made by the Mann–Whitney U test. The results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Preparation of Microcapsules and Physical Parameters Evaluation

Core material (red clover, liquorice extract and nutmeg essential oil) for spray-drying was obtained as explained in section "materials and methods", using extraction techniques that have been developed previously to produce the largest numbers of isoflavones daidzein and genistein, saponin glycyrrhizin and main volatile monoterpenes found in nutmeg essential oil α-pinene, sabinene, β-myrcene and β-terpinene.

For lyophilization and spray drying the exact formulations of emulsions were used to prepare microcapsules.

3.1.1. Evaluation of Differently Prepared Microcapsules Yield, Moisture, and Quality

After the production of microcapsules LM and SDM, the powder's yield, moisture content and technological properties were evaluated (Table 4). The formulation of the microcapsules was the same.

Table 4. Physical microcapsules' parameters.

Samples	Yield, %	Moisture, %	Carr Index, %	Hausner Ratio Value
LM	85.34 ± 3.17 *	4.45 ± 0.23 *	20.83	1.263
SDM	45.12 ± 7.81	3.24 ± 0.36	38.71 *	1.632 *

* The difference between samples' parameters statistically significant at $p < 0.05$.

The yield was 85.34% when preparing microcapsules using the lyophilization method and using spray-drying—45.12%. Results from the lyophilization method were much better. According to the findings, a severe buildup of the material on the cyclone walls of the spray dryer caused lower yields to be obtained by spray-drying than by lyophilization. Nevertheless, the moisture content in the SDM samples were significantly lower than LM's. The moisture parameter is crucial because it can influence powder's other physical parameters like flowability. According to published research, moisture content ought to fall below 4–5% and water activity should be below 0.20–0.25 to ensure stability [26]. Therefore, even though SDM moisture is lower than LM, it does not exceed the possible moisture limit.

The flowability of LM and SDM samples were different. According to compressibility index and Hausner ratio LM microcapsules' powder was of fair quality, but SDM has very poor flowability (Table 4). Index's and Hausner ratio values are presented in the Monograph of the European Pharmacopoeia [27]. Powders with higher moisture and irregular form have worse flowability [28]. In the pharmaceutical industry, powder flowability is a crucial parameter that affects manufacturing efficiency because of layer generation capabilities and content uniformity [29,30]. Consequently, preparing the powders flowability parameter should be a priority to produce quality product.

The shape of LM and SDM microcapsule powders differed. Both samples' microcapsules shapes and sizes were unequal. However, LM sample shapes were bigger compared with the SDM sample. Whereas spray-dried microcapsule powder featured small splinter-shaped particles and a spherical shape, freeze-dried microcapsule powder had an irregular form, like shattered glass (Figure 1). These irregularities in shape can be one of the reasons why powders did not have had excellent flowability.

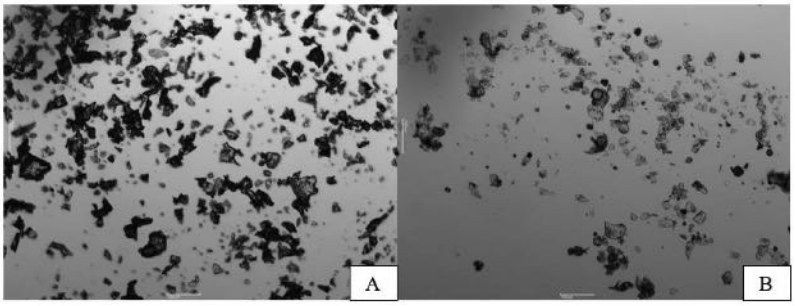

Figure 1. Structure of microcapsules (microscope magnification 100 times, (**A**)—LM microcapsules, (**B**)—SDM microcapsules).

3.1.2. Microcapsules' Solubility and Essential Oil Particles' Distribution

Spray-dried microcapsules had a solubility of 95.16%, while lyophilized microcapsules had a solubility of 94.69%. Spray-dried microcapsules were more soluble, nevertheless, the differences were statistically insignificant ($p > 0.05$). It is possible that particle size affected solubility.

Encapsulated essential oil (*Myristica fragrans* Houtt.) particle size and distribution were determined using a Mastersizer 3000. The particles produced using lyophilization and spray drying were 13.51 μm and 7.33 μm, respectively, expressed as D [4,3] (De Brouckere mean diameter).

From the graph provided in Figure 2, part A, uniformity in essential oil is irregular in LM microcapsules, while in part B it is more even. Uniformity is a measure of absolute deviation from the median, LM sample uniformity was 0.533 and SDM 0.379. Therefore, LM essential oil particles' sizes (in microcapsules) had a wider distribution range compared to the SDM samples. As mentioned before, this is influenced by the fact that the microcapsules obtained during lyophilization were broken glass-shaped, while spray-dried microcapsules were more rounded.

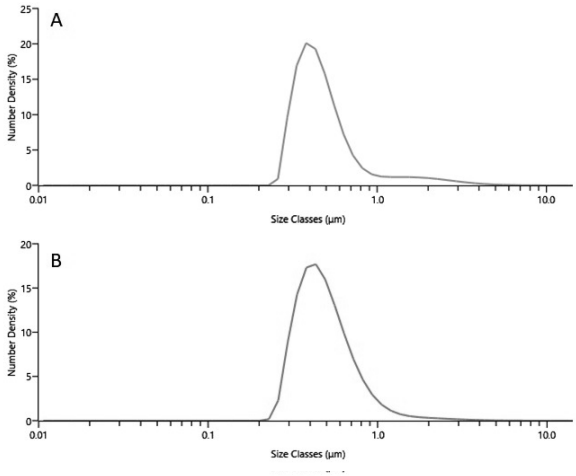

Figure 2. Essential oil particles' distribution in microcapsules (**A**)—LM microcapsules, (**B**)—SDM microcapsules.

3.2.1. Antioxidant Activity of Microcapsules

Red clover extracts, that are used in both microcapsules' formulation, contains phenols—isoflavones, which have been shown to have wide varieties of biological activities such as antibacterial, antiviral, antioxidant and anti-inflammatory [35]. Glycyrrhizin found in liquorice extract has been reported to possess anti-inflammatory, and antioxidant activities and can stimulate endogenous production of interferons [36]. These two extracts also contain a variety of other phenolic compounds, which may be lost to evaporation, thermal decomposition, or chemical and enzymatic degradation. According to studies, the main process causing the reduction in polyphenol concentration is due to the latter factors [37].

All the results obtained with LM samples were significantly higher compared to SDM samples obtained using spray-drying. Total phenolic content determined in the LM sample was 11.47 ± 0.01 mg GA/g dw and SDM 8.56 ± 0.42 mg GA/g dw. Therefore, the freeze-drying process was less harmful for phenols than spray-drying. These results agree with those reported in the literature; however, it was stated that both drying processes led to a partial loss of compounds like small molecular carbonyls, furans, and phenols [38]. In another study it was reported that freeze drying can generate a more significant amount of isoflavone powder from soybean cake than vacuum drying and spray drying [39].

Similar results for phenolic content was observed for total flavonoid content. LM results were higher compared to the same formulation sample SDM (Table 5). All the phenolic content results correlated with antioxidant activity determined using three different methods (DPPH, ABTS and FRAP). All the results obtained with the LM samples were statistically higher than SDM ($p < 0.05$). Comparing both samples' preparation methods, antioxidant activity of samples prepared using freeze drying (LM) were 29.48% higher than SDM (ABTS method); the FRAP method resulted in LM being 31.34% higher than SDM; the DPPH method showed that LM samples were 40.45% higher. These results were similar to other research. In the study of Koffi et al., it was found that samples prepared using lyophilization and freeze-drying methods from Justicia secunda leaves' polyphenols content and the antioxidant capacity of freeze-dried extract were higher than those of spray-dried extract. Additionally, freeze-dried powder gave better recovery yields [40].

Table 5. Total phenolics, flavonoids content and antioxidant activity of microcapsules.

Sample	Total Phenolic Content, mg GA/g dw	Total Flavonoids Content, mg RU/g dw	DPPH, µg TE/g dw	ABTS, µg TE/g dw	FRAP, mg FS/g dw
LM	11.47 ± 0.01 *	7.56 ± 0.02 *	8.85 ± 0.01 *	136.73 ± 0.73 *	252.62 ± 0.01 *
SDM	8.56 ± 0.42	5.21 ± 0.22	5.27 ± 0.02	96.41 ± 0.93	173.25 ± 0.25

* The difference between samples parameters statistically significant at $p < 0.05$.

3.2.2. In Vitro Release of Active Compounds from Microcapsules

Since the lyophilized powder's yield and encapsulation were more efficient than the spray-dried powder, further studies were performed with only LM microcapsules' powder. The release was performed for 180 min (90 min in simulated gastric medium and 90 min in simulated intestine medium). Nevertheless, microcapsules fully dissolved after 30 min in intestine media, therefore, the results of main compound release in graphs are present until 120 min.

Although genistein has moderate intestinal absorption, larger doses could not be absorbed because of their poor solubility, necessitating the requirement of appropriate nutraceutical formulations. One of the main causes of genistein's limited oral bioavailability is likely to be extensive metabolism [41]. However, orally administered genistein is more bioavailable than daidzein in foods and drinks. Regardless, different pharmacokinetics of daidzein have been reported. Still, these limitations of interpreting the findings relate to whether data from administration of pure compounds can really be translated to isoflavones contained within the food matrix [42–44].

It was suggested that glycyrrhizin from liquorice roots can be a potential bioactive constituent for managing oral diseases, when applied locally. However, it also can be administered orally, but it is metabolized to glycyrrhetinic acid by intestinal bacteria which contain β-D-glucuronidase, and that can cause side-effects as hypokalemia and hypertension [45,46].

The rate at which essential oils are required to be released from encapsulations differs depending on the application.

Microencapsulation protects volatile compounds from environmental exposure for a more extended period of time [47,48]. Spray-dried microcapsules with nutmeg essential oil did not lose their characteristic aroma when stored at room temperature for a month, and 4 active volatile compounds out of 11 (identified compounds in the essential oil) were identified [49]. The main volatile compounds identified in microcapsules (in this study) were: α-pinene, which modulates antibiotic resistance, by reducing the MIC value of ciprofloxacin, erythromycin, and triclosan, up to 512 times [50]; sabinene, which has anti-oxidant and anti-inflammatory properties and could be a potential compound for skeletal muscle atrophy prevention [51]; β-myrcene, which has potential analgesic, sedative, anti-diabetic, antioxidant, anti-inflammatory, antibacterial, and anticancer effects [52] and β-terpinene, which has an anti-inflammatory effect and inhibits the production of pro-inflammatory mediators [53].

After evaluating the release of the active compounds from microcapsules, it was found that microcapsules dissolve fully in intestinal medium after 60 min and glycyrrhizin, daidzein and genistein also dissolve. The concentrations of compounds after 120 min after release was 428.25 µg/mL, 235.63 µg/g and 91.36 µg/g (glycyrrhizin, daidzein and genistein, respectively) (Figure 3). The lowest concentration of all the compounds was genistein. In gastric medium, isoflavones, daidzein, and genistein were released gradually as well as in the intestinal medium. Regardless, glycyrrhizin release was most intense between 60 and 90 min (Figure 3).

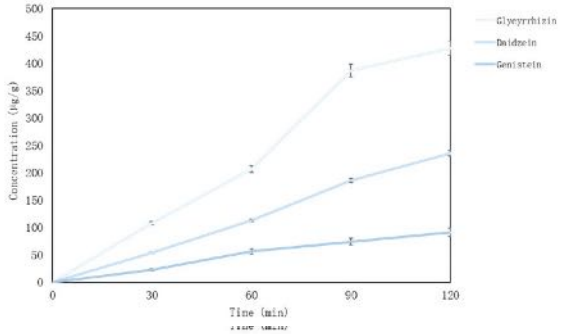

Figure 3. Main compounds (glycyrrhizin, daidzein and genistein) released in vitro from red clover and liquorice microcapsules.

The percentage of monoterpenes is calculated based on the concentrations of compounds identified in the essential oil. The maximum number of essential oil compounds was determined after 30 min—later their amounts decrease. Sabinene is released the most—58.51% (after 30 min), the results are presented Figure 4.

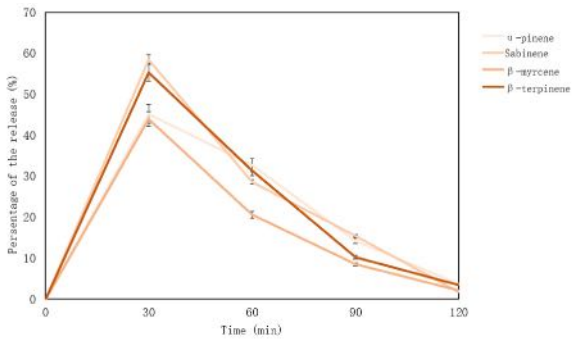

Figure 4. Monoterpenes' release from nutmeg essential oil-loaded lyophilized microcapsules (%).

The spray-drying method is more popular than lyophilization, the release of active compounds of nutmeg essential oil loaded microcapsules is determined for powders produced by the spray-drying method [49,54,55].

3.3. Gel Tablets Preparation and Physical Parameters' Determination and Release in Simulated Media

Gelatin base chewable gel tablets were prepared based on previous studies' results [22,56] and the microcapsules were added. For the pectin gel tablets, five compositions were modelled and one (C, Table 3) was chosen for the following research.

The best composition was selected by the appearance and flavor of the tablets (E tablets were very hard, A and B were soft and were not taken out from the form without mass loss, and D lacked flavor intensity; compositions are shown in Table 3). Both tablets' bases were prepared without added sugar. Gel tablets had appearance differences: gelatin tablets had smooth surface, while pectin was rough (Figure 5).

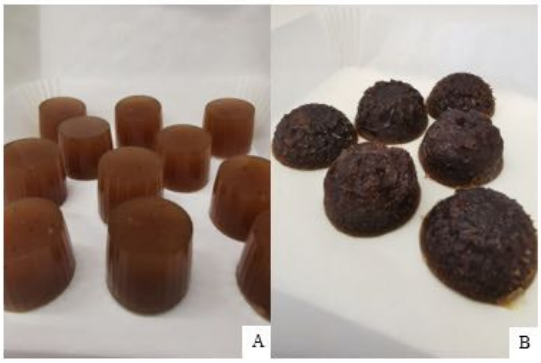

Figure 5. Gel tablets' appearance (**A**) gelatin gel tablets; (**B**) pectin gel tablets.

Citric acid was used as a flavor corrigent in gelatin base tablets, however, it was used as a gelification initiator [57] in pectin base tablets. HM pectin forms physical gels at pH < 3.5 [58]. Solid materials in the composition also influenced gel texture [59].

3.3.1. Physical Parameters' Comparisons of Gel Tablets with Different Bases

The texture of tablets is significantly affected by different polymers. Compared to pectin tablets, gelatin tablets were less firm and harder, but more elastic. Pectin base tablets with lyophilized microcapsules were firmest and hardest compared with other samples (Table 6). Microcapsules reduced the stickiness of gelatin base tablets by 68% after the addition of microcapsules.

Table 6. Chewable gel tablets texture properties depending on the base.

Samples	Mass of the Sample	Firmness (g)	Stickiness (g)	Hardness (g)	Springiness (%)
P [a]	5.51 ± 0.67	1027.8 ± 179.04 ***	−24.39 ± 1.85 ***	609.3 ± 233.03 ***	41.79 ± 0.74 ***
PLM	6.04 ± 0.58	1303.3 ± 377.28 *	−27.25 ± 15.15	798.09 ± 201.12	38.38 ± 0.23 *
G [b]	5.73 ± 0.25	895.12 ± 62.78	−20.23 ± 5.92	738.27 ± 27.24	64.58 ± 1.35
GLM	5.77 ± 0.36	548.69 ± 12.63 **	−34.04 ± 2.91 **	549.51 ± 11.36 **	68.02 ± 2.53

[a]—control pectin base gel tablet, [b]—control gelatin base tablet. * $p < 0.05$ versus pectin base tablets, ** $p < 0.05$ versus gelatin base tablets, *** $p < 0.05$ versus gelatin base tablet.

Compared microcapsules' influence on tablet texture was evaluated and microcapsules increased the firmness and hardness of tablets. Nevertheless, the stickiness decreased, and tablets were less sticky. The correlation of springiness was not determined, pectin tablets were less elastic than gelatin (compared with pectin and gelatin base control tablets, respectively).

Microcapsules' tablets increased firmness and hardness (about 27% and 31%, respectively) in the pectin base, nevertheless, gelatin tablets were soft (about 63% and 34%, respectively).

In other studies, the firmness and springiness of chewable gel tablets with gelatin was 375.45 g and 74.09% (27% of the sugar was in composition) [56]. Gel tablets with 7% of gelatin and 70% of sweeteners springiness was 97.5% [60]. The texture results of gel tablets are compared with those of tablets of similar composition. It can be concluded that natural fruit powder significantly changed the texture of gel tablets.

Pectin base gummies had about 612 N of hardness (1.5% of pectin and 50% of sweeteners were in composition; 100 g of sample was pressed) [61]. It is too difficult to compare this study's results with other data because a different texture test was applied and gel tablets with pectin are less popular than gelatin.

3.3.2. Release of Active Compounds from Different Bases Gel Tablets

Two different methods evaluated the concentrations of active compounds. Isoflavones and glycyrrhizin were determined by HPLC and monoterpenes—HS-GC–MS.

The highest concentrations of active compounds were determined in pectin base tablets: 9.1% of glycyrrhizin, 30.66% of daidzein and 53.32% of genistein. These compounds released gradually from the tablets and their amount increased depending on the duration of the study (Figure 6).

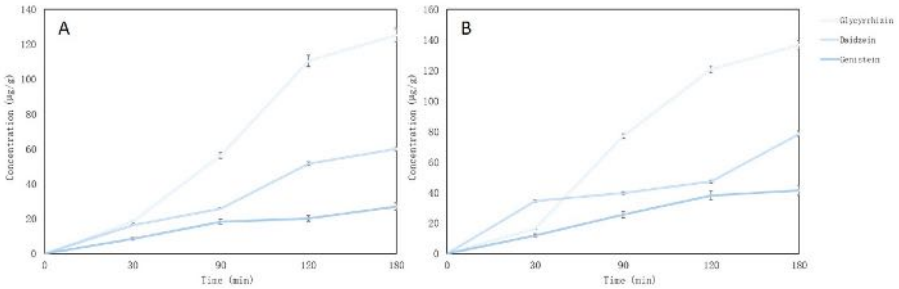

Figure 6. Isoflavones and saponin release from nutmeg essential oil-loaded lyophilized microcapsules (%); (**A**) gelatin gel tablets; (**B**) pectin gel tablets.

The last test sample was obtained after 360 min when the chewable tablets in the gut juice disintegrated. This test was performed to calculate the effectiveness of gel tablets. The effectiveness of the pectin tablet encapsulation was 71.36 ± 4.22% for glycyrrhizin, 72.03 ± 6.84% for daidzein and 64.83 ± 2.37% for genistein. In the gelatin tablet, active compounds' release effectiveness was 63.41 ± 3.64% for glycyrrhizin, 61.78 ± 4.23% for daidzein and 60.11 ± 3.82% for genistein. The concentration of active compounds was lower in gelatin tablets compared to pectin. Incomplete relaxation of the compounds may be due to ballast materials (used puree, pectin, gelatin), temperature during production, and incomplete solubility of compounds in gut media.

Most of the monoterpenes were released after 30 min. (they are volatile compounds, so during a long experiment, their evaporation is not prevented) (Figure 7). The highest amount was of β-terpinene. The highest amount of compounds were released from pectin base tablets ($p < 0.05$) except for sabinene, as its maximum amount was released from the gelatin base ($p > 0.05$).

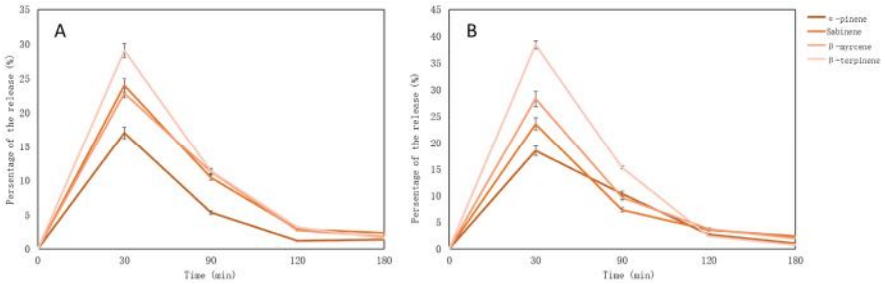

Figure 7. Monoterpenes' release from nutmeg essential oil-loaded lyophilized microcapsules (%); (**A**) gelatin gel tablets; (**B**) pectin gel tablets.

According to other studies, the maximum release of essential oil compounds was found after 30 min. (gelatin-based chewable gel tablets), however, other compounds had been identified [56]. The amount of volatile compounds from essential oils after 360 min was not investigated, because already after 60 min in gut media, the amount of compounds decreased strongly, and due to evaporation of the compounds, this study was not appropriate.

This technology with pectin based chewable tablets with lyophilized microcapsules can be applied in the development of pharmaceutical products. They can be adapted according to extracts and essential oils that are microencapsulated, and for biological activity (antiviral, antibacterial and antioxidant [17]). This technology is easily applicable to other essential oils and extracts, as the active compounds are preserved, including thermolabile compounds.

4. Conclusions

The microcapsules obtained by the lyophilization, and spray-drying methods showed different properties, although they were produced using the same formulation. Significant differences were found between the form, yield, and antioxidant activity. Lyophilized microcapsules had better properties, which led to their further use in the study.

The polymer Influenced tablet texture and active compounds' release. Chewable gelatin tablets were soft and elastic, while pectin tablets were hard, firm, and less sticky. Lyophilized microcapsules had a statistically significant influence on tablet parameters compared with control pectin tablets. Active compounds were released in larger quantities from pectin-based tablets. There was no statistically significant difference in the amount of compounds released in the gastric and intestinal media, the release of the compounds was significantly affected by the duration of the study. The method and formulations of freeze-dried microcapsules in pectin or gelatin base are appropriate for use in the pharmaceutical or food industry for effective flavor (with no added sugar) or active substances' release during oral administration. Additionally, the chewable tablet with lyophilized microcapsules technology is easily applicable for other substances sensitive to environmental factors.

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Paper 6

Title: Impact of Polyvinylpyrrolidone-Vinyl Acetate Copolymer and Sodium Starch Glycolate Excipients on Phenolic Extraction from Red Clover: Enhancing Biological Activity and Antioxidant Potential

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Article

Impact of Polyvinylpyrrolidone-Vinyl Acetate Copolymer and Sodium Starch Glycolate Excipients on Phenolic Extraction from Red Clover: Enhancing Biological Activity and Antioxidant Potential

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Abstract: Adding certain excipients during the extraction process can enhance the concentration of target compounds, leading to potentially increased biological properties of the plant extract. This study explores the impact of PVP/VAC and SSG excipients on red clover bud extracts, aiming to enhance their concentration of target compounds and, consequently, their biological properties. The antioxidative potential was evaluated using DPPH, ABTS, and FRAP methods, and the chemical profile was determined using mass spectrometry. Antibacterial activity against various strains was determined through the minimal inhibitory concentration (MIC) method. The results revealed that the excipient-enriched samples exhibited significantly elevated antioxidant activities as well as phenolic and flavonoid contents compared to control samples. Notably, sample V1E3 demonstrated the highest antioxidant potential, with 52.48 ± 0.24 mg GAE/g dw (phenolic content), 463 ± 6.46 μg TE/g dw (ABTS), 12.81 ± 0.05 μg TE/g dw (DPPH), and 29.04 ± 1.16 mg TE/g dw (post-column ABTS). The highest flavonoid content was found in the S1E3 sample—24.25 ± 0.17 mg RU/g dw. Despite the increased antioxidant potential, no significant variance in antimicrobial activity was noted between the test samples and controls. This implies that excipients may hold the potential to enhance the biological properties of red clover extracts for pharmaceutical applications. These findings contribute valuable insights into optimizing extraction processes for improved functionality and application of plant-derived compounds in therapeutic formulations.

Keywords: excipients; red clover; *Trifolium pratense*; isoflavones; polyphenols; polyphenolic activity; extraction

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1. Introduction

Polyphenolic compounds in plant extracts are known for their radical scavenging capacity and antioxidant activity, with flavonoids being one of the phenol groups that possess a wide range of biochemical and pharmacological actions such as anticarcinogenic, antiviral, antimicrobial, antithrombotic, anti-inflammatory, and antimutagenic activities [1]. Flavonoids act as free radical scavengers, making them effective antioxidants in oils, fats, and emulsions [2]. Isoflavones, especially genistein and daidzein, like flavonoids, also possess antioxidant activities and are found mainly in legumes. In the past decade, extensive research has focused on various plant extracts that are rich in isoflavones due to their potential protective effects against a range of disorders. These include cardiovascular disease, cancer, hyperlipidemia, menopause symptoms, osteoporosis, and diabetes. Additionally, these extracts exhibit diverse bioprotective properties such as antioxidant, antimutagenic, anticarcinogenic, and antiproliferative activities [3–6].

Nowadays, soybeans and soy-derived foods are a major source of isoflavonoids in human nutrition. However, due to the allergenic nature of many soy products and the difficulty in obtaining genetically modified organism-free soybeans, there is a need for alternative sources of isoflavonoids. Among the alternative sources of isoflavones is red clover (*Trifolium pratense* L.), which has recently received considerable interest as a potential source of health-enhancing isoflavones [7–10]. A study found that 36% of the total red clover extract was identified. Within this analysis, 22 compounds were identified. Notably, the extract consisted of 35.54% isoflavones, 1.11% flavonoids, and 0.06% pterocarpans, while coumarins and tyramine were detected in trace amounts ($\leq$0.03%) [11]. It is of the utmost importance to study the antibacterial and antioxidant properties of red clover flower samples' phenolic components, particularly isoflavones, which are known to protect against several conditions. With antibiotic resistance becoming a global concern, plant extracts and phytochemicals with known antimicrobial properties are significant for treatment and prevention [12].

Plants accumulate a multitude of phenolic compounds that may interact synergistically or antagonistically. Phenolic compounds exhibit synergistic or antagonistic interactions due to their diverse structures and functional groups. Similar structures and functional groups lead to cooperative effects, especially when compounds target the same receptors, enhancing their overall effects; e.g., isoflavone phytoestrogens have estrogen receptor binding abilities [13]. Conversely, antagonistic interactions may occur when compounds compete for binding sites. Phenolic compounds, known for their antioxidant properties, can demonstrate synergy in combined effects, while antagonistic interactions may result from interference with their antioxidant activity. These compounds also modulate enzymatic activity, showing synergy in collectively enhancing or inhibiting enzyme pathways. However, antagonistic interactions may arise if compounds interfere with each other's impact on enzymatic processes. Additionally, phenolic compounds can disrupt or enhance cellular metabolic pathways, with synergistic or antagonistic effects depending on their collective impact [14–16].

However, the extraction conditions could potentially degrade these compounds, or the properties of the solvent employed might hinder their efficient extraction from the plant. Using excipients in extraction media can help increase the yields of target compounds depending on their mechanism of action.

Previous research has shown that the use of sodium starch glycolate (SSG) and polyvinylpyrrolidone-vinyl acetate copolymer (PVP/VAC) increases the yield of the isoflavones daidzein and genistein; therefore, the excipient's effect was determined by comparing the antioxidant potential and microbial activity of the red clover extracts [17]. This was due to the fact that modern carriers with a large surface area and high absorption capacity can help improve the oral bioavailability of poorly water-soluble drugs. Incorporating higher doses of these compounds into liquid–solid systems makes them more soluble and can improve bioavailability.

SSG, a commonly used super-disintegrant, accelerates the disintegration and dissolution of solid dosage forms, being FDA-approved for both prescription and over-the-counter medications [18,19]. SSG is produced through the chemical modification of starch, specifically carboxymethylation, for heightened hydrophilicity and cross-linking to decrease solubility [20].

SSG functions as a super-disintegrant by swiftly absorbing water, swelling, and exerting mechanical stress on the tablet matrix, facilitating rapid disintegration. Its swelling properties improve water penetration, aiding active ingredient dissolution. Optimization involves considerations such as particle size, degree of substitution, and concentration, while factors like pH, temperature, and compression force offer insights into disintegration and dissolution, with higher pH values and temperatures enhancing its effectiveness [19,21].Thus, reflux (high temperatures) and ultrasound aid in faster solvent dissipation.

PVP/VAC is a commonly used excipient in the pharmaceutical industry. It is soluble in both water and alcohol and is valued for its ability to improve the solubility of poorly soluble compounds by reducing particle size and increasing surface area for dissolution [22]. The copolymer can serve as a carrier or stabilizer for drug molecules, averting aggregation and maintaining an amorphous state that is conducive to dissolution and absorption in the body [23]. Daily dosage recommendations stand at 100 mg per tablet (10% in a 1000 mg tablet) for high consumers, with anticipated exposures of 200 mg PVP/VAC/day for children and 700 mg PVP/VAC for adults [22]. Comprising 1-vinyl-2-pyrrolidone and vinyl acetate in a 6:4 mass ratio, the current literature on PVP/VAC indicates solid dispersion preparation as a predominant approach to enhance the solubility and bioavailability of water-insoluble drugs [24–26].

By conducting a comparative analysis with a control group lacking these excipients, we investigated whether the inclusion of PVP/VAC and SSG could enhance the biological and microbial activity of the plant extract. This is the first study to investigate the effect of PVP/VAC and SSG excipients on plant biological activity. Consequently, it contributes valuable insights to the scientific understanding of the role played by these excipients in the development of red clover-based medicinal applications.

Therefore, this study aimed to determine the excipient's effect by comparing the antioxidant potential and microbial activity of the red clover extracts prepared using excipients and without them.

2. Materials and Methods

2.1. Plant Material and Reagents

Red clover samples were harvested in Laičiai, Kupiškis district, Lithuania, on 31 July 2021. Prior to extraction, the flower buds were ground to a fine powder using an Ultra-Centrifugal Mill ZM 200 (Retsch, Haan, Germany). The grinding parameters included a force of 4025 g and a 0.5 mm trapezoid-hole sieve. Subsequently, the moisture content of the milled red clover was assessed using an MLB apparatus (KERN & Sohn GmbH, Balingen, Germany).

In this study, purified water was prepared using GFL2004 (GFL, Burgwedel, Germany), while deionized water was obtained from Millipore, SimPak 1 (Merck, Darmstadt, Germany). The excipients utilized in the samples included a vinylpyrrolidone-vinyl acetate copolymer (PVP/VAC) (Molecular weight~50,000) sourced from Yigitoglu Kimya, Istanbul, Turkey, and sodium starch glycolate (SSG) (Molecular weight~515.6862) acquired from Sigma-Aldrich, Taufkirchen, Germany.

To obtain standardized isoflavone samples, genistein, genistin, daidzein, and daidzin (Sigma Aldrich, Steinheim, Germany) were employed. A mixture of 2.21 mg of genistein and 2.18 mg of daidzein was prepared in 96% ethanol, while 3.30 mg of genistin and 3.03 mg of daidzin were mixed with 1 mL of DMSO and then diluted up to 10 mL using 96% ethanol.

Various reagents were employed in the experiment, including 96% ethanol (Vilniaus Degtinė, Vilnius, Lithuania), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), aluminum chloride, hexaethylenetetramine, dimethyl sulfoxide (DMSO), acetic acid, and Sabouraud dextrose agar (dehydrated) obtained from Sigma-Aldrich (Buchs, Switzerland). Additionally, potassium persulfate was procured from Alfa Aesar (Karlsruhe, Germany), while monosodium phosphate, ferrous sulfate heptahydrate, saline phosphate buffer, and hydrogen peroxide were sourced from Sigma Aldrich (Schnelldorf, Germany). Furthermore, disodium hydrogen phosphate was obtained from Merck (Darmstadt, Germany), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and Mueller-Hinton Agar (BBL, Baltimore, MD, USA) were also utilized.

2.2. Preparation of Red Clover Extracts and Sample Coding System

The extraction procedures followed the methodology outlined by Kazlauskaite et al. in a previous study [17]. Two extraction methods were employed: ultrasound combined with thermal hydrolysis, with varying ultrasound processing times, and heat-reflux extraction.

For the heat-reflux extraction, dried and milled flower heads were combined with solvent (either 50% ethanol v/v or purified water) in a round-bottom flask. The sample was refluxed in a sand bath at a solvent boiling temperature for 1 h and then cooled to room temperature. Following cooling, the sample underwent centrifugation, decantation, and filtration through PVDF syringe filters (pore size 0.22 µm, Frisenette, Knebel, Denmark).

Ultrasound-assisted extraction with thermal hydrolysis involved the use of an ultrasound bath (38 kHz) (Grant Instruments™, XUB12 Digital, Cambridge, UK) and a procedure similar to the heat-reflux method. The extraction involved macerating dried and milled flower heads in a solvent. Different conditions were applied, including the solvent type (50% v/v ethanol and purified water) and extraction time (10 or 30 min), with a processing temperature of 40 ± 2 °C. After processing, the sample was transferred to a round-bottom flask and refluxed in a sand bath for 1 h. Subsequently, the supernatant was treated as in the heat-reflux method.

The main samples in the experiment were prepared with excipients, PVP/VAC and SSG under the same conditions as previously mentioned, using either heat-reflux or ultrasound-assisted extraction with thermal hydrolysis. Purified water or 50% ethanol (v/v) served as the solvent, and the excipient was added to the extraction mixture along with the plant material. The PVP/VAC concentrations in the samples ranged from 1–5% (w/v in water) and 1% (in ethanol), while the SSG concentration was maintained at 1% (w/v in water and ethanol). Although the SSG concentration was increased to 5%, the resulting samples were too viscous for experimentation. Notably, all the excipients were removed from the extract during filtration, ensuring the absence of PVP/VAC or SSG in the final products.

The sample names comprised 4 characters: the first being a letter indicating the excipient (V for PVP/VAC and S for SSG), followed by the excipient concentration (V1 or V5 for PVP/VAC and S1 for SSG). The last letter indicated the solvent used (E for ethanol and W for water), with the number indicating the extraction conditions: 1 for reflux alone, 2 for reflux combined with 10 min ultrasound processing, and 3 for reflux combined with 30 min ultrasound processing.

Thus, if the sample is prepared with PVP/VAC 5% in water using reflux, the sample code will be V5W1. If these are control samples, there is no excipient letter in the code. Figure 1 shows a simplified coding scheme.

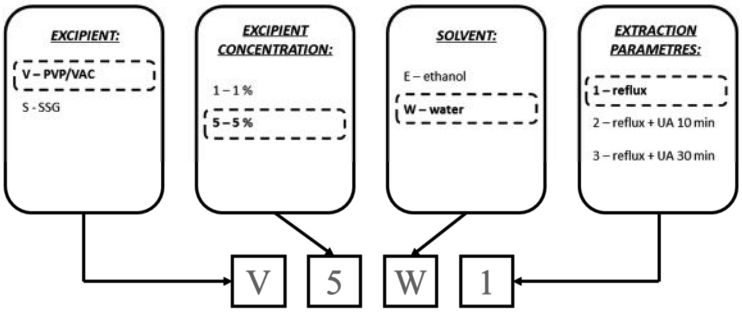

Figure 1. Schematic coding system of the samples.

2.3. Determination of Total Phenolic and Flavonoid Contents

The determination of the total phenolic content was carried out according to Dewanto et al. [27]. Folin–Ciocalteu's phenol reagent, along with 7% (*w*/*v*) sodium carbonate, was employed for the reaction. The absorbance was measured after 1 h using a spectrophotometer (765 nm) (Shimadzu UV-1800, Kyoto, Japan). The calibration curve used gallic acid (0–0.1 mg/g; y = 11.108; R^2 = 0.9981). The results were reported as gallic acid equivalent per gram dry weight (mg GA/g dw).

For flavonoid content determination, the extract was mixed with 96% (*v*/*v* ethanol, 33% acetic acid, 10% aluminum chloride, and 5% hexaethylenetetramine solutions in a test tube. A spectrophotometric analysis was conducted after 30 min (475 nm) using a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The flavonoid content was expressed as rutin equivalent mg RE/g dw (milligrams of standard antioxidant Rutin equivalents per gram of dry weight) and calculated using the following formula: TFC = C $\times$ Ve $\times$ F/M, where TFC represents the total flavonoid content in mg RE/g dw, C is the concentration of the standard used in mg/L, Ve is the volume of the solvent used in L, F is the dilution coefficient of the sample, and M is the mass of the sample in g. The calibration curve was established with rutin (0–0.5 mg/g; y = 5.0867; R^2 = 0.9985).

2.4. Antioxidant Activity

2.4.1. ABTS and DPPH Radical Scavenging Activity Assays

The ABTS radical was generated through the oxidation of ABTS with potassium persulfate [28]. The results were expressed as Trolox equivalent μg TE/g dw (micrograms of standard antioxidant Trolox equivalents per gram of dry weight). The calibration curve was obtained using Trolox (0–0.5 mg/g; y = 0.0001728x; R^2 = 0.9832).

A DPPH working solution (0.1 mM in ethanol) was prepared, and the reaction procedure followed the method described in a study by Gómez-Alonso et al. [29]. The calibration curve was obtained using Trolox (0–0.016 mg/g; y = 0.00623x; R^2 = 0.9923). The results were expressed as Trolox equivalent μg TE/g dw (micrograms of standard antioxidant Trolox equivalents per gram of dry weight).

2.4.2. Ferric Reducing Antioxidant Power (FRAP)

The sample was mixed with FRAP reagent as described in a previous study [30]. The calibration curve was obtained using ferrous sulfate (0–1 mg/g; y = 2.6272; R^2 = 0.9985). The results were expressed as ferrous sulfate equivalent mg FS/g dw (milligrams of standard ferrous sulfate equivalent per gram dry weight).

2.5. Antimicrobial Activity

The antimicrobial activity was assessed using the diffusion method on solid nutrient media, specifically Mueller–Hinton agar (BBL, Baltimore, MD, USA). Standard reference microorganisms from the ATCC (American Type Culture Collection) were employed to evaluate the antimicrobial efficacy of the tested extracts. Cultures of non-spore bacteria, including *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), and *Proteus vulgaris* (ATCC 8427), were cultivated for 20–24 h at 35–37 °C on the agar.

Bacterial suspensions were prepared from these cultivated cultures in sterile physiological sodium chloride (0.9%) solution and standardised using a McFarland standard indicator. Standardisation was achieved when the indicator value reached 0.5, indicating that 1 mL of the bacterial suspension contained approximately 1.5 $\times$ 10^8 cells of the microorganism.

Standard cultures of *Bacillus cereus* (ATCC 11778) spore bacteria were cultivated for 7 days at 35–37 °C on Mueller–Hinton agar. Following bacterial growth, the spore bacteria culture was carefully rinsed from the surface of the medium using a sterile physiological solution. The resulting suspension underwent a 30 min heat treatment at 70 °C and

was subsequently diluted with physiological saline until the spore concentration reached 10×10^6–100×10^6 per 1 mL.

Candida albicans (ATCC 10231) fungus standard cultures were grown for 20 to 24 h at 30 °C over a span of 72 h on Sabouraud dextrose agar. A fungal suspension was prepared from these cultivated cultures in physiological saline and standardised using a McFarland standard.

Different concentrations of red clover extract were dispensed into Petri dishes. Subsequently, 5 mL of 45 °C liquid Mueller–Hinton agar was added to each sterile Petri dish and thoroughly mixed with the added sample volumes. Once the agar solidified, suspensions prepared from reference microorganisms were inoculated into segments within the Petri dishes. The bacterial cultures were then incubated with samples in a thermostat for 20–24 h at 35 °C, followed by an additional 24 h of storage at room temperature.

The antimicrobial activity of the red clover flowers' ethanolic and aqueous extracts and antibiotics such as penicillin, ciprofloxacin, ampicillin, amoxicillin, and daptomycin were assessed.

2.6. HPLC–PDA Conditions for Isoflavone Quantification

Isoflavone quantification was conducted using the high-performance liquid chromatography with photodiode array detection (HPLC-PDA) method, following the exact procedures outlined in a study by Kazlauskaite et al. [31].

For the determination of the isoflavones daidzein, genistein, daidzin, and genistin, an ACE 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase of solvent A consisted of acetic acid, methanol, and deionised water (using the ratio of 1:10:89 $v/v/v$). Solvent B was made using acetic acid and methanol (1:99 $v/v/v$). The linear gradient elution profile was as follows: 80% A/20% B at 0 min; 30% A/70% B at 30 min; 90% A/10% B at 39 to 40 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. The absorption was measured at 260 nm.

2.7. HPLC Post-Column Antioxidant Activity

The HPLC post-column method was carried out using ABTS reagent according to the methodology described by Marksa et al. [32].

Following the introduction of samples into the HPLC detection system, the mobile phase, along with the sample, traversed through the reaction loop to a mixing tee. Concurrently, a Gilson pump 305 (Middleton, WI, USA) delivered 0.5 mL/min of ABTS reagent solution. The HPLC-ABTS system's reaction loop was maintained at a constant temperature of 50 °C. Upon interaction with ABTS, the antioxidants triggered a color change in the reagent, which was quantified using a Waters 2487 UV/VIS detector (Waters Corporation, Milford, MA, USA). The test solutions were detected at a wavelength of 650 nm, with the signal strength depicted as the peaks of negative active compounds.

The antioxidant activity of the extract compounds was assessed relative to the Trolox standard equivalent. A calibration curve was generated using a Trolox/ethanol solution at five dilutions within the 8.359–133.750 g/mL range, $R^2 = 0.999565$.

2.8. Qualitative Analysis of Red Clover Extracts Using LC-MS

Following the reverse phase liquid chromatography (RP-LC) separation, electrospray ionisation (ESI) (in the negative and positive modes) and mass spectrometric (MS) analyses were performed to determine the qualitative profile of the red clover extract. The LC/MS system was composed of a Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan) equipped with an LCMS-2020 mass spectrometer (Shimadzu, Tokyo, Japan).

The chromatographic separation was performed using a YMC-Triart C18 (YMC Karasuma-Gojo, Kyoto, Japan) (150 mm × 3.0 mm, 3 µm) analytical column, and its temperature was 40 °C.

Mobile phase A consisted of 0.1% formic acid in the water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. For each clover sample, 1 µL aliquot volumes

were injected into the chromatographic column. The linear gradient elution profile was as follows: 10% B at 0.01 min; 10% B at 1.0 min; 50% B at 10 min; 70% B at 20 min; 90% B at 23 min; 90% B at 26 min; 10% B at 27 min; 10% B at 30 min. The HPLC was run at a flow rate of 0.4 mL/min.

The optimum ESI conditions were set as 350 °C for the interface temperature, 250 °C for the DL temperature, 400 °C for the heat block temperature, 1.5 L/min for the nebulising gas flow, and 10 L/min for the drying gas flow. The positive ion and negative ion measurements were performed while switching alternately between the positive and negative ionisation modes. The m/z ranges for the positive and negative modes were 50–1000, the scan speed was 15.000 μ/s, and a step size of 0.1 m/z was used.

The quantitative analysis of the identified compounds was conducted utilising hyperoside as a reference standard. The compounds' concentrations were determined by applying a hyperoside calibration curve. The results were expressed as micrograms of hyperoside equivalent per milliliter (μg/mL). The calibration curve was prepared from a hyperoside solution at eighth dilutions in the range of 0.21–27.04 μg/mL, y = 22,498.4x + 2508.57, R^2 = 0.9998.

2.9. Statistical Data Analysis

The data were analysed using SSPS version 20.0 (IBM Corporation, Armonk, NY, USA). All the antioxidant experiments were performed 4 times. The data are expressed as mean ± standard deviation (S.D.). The antimicrobial experiments were repeated 3 times. Comparisons between the three different measurements were made using Friedman and Wilcoxon tests. Comparisons between the two groups were made using the Mann–Whitney U test. The results were considered statistically significant at $p < 0.05$. Spearman's correlations were performed at a significance level of 0.01 (two-tailed). The strength of association was judged based on the following scores: $|r| \geq 0.9$—very strong; $0.7 \leq |r| < 0.9$—strong; $0.5 \leq |r| < 0.7$—moderate; $0.3 \leq |r| < 0.5$—weak; $|r| < 0.3$—very weak.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Content

The total phenolic and flavonoid content results were obtained by applying spectrophotometry. This technique allows for quantitative composition determination of groups of biologically active compounds. Additionally, PVP/VAC (1–5%) and SSG (1%) were incorporated into the samples to analyze and determine their total phenolic content, flavonoid content, and antioxidant activity. It is important to note that these excipients alone do not possess antioxidant properties [17].

The methodologies chosen for studying raw plant materials were selected to assess the variability of phenolic compounds and flavonoids in red clover extracts prepared using different excipients (SSG and PVP/VAC). The red clover extracts with excipients were prepared in 50% ethanol and 70 and 96% ethanol solutions. Following testing, the differences in the results among the solvents were not statistically significant. Consequently, the decision was made to use the ethanol solution with the lowest concentration (50%). A previous study guided the selection of the extraction parameters mentioned earlier [17].

The total amount of phenolic compounds in the red clover aqueous flower samples with excipients was determined to vary from 35.79 ± 0.12 to 45.38 ± 0.19 mg GAE/g dw (Figure 2), and in the ethanolic samples, it varied from 43.11 ± 0.24 to 52.48 ± 0.24 mg GAE/g dw (Figure 3).

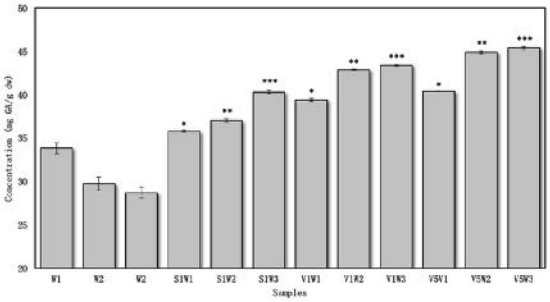

Figure 2. The total amount of phenolic compounds in the aqueous extracts, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. W1; ** $p < 0.05$ vs. W2; *** $p < 0.05$ vs. W3 (the samples were compared with their respective control groups).

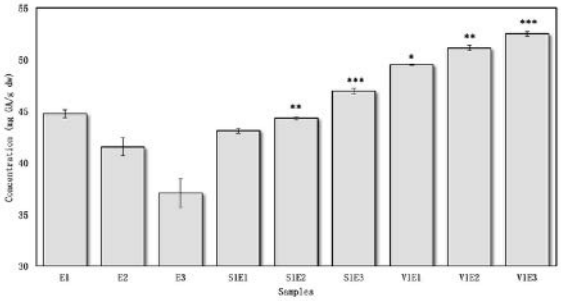

Figure 3. The total amount of phenolic compounds in ethanolic extracts, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. E1; ** $p < 0.05$ vs. E2; *** $p < 0.05$ vs. E3 (the samples were compared with their respective control groups).

Among the aqueous samples, V5W3 exhibited the highest concentration of phenolic compounds, measuring 45.38 ± 0.19 mg GAE/g dw. In contrast, the sample S1W1 displayed the lowest phenolic compound content, amounting to 35.79 ± 0.12 mg GAE/g dw (Figure 2).

Increasing the concentration of PVP/VAC excipient in the extraction media from 1% to 5% led to overall increases. However, a statistically significant difference ($p < 0.05$) was specifically observed in the samples prepared through a combination of reflux and ultrasound processing. Importantly, across all the samples prepared with excipients in both 50% ethanol and water solvents, there were statistically significant differences ($p < 0.05$) compared to the control samples.

The highest total phenolic content in the ethanolic extracts was determined for the sample V1E3 (52.48 ± 0.26 mg GAE/g dw), and lowest was found for S1E1—43.11 ± 0.24 mg GAE/g dw (Figure 3). PVP/VAC demonstrated a greater extraction efficacy than the SSG excipient, even without increasing the concentration from 1% to 5%. Specifically, when employing PVP/VAC, there was an average increase of 11% in the amount of phenolic compounds compared to using SSG.

In research by Küçükboyaci et al., the total phenolic content was determined to be 52.30 ± 1.20 mg GAE/g [33]. This yield is higher than that found in our study without and with excipients. Esmaeili et al. investigated in vivo grown red clover, which varied from 27.57 to 46.88 mg GAE/g dw. These results are similar to the results obtained in

this study without using toxic solvents but using green extraction with excipients [34]. However, the study by Küçükboyaci et al. used methanol for extraction, an effective solvent based on medicinal plant research [33]. The second study used different organic solvents (methanol, ethyl acetate, chloroform). Notably, the results obtained using ethyl acetate were found to be ineffective. While all the solvents used in the studies are capable of extracting both polar and non-polar secondary metabolites, it is important to highlight that methanol and chloroform present challenges due to their toxicity, rendering them unsuitable for studies involving organisms and animal models. The procedural step of solvent evaporation, although implemented, may still leave trace amounts behind [35]. There is also the possibility that differences in the composition of phenolic compounds can be influenced by biotic and abiotic factors, plant parts, harvesting time, and inaccuracies in taxonomic identification and chemical analysis [36,37]. It is not easy to compare chemical data because there is a great deal of inconsistency, but it can provide an overall picture of a species' chemical profile.

Using both excipients (PVP/VAC and SSG) in ethanol/water extraction was more effective than extraction using the solvent alone. However, regardless of the effectiveness of both excipients, the use of PVP/VAC in the extraction helped to extract higher amounts of phenolic compounds in both water and ethanol (Figures 2 and 3).

As solutions cool down, both excipients gradually settle into sediments. The sedimentation of these excipients, rather than being a hindrance, present an opportunity for resource optimization. The settled excipients can be effectively recovered and reused in extraction processes. This sustainable approach not only minimizes waste but also contributes to cost-effectiveness in various applications. Industries can enhance their efficiency and reduce their environmental impact by harnessing the recoverable excipients.

The phytochemical profile of red clover includes a wide range of polyphenolic compounds, such as numerous flavonoids (including isoflavones) [38]. Most flavonoids exist naturally as glycosides. The presence of sugars and hydroxyl groups makes them water soluble, while the presence of methyl groups makes them lipophilic.

The content of total flavonoids was expressed as milligrams of rutin equivalents per gram of dry sample, ranging from 20.38 ± 0.23 to 23.10 ± 0.11 mg RU/g dw in the aqueous test extracts (Figure 4) and 24.57 ± 0.13 to 26.98 ± 0.28 mg RU/g dw in the ethanolic extracts with excipients (Figure 5).

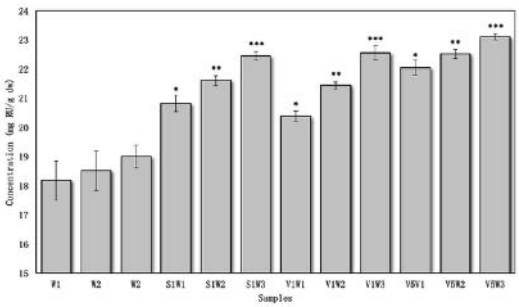

Figure 4. The total amount of flavonoids in aqueous extracts, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. W1; ** $p < 0.05$ vs. W2; *** $p < 0.05$ vs. W3 (the samples were compared with their respective control groups).

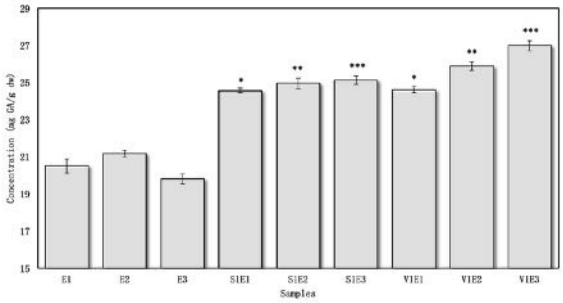

Figure 5. The total amount of flavonoids in ethanolic extracts, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. E1; ** $p < 0.05$ vs. E2; *** $p < 0.05$ vs. E3 (the samples were compared with their respective control groups).

The highest amount of flavonoids from the aqueous samples was found in V5W3 (23.10 ± 0.11 mg RU/g dw) (Figure 4). The lowest content of flavonoid compounds was found in the S1W1 and V1W1 samples (20.82 ± 0.27 and 20.38 ± 0.23 mg GAE/g dw, respectively). All the samples prepared using the excipients in water yielded statistically significant differences ($p < 0.05$) compared to the controls.

Observing the results, it was determined that when using the PVP/VAC excipient, the lowest outcomes were observed with reflux alone, but an increase in flavonoid yield occurred when ultrasound was incorporated and the processing time was extended from 10 to 30 min. Similarly, a parallel pattern of increased flavonoid yield was observed when using the SSG excipient in the extraction process.

The investigation revealed a direct correlation between the concentration of PVP/VAC and the increase in flavonoid levels, specifically from 1% to 5%. A significant ($p < 0.05$) rise in flavonoid yield was noted when comparing samples with PVP/VAC concentrations of 1% and 5%. While a comparable effect was observed when comparing the results from both excipients, it is noteworthy that adjusting the PVP/VAC concentration in the extraction media demonstrated the potential for further increases in flavonoid concentrations.

Using ethanol as a solvent, the total flavonoid amount was the highest in the sample V1E3 prepared with PVP/VAC excipient (1%) (26.98 ± 0.28 mg RU/g dw) (Figure 5). The lowest flavonoid amount was found in the S1E1 and V1E1 samples (24.57 ± 0.13 and 24.61 ± 0.17 mg RU/g dw). The results obtained using ethanol and excipients were better than those obtained using water. Still, the same trends remained: the extracts prepared with PVP/VAC and SSG using only reflux yielded the lowest flavonoid concentrations, but when combining this method with ultrasound and increasing the processing time, the concentrations increased. The results were statistically significantly higher than the controls prepared without excipients ($p < 0.05$).

3.2. Excipients' Influence on Radical Scavenging Activity

Due to the fact that the electron reduction potential of phenolic radicals is lower than that of oxygen radicals and that phenoxyl radicals are less reactive than oxygen radicals, phenolic compounds are effective scavengers of oxygen radicals [39]. Many of polyphenolics' biological functions have been associated with their ability to scavenge and neutralize free radicals and their antioxidant activity, including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic, and vasodilator effects [40,41].

Many factors, such as the plant growth environment, the composition of the extract, and the test system, influence the antioxidant capacity of the extract from the plant. Therefore, it cannot be fully described using one single method to obtain objective results.

A reliable antioxidant assay protocol requires measuring more than one property using several different methods, since most natural antioxidants are multifunctional [42,43]. The antioxidant activity of phenolic-rich plant materials correlates with their antioxidant capacity, which depends on the structure of the compounds.

The antioxidant activity was evaluated using ABTS and DPPH in vitro techniques that have different experimental conditions but belong to single electron transfer-based assays. The results in the samples with excipients investigated using the DPPH method varied from 10.17 ± 0.02 to 11.25 ± 0.11 µg TE/g dw (Table 1) in the aqueous samples and 11.26 ± 0.06 to 12.81 ± 0.05 µg TE/g dw (Table 1) in the ethanolic samples.

Table 1. DPPH and ABTS radical scavenging activity of extracts with different excipients, $n = 4$ (the codes of the samples are provided in Figure 1).

	DPPH, (µg TE/g dw) *	SD, $n = 4$	ABTS, (µg TE/g dw) *	SD, $n = 4$
W1	9.76	0.39	353.45	9.19
W2	9.04	0.22	348.14	4.18
W3	8.52	0.78	344.28	7.06
S1W1	10.50	0.15	389.06	6.45
S1W2	10.29	0.08	383.63	7.73
S1W3	10.17	0.02	388.60	6.40
V1W1	10.67	0.04	395.45	2.51
V1W2	10.98	0.10	397.70	6.54
V1W3	10.76	0.01	405.61	2.54
V5W1	10.76	0.06	402.55	8.12
V5W2	11.10	0.08	405.49	7.57
V5W3	11.25	0.11	419.29	7.00
E1	10.98	0.29	393.05	5.82
E1	10.73	0.25	390.39	7.41
E3	10.49	0.22	385.56	6.51
S1E1	11.84	0.11	433.48	3.84
S1E2	11.26	0.06	425.04	4.38
S1E3	12.02	0.13	436.06	8.17
V1E1	11.92	0.32	447.68	4.46
V1E2	12.25	0.10	460.67	5.08
V1E3	12.81	0.05	463.92	6.46

* All samples had statistically significantly higher antioxidant activity than the controls ($p < 0.05$).

Analyzing the antioxidant results of the aqueous samples obtained using the DPPH method, it was determined that the highest antioxidant activity was found in the V5W3 sample (11.25 ± 0.11 µg TE/g dw) that was prepared using an enhanced amount (5%) of PVP/VAC. The lowest activity was determined in the sample S1W3 (10.17 ± 0.08 µg TE/g dw). All the aqueous samples were statistically significantly different ($p < 0.05$) compared with controls that were prepared without the excipients (Table 1). It was determined that the samples prepared using PVP/VAC had antioxidant activities that were significantly ($p < 0.05$) higher than those prepared using the SSG excipient.

The highest antioxidant activity in the ethanolic samples was found in the V1E3 sample (12.81 ± 0.05 µg TE/g dw), and the lowest was found in the S1E2 sample (11.26 ± 0.06 µg TE/g dw) (Table 1). All the samples were statistically significantly different compared with the controls. In ethanol, using the SSG excipient, ultrasound processing for 10 min, and reflux, the antioxidant activity was lower than when using reflux alone, but the activity increased with increasing processing times. However, using PVP/VAC in extraction reflux resulted in the lowest antioxidant activity.

Limited literature is available on the assessment of phenolic compounds and antioxidant activity in red clover flowers. Most studies focus on extracts from either the entire plant or, specifically, the leaves. In research by Jakubczyk et al., red clover flowers were extracted using a Soxhlet apparatus and 96% ethanol as a solvent [44]. The determined

antioxidant activity (DPPH) of the red clover extract was lower compared with the results in this study by approximately 30% (controls) and 33–39% compared with samples prepared using the excipients. In other research by Horvat et al., extracts were prepared from red clover leaves of different breeding populations [45]. The results showed that on average, DPPH inhibition was 18% lower compared to our controls and 24–27% lower compared with the samples prepared using the excipients. The right extraction method of phenolic compounds can increase antioxidant activity, and the right excipient can also increase antioxidant potential from 3 to 9%.

An ABTS assay offers the advantages of simplicity and speed, and it is a well-established method for estimating the antioxidant activity of test samples [46]. The radical scavenging ability measured using the ABTS assay is given in Table 1 and expressed as for the DPPH assay. The ABTS radical activity followed a similar pattern to the DPPH assay in both the aqueous and ethanolic extracts with excipients. Using the ABTS method, the results varied from 383.63 ± 7.73 to 419.29 ± 7.00 μg TE/g dw (Table 1) in the aqueous samples and 425.04 ± 4.38 to 463.92 ± 6.46 μg TE/g dw (Table 1) in the ethanolic samples.

The lowest antioxidant activity in the aqueous samples was detected in sample S1W2 (383.63 ± 7.73 μg TE/g dw) prepared with SSG excipient using reflux and ultrasound processing for 10 min. Among samples prepared using SSG antioxidant activity, adding ultrasound for the extraction decreased, but prolonging the time to 30 min (S1W3 sample, 388.60 ± 6.40 μg TE/g dw) (Table 1) increased the activity. However, it was still lower than when using reflux alone (S1W1 sample, 389.06 ± 6.45 μg TE/g dw). The highest antioxidant activity was determined in the V5W3 extract (419.29 ± 7.00 μg TE/g dw) that was prepared using 5% PVP/VAC and reflux combined with 30 min of ultrasound processing. This result was significantly higher ($p < 0.05$) than all the results from the aqueous extracts. However, even when using only 1% PVP/VAC, the antioxidant activity was significantly higher than when using SSG (Table 1).

The highest antioxidant activity in the ethanolic samples was determined in sample V1E3 (463.92 ± 6.46 μg TE/g dw). The lowest activity was found in the S1E2 (425.04 ± 4.38 μg TE/g dw) sample. Tendencies like those in the aqueous extracts were also observed in the ethanolic samples. All the results were statistically significantly higher compared to the controls.

Both the ABTS and DPPH methods showed similar results, which demonstrate that the results are reproducible and that these methods are complementary. The extracts prepared in ethanol were more effective, and when using ethanol with PVP/VAC, the antioxidant activity increased by approximately (not including the results obtained using 5% PVP/VAC) 14% (compared to aqueous extracts). Using SSG excipient in ethanol, the antioxidant activity increased by 10%.

Correlation analyses were performed to observe the relationship between the antioxidant activity and the chemical composition of the extracts for DPPH and ABTS, as well as the two major groups of compounds—the total amount of phenolic compounds and flavonoids. The results show a positive correlation between the total phenolic content of all the extracts (controls and test samples) and the antioxidant capacity measured using DPPH (r = 0.917 in ethanolic extracts, r = 0.939 in aqueous extracts) ($p < 0.01$) as well as ABTS (r = 0.932 ethanolic samples, r = 0.951 aqueous samples) ($p < 0.01$). This confirms that the phenolic compounds are the primary source of inherent antioxidant activity, which has additionally been stated in other publications [47,48].

Flavonoids also showed a positive correlation with both radical scavenging methods: r = 0.933 in ethanol, r = 0.753 in water ($p < 0.01$) (DPPH); r = 0.899 in ethanol, r = 0.832 in water ($p < 0.01$) (ABTS). The correlations were higher in the ethanol samples than in water because flavonoids are poorly soluble [49]. Nevertheless, excipient use increases flavonoid solubility using both excipients in water and ethanol.

The results obtained using both antioxidant activity methods, ABTS and DPPH, were in agreement, which was also reflected in the correlation coefficients of r = 0.953 for the aqueous samples and r = 0.982 for the ethanolic samples.

3.3. Reducing Power Activity of Extracts Prepared Using Excipients

The FRAP method is a simple, inexpensive, reproducible, and sensitive method to assess antioxidant power [46]. The obtained results were expressed as ferrous sulfate equivalent mg FS/g dw. The FRAP values of the studied extracts varied from 144.33 ± 4.60 to 167.45 ± 2.06 mg FS/g dw in the aqueous samples (Figure 6) and 189.03 ± 2.35 to 192.49 ± 1.46 mg FS/g dw in the ethanolic samples (Figure 7).

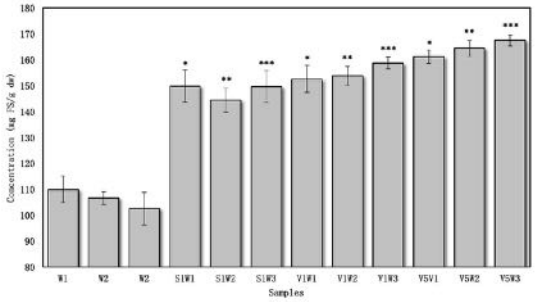

Figure 6. Antioxidant activity of aqueous samples using the FRAP method, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. W1; ** $p < 0.05$ vs. W2; *** $p < 0.05$ vs. W3 (the samples were compared with their respective control groups).

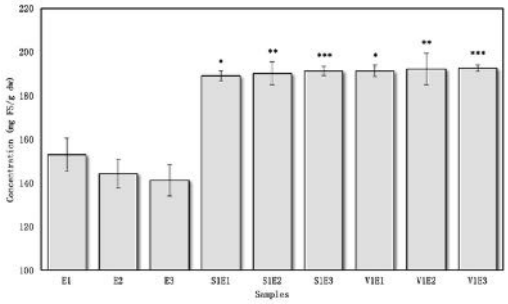

Figure 7. Antioxidant activity of ethanolic samples using the FRAP method, $n = 4$ (the codes of the samples are provided in Figure 1). * $p < 0.05$ vs. E1; ** $p < 0.05$ vs. E2; *** $p < 0.05$ vs. E3 (the samples were compared with their respective control groups).

The highest value obtained using the FRAP method for the aqueous samples was found for the V5W3 sample—167.45 ± 2.06 mg FS/g dw (Figure 6), while the lowest value was noted in S1W2 at 144.33 ± 4.60 mg FS/g dw. The outcomes obtained using the FRAP method were consistent with those obtained using the ABTS and DPPH methods. Notably, all the test samples exhibited statistical superiority over the controls ($p < 0.05$). However, it is important to highlight that the results with PVP/VAC were significantly higher than those with SSG only when the former was used at a 5% concentration.

The antioxidant activity in the ethanolic test samples was very similar (Figure 7). All the samples had statistically higher activities than the controls, but no significant difference existed between the samples prepared using PVP/VAC and SSG. Nevertheless, the results obtained in this study were higher than those of Zawislak et al., who used different solvents for the extracts and different red clover drying conditions [50].

The previously presented results show a positive correlation between the total phenolic content and the ABTS/DPPH methods. These results were confirmed by the reducing power activity method FRAP, which also showed a positive correlation between the total amount of phenolic compounds: r = 0.950 ($p < 0.01$) in ethanol and r = 0.944 ($p < 0.01$) in the aqueous samples. The same correlation was observed between flavonoids and the FRAP method, but it was weaker in the aqueous samples r = 0.804 ($p < 0.01$). In the ethanolic samples, r = 0.950 ($p < 0.01$).

3.4. Phenolic Compounds' Antioxidant Potential

Studies have shown that red clover contains the following isoflavones: genistein, daidzein, biochanin A, and formononetin [7,51]. Isoflavonoids have a variety of bioprotective effects, including antioxidant, antimutagenic, anticarcinogenic, and antiproliferative activities. In a previous study by Kazlauskaite et al. [17], the concentrations of the isoflavonoids daidzein and genistein, depending on the extraction conditions, were determined in the red clover extracts with PVP/VAC and SSG excipients. In this study, we used a mixture of the isoflavones daidzein, genistein, daidzin, and genistin and determined the antioxidant activity of the known sample. The preparation of the isoflavones mix from standards is described in Section 2.1—"Plant Material and Reagents".

The obtained concentrations of isoflavones based on the HPLC analysis were as follows: genistein 97.29 ± 3.89 μg/g, daidzein 95.74 ± 3.83 μg/g, genistin 125.41 ± 5.01 μg/g, and daidzin 139.45 ± 5.57 μg/g. These concentrations were used to determine the antioxidant activity (total content of these isoflavones: 457.91 ± 18.31 μg/g).

In this study, the total amount of phenolic compounds, flavonoid content, and antioxidant activity of the samples were determined using the ABTS, DPPH, and FRAP methods. The total phenolic content was 7.55 ± 0.16 mg GA/g dw, and the determined flavonoid content was 12.77 ± 1.24 mg RU/g dw. We determined that antioxidant activities of the isoflavones samples were relatively low: 1.93 ± 0.05 μg TE/g dw (DPPH), 74.25 ± 0.65 μg TE/g dw (ABTS), and 24.42 ± 1.62 mg FS/g dw. Although glycosides (daidzin, genistin) are inactive forms, studies have shown that they have a similar antioxidant activity to aglycones in vitro [52,53].

A similar total concentration of isoflavones was found in sample S1E1 (464.80 μg/g: genistein 187.10 μg/g; daidzein 216.67 μg/g; genistin 61.03 μg/g; and daidzin 0 μg/g). Even though the isoflavone contents were similar, as mentioned before, the glycosides' antioxidant action in vitro showed the same results. The results showed that the extract sample had 6 times more phenolic compounds but only 1.92 times more flavonoids. Though phenolic acids have been studied both in vitro and in vivo for their antioxidant properties, the mechanisms of their action remain unclear and/or undefined. An essential factor that should be considered is their mutual interactions, which can be synergistic, antagonistic, or additive (no interaction). These interactions were investigated by several studies using a variety of antioxidant assays, and both synergistic and antagonistic interactions were confirmed [15,54]. Although the antioxidant responses of the isoflavones tested in this study were small, they may act synergistically with other compounds in the extract, thus increasing the response of the whole product. Various compounds were investigated to act in synergy with daidzein or genistein [55–58]. The use of the red clover extract as a source of phenolics, including isoflavones, seems to be promising due to the presence of several molecules that can act in synergy with each other, promoting and enhancing antioxidant capability.

3.5. Post-Column ABTS Antioxidant Assay and RP-LC/PDA/MS Qualitative Analysis

Additionally, the best results were obtained for the samples that were investigated using the post-column ABTS method. The highest antioxidant activity using the post-column ABTS method in the aqueous samples was V1W3 20.90 ± 0.83 mg TE/g dw, and in the ethanolic samples, it was V1E3—29.04 ± 1.16 mg TE/g dw (Table 2). All the samples had statistically higher values compared with the controls prepared using the same method.

Table 2. ABTS radical scavenging activity of extracts with different excipients, $n = 4$ (the codes of the samples are provided in Figure 1).

Samples Codes	ABTS Radical Scavenging, mg TE/g dw
W3	4.00 ± 0.16
S1W3	15.06 ± 0.60 [b]
V1W3	20.90 ± 0.83 [b]
E3	11.46 ± 0.45
S1E3	16.91 ± 0.83 [a]
V1E3	29.04 ± 1.16 [a]

[a] $p < 0.05$ vs. E3; [b] $p < 0.05$ vs. W3. The control in water is compared only with samples prepared in water. Moreover, the controls prepared in ethanol are compared only with the ethanol samples.

An LC-MS analysis of all the post-column samples was performed, and the determined compounds were compared to published red clover profiles. As a result, nine compounds were identified in the red clover extracts with and without the excipients (Figure 8A–F).

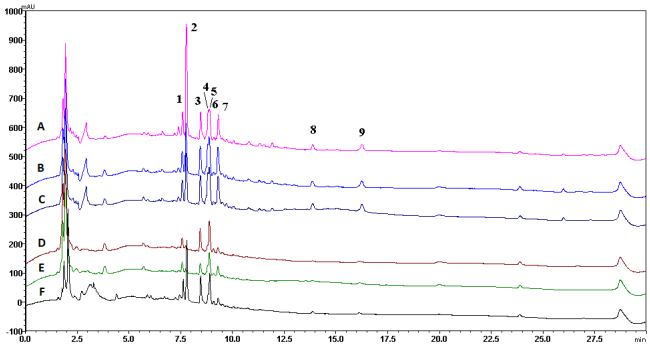

Figure 8. Profiles of control and test samples: A—V1E3 sample profile; B—S1E3 sample profile; C—E3 sample profile; D—S1W3 sample profile; E—W3 sample profile; F—V1W3 sample profile. The wavelength used to determine the compounds was 330 nm.

It was determined that peak 1 (m/z 295.0 [M-H]$^-$) was caffeoylmalic acid, peak 2 (m/z 358.0 [M-H]$^-$) was cis-clovamide, peak 3 (m/z 463.1 [M-H]$^-$) was hyperoside, peak 4 (m/z 505.1 [M-H]$^-$) was quercetin-O-hexoside-acetate, peak 5 (m/z 447 [M-H]$^-$) was kaempferol-O-hexoside, peak 6 (m/z 477.1 [M-H]$^-$) was methylquercetin-O-hexoside, peak 7 (m/z 489.1 [M-H]$^-$) was ellagic acetyl rhaminoside, peak 8 (m/z 267 [M-H]$^-$) was formononetin, and peak 9 (m/z 283 [M-H]$^-$) was biochanin A [38,59] (Figure 8).

Many of the detected compounds like hyperoside, clovamide, and caffeomalic acid contribute to stress tolerance in numerous plant species due to their high antioxidant activity, increased cell viability, and antimicrobial activity [60–62]. Comparing the aqueous samples with the ethanolic extracts prepared using water demonstrated that the former had a narrower compound profile. Formononetin and biochanin A were not found.

The V1E3 sample, prepared using the PVP/VAC excipient, exhibited the highest antioxidant activity. However, the total concentration of identified compounds was higher in the S1E3 sample (Table 3). This phenomenon can be attributed to variations in the antioxidant properties of the compounds present. Consequently, compounds with higher concentrations in S1E3 displayed lower antioxidant capacities.

Table 3. Identified compound concentrations expressed as hyperoside equivalent μg/mL.

Compounds nr.	W3, μg/mL	S1W3, μg/mL	V1W3, μg/mL	E3, μg/mL	S1E3, μg/mL	V1E3, μg/mL
1	19.25 ± 0.58	1.94 ± 0.058	2.7 ± 0.081	78.06 ± 2.34	25.81 ± 0.77	75.41 ± 2.26
2	36.33 ± 1.09	19.42 ± 0.58	49.41 ± 1.48	118.55 ± 3.56	38.77 ± 1.16	69.92 ± 2.10
3	77.3 ± 2.32	72.73 ± 2.18	25.71 ± 0.77	45.29 ± 1.36	97.64 ± 2.93	56.97 ± 1.71
4	18.91 ± 0.57	15.18 ± 0.46	16.61 ± 0.50	45.18 ± 1.36	88.5 ± 2.66	40.31 ± 1.21
5	46.13 ± 1.38	61.94 ± 1.86	45.16 ± 1.35	48.28 ± 1.45	75.66 ± 2.27	62.76 ± 1.88
6	10.3 ± 0.31	13.53 ± 0.41	3.75 ± 0.11	4.53 ± 0.14	10.3 ± 0.31	6.52 ± 0.20
7	17.65 ± 0.53	15.55 ± 0.47	23.42 ± 0.70	41.68 ± 1.25	46.13 ± 1.39	54.83 ± 1.65
8	15.83 ± 0.47	7.11 ± 0.21	0.28 ± 0.01	1.15 ± 0.04	44.19 ± 1.33	1.29 ± 0.04
9	0.33 ± 0.01	3.22 ± 0.10	0.79 ± 0.02	7.74 ± 0.23	73.02 ± 2.19	8.71 ± 0.26

The lowest concentrations of the identified compounds were found in the aqueous samples as opposed to the ethanolic ones. Despite the samples prepared using PVP/VAC exhibiting better biological properties, the determined compounds' concentrations were lower than those prepared with SSG, ethanolic, and aqueous solutions. However, the control samples showed very minimal concentrations of active compounds.

3.6. Antimicrobial Activity of Red Clover Extracts

The samples' antimicrobial activities were investigated based on their diffusion into solid nutrition agar. The samples prepared with excipients (PVP/VAC and SSG in water or ethanol) and the controls (prepared without excipients in water or ethanol) were investigated against pathogenic bacteria. Still, there was no significant difference between the test samples and controls without excipients.

Comparing the samples prepared using ethanol and water, differences between their inhibition were found. The water samples inhibited five microorganisms, and the ethanol samples inhibited four (Table 4). Although the maximum concentration used in this study of extracts was 210 mg/mL, this concentration did not inhibit *Enterococcus faecalis*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* in both the water and ethanolic samples. Additionally, the ethanolic extract did not inhibit *Bacillus cereus*. Nevertheless, the ethanolic samples inhibited microorganisms in much lower concentrations than the aqueous samples. Generally, Gram-positive bacteria were more sensitive to the aqueous extracts tested than Gram-negative bacteria. However, the ethanolic extract had a similar effect on Gram-positive and Gram-negative bacteria.

Table 4. The antimicrobial activity (MIC) of red clover aqueous and ethanolic extracts, $n = 3$.

Solvent of Sample **	**Gram-Positive Bacteria**				**Gram-Negative Bacteria**			**Yeast**
	Staphylococcus aureus ATCC 25923	*Staphylococcus epidermidis* ATCC 12228	*Enterococcus faecalis* ATCC 29212	*Bacillus cereus* ATCC 11778	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumoniae* ATCC 13883	*Candida albicans* ATCC 10231
Water	125.51 mg/mL	65.92 mg/mL	NA	99.34 mg/mL	99.34 mg/mL	NA	NA	210.16 mg/mL
Ethanol	34.23 mg/mL	25.92 mg/mL	NA	NA mg/mL	34.23 mg/mL	NA	NA	34.23 mg/mL
Penicillin	>0.25 μg/mL	>0.25 μg/mL	>16 μg/mL	-	-	-	-	-
Ciprofloxacin	>2 μg/mL	>2 μg/mL	>4 μg/mL	-	-	-	-	-
Ampicillin	-	-	-	-	>61.9 μg/mL	NA	>32 μg/mL	-
Amoxicillin,	-	-	-	-	>32 μg/mL	NA	NA	-
Daptomycin	>1 μg/mL	>1 μg/mL	NA	-	-	-	-	-

NA means "no activity". The extracts did not inhibit the reference microbial strains in their ≤ 210 mg/mL concentration. - means it was not tested with the bacteria. ** The antimicrobial activity was evaluated against both solvents. The solvents (water and 50% ethanol) alone did not inhibit the reference microbial strains.

The antimicrobial activity was assessed against key antibiotics, including penicillin, ciprofloxacin, ampicillin, amoxicillin, and daptomycin, targeting Gram-positive and Gram-negative bacteria. Penicillin exhibited potent activity against all Gram-positive bacteria tested, including *Staphylococcus aureus* (MIC > 0.25 μg/mL), *Staphylococcus epidermis* (MIC > 0.25 μg/mL), and *Enterococcus faecalis* (MIC > 16 μg/mL). Ciprofloxacin, while effective, required higher concentrations compared to penicillin for *Staphylococcus aureus* (MIC > 2 μg/mL), *Staphylococcus epidermis* (MIC > 2 μg/mL), and *Enterococcus faecalis* (MIC > 4 μg/mL). Daptomycin showed no inhibition for *Enterococcus faecalis*, and its MIC for *Staphylococcus aureus* and *Staphylococcus epidermis* exceeded 1 μg/mL.

Ampicillin and amoxicillin were tested against Gram-negative bacteria. Ampicillin failed to inhibit *Pseudomonas aeruginosa*. Against *Escherichia coli*, ampicillin had an MIC value of >32 μg/mL, and for *Klebsiella pneumonia*, it was ≥32 μg/mL. Amoxicillin did not inhibit *Klebsiella pneumonia* or *Pseudomonas aeruginosa* but demonstrated inhibitory activity against *Escherichia coli* with a MIC value of ≥32 μg/mL.

Despite certain antibiotics demonstrating higher inhibitory concentrations, this study underscores the considerable potential of red clover flower extracts. The results suggest that red clover extract, whether ethanolic or aqueous, should be employed in concentrated formulations to optimize its effects. This finding advocates for the strategic utilization of red clover in product development, emphasizing its unique attributes in achieving optimal therapeutic outcomes. Even though *Fabaceae* is the largest family with about 19.580 species, only 93 individual species of Fabaceae were reported for their antibacterial activities. This represents a relatively small percentage (0.5%) of the total diversity in this family [63]. Nevertheless, it was reported that isoflavone-containing plants like soybeans and alfalfa possess antioxidant activities. These plants especially inhibit *Escherichia coli* and *Staphylococcus aureus* [64,65].

The main isoflavone compounds found in red clover are daidzein and genistein. In the literature, it was reported that the metabolites of isoflavones can be even more potent antibacterial agents than their precursors. However, the same precursors (daidzein and genistein) possess antimicrobial activity, especially against Gram-positive bacteria and can act synergistically with other phenolic compounds in order to downregulate the resistance mechanisms of bacteria [66–68].

Even though there are limited studies regarding the antimicrobial effects of red clover flowers, some suggest that essential oil and extract do not possess antimicrobial and antifungal properties [69,70]. Nevertheless, this can be due to ineffective extraction methods. Gligor et al. [71] concluded that the samples' biological activity depends on the phytochemical profile determined by the extraction technology.

4. Conclusions

The extract of red clover flowers is a promising natural alternative for the development of pharmaceuticals due to its high levels of phenolics and flavonoids as well as its antioxidant and antibacterial properties. The addition of the excipients SSG and PVP/VAC demonstrated a significant increase in the phenolic and flavonoid content in the extraction media, which enhanced their antioxidant activity. Although there was no difference in antimicrobial activity between the extracts with or without excipients, there were differences in the extraction solvents. Using excipients can safely enrich the extract with valuable compounds, particularly the PVP/VAC excipient, which showed greater results in both water and ethanol than the SSG excipient.

The extracts prepared using 5% PVP/VAC in water, as opposed to 1%, exhibited notably superior results across all the test samples. Specifically, sample V5W3 demonstrated the highest DPPH (11.25 ± 0.11 μg TE/g dw), ABTS (419.29 ± 7.00 μg TE/g dw) radical scavenging activity, and FRAP (167.45 ± 2.06 mg FS/g dw) reducing power activity among the aqueous samples. Additionally, V5W3 displayed the highest concentrations of phenols and flavonoid contents among the aqueous samples (45.88 ± 0.19 mg GAE/g dw and 23.10 ± 0.10 mg RU/g dw).

Regarding the ethanolic samples, the highest activity was observed in sample V1E3, boasting the highest phenol content (52.48 ± 0.24 mg GAE/g dw), flavonoid content (26.98 ± 0.28 mg RU/g dw), and antioxidant activity, including DPPH (12.81 ± 0.05 µg TE/g dw), ABTS (463.92 ± 6.46 µg TE/g dw), and FRAP (192.49 ± 1.45 mg FS/g dw. In the future, further research is needed to explore the potential applications of red clover flower extract in various pharmaceutical products.

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Paper 7

Title: Technological Functionalisation of Microencapsulated Genistein and Daidzein Delivery Systems Soluble in the Stomach and Intestines

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Article

Technological Functionalisation of Microencapsulated Genistein and Daidzein Delivery Systems Soluble in the Stomach and Intestines

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Abstract: Encapsulating antioxidant-rich plant extracts, such as those found in red clover, within microcapsules helps protect them from degradation, thus improving stability, shelf life, and effectiveness. This study aimed to develop a microencapsulation delivery system using chitosan and alginate for microcapsules that dissolve in both the stomach and intestines, with the use of natural and synthetic emulsifiers. The microcapsules were formed using the extrusion method and employing alginate or chitosan as shell-forming material. In this study, all selected emulsifiers formed Pickering (β-CD) and traditional (white mustard extract, polysorbate 80) stable emulsions. Alginate-based emulsions resulted in microemulsions, while chitosan-based emulsions formed macroemulsions, distinguishable by oil droplet size. Although chitosan formulations with higher red clover extract (C1) concentrations showed potential, they exhibited slightly reduced firmness compared to other formulations (C2). Additionally, both alginate and chitosan formulations containing β-CD released bioactive compounds more effectively. The combined use of alginate and chitosan microcapsules in a single pill offers an innovative way to ensure dual solubility in both stomach and intestinal environments, increasing versatility for biomedical and pharmaceutical applications.

Keywords: microcapsules; drug delivery; natural compounds; isoflavones; red clover; chitosan; sodium alginate

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1. Introduction

Various medicinal applications have been attributed to isolated natural bioactive substances, including pure phytochemicals, botanical extracts, and essential oils. Among these, red clover stands out for its phenolic content, especially isoflavones, which are compounds with estrogenic properties. Isoflavones exhibit promise in addressing diverse menopausal symptoms like hot flashes, supporting cardiovascular health, and combating osteoporosis [1–3].

Natural compounds, such as phenolic compounds, offer beneficial properties like antioxidants, antimicrobial, and anti-inflammatory effects, which are valuable for various applications. However, they are susceptible to environmental factors such as heat and light, have low water solubility, undergo rapid metabolism, and are quickly eliminated from the body. Additionally, they can degrade in water, undergo oxidation, and lose activity over time [4,5]. Encapsulating antioxidant-rich plant extracts within microcapsules safeguards them from degradation, boosting stability, shelf life, and efficacy. Additionally, plant-derived compounds demonstrate excellent solubility in both water and oil phases, facilitating the creation of stable, homogeneous emulsions with uniform dispersion of

natural components, crucial for desired functionalities [6]. Recently, there has been an extensive interest in research focusing on emulsions stabilised by natural compounds [7]. In this study, emulsifiers and polymers known for their stability-enhancing and solubilising properties of bioactive compounds were carefully selected. Mustard extract, β-cyclodextrin (β-CD), and polysorbate 80 have been chosen for their emulsifying capabilities.

The white mustard extract plays a crucial role in forming traditional oil-in-water emulsions. This extract contains lecithin, the primary emulsifier in egg yolks, contributing to emulsion stability [8]. Studies have demonstrated that mustard powder can also effectively stabilise emulsions, with research showing stability for up to one month when stabilised with mustard or cinnamon. Additionally, mustard extract's capacity to absorb and retain liquid further enhances its effectiveness as an emulsifying agent [9,10]

β-CD can enhance emulsion stability and antioxidant properties when combined with conventional emulsifiers. Its ability to bring antioxidant-active substances to the oil-water interface makes it valuable in emulsions seeking antioxidant effects [11,12]. Additionally, β-CD forms inclusion complexes with compounds, allowing for targeted ingredient delivery. Emulsions with β-CD as an emulsifier are Pickering emulsions, offering better stability and droplet size control [13]. Pickering emulsion differs from traditional emulsions as solid particles rather than surfactants stabilise it. These solid particles, typically amphiphilic, possess both hydrophilic and hydrophobic traits, allowing them to attach to the oil-water interface and uphold the stability of the emulsion [14].

Polysorbate 80 is a synthetic non-ionic surfactant widely used as an emulsifier in emulsions. It has several functions, including emulsification, dispersion, wetting and stabilisation. It is also known for its solubilising properties, allowing it to enhance the solubility of poorly soluble substances. It acts as an emulsifier in pharmaceutical formulations, ensuring the uniform distribution of active ingredients and improving their stability. This makes it useful in various oral, parenteral, and topical formulations where increased solubility is desired [15–17]. Due to its viscous nature, it contributes to the texture and stability of products.

Besides overcoming stability problems, microencapsulation of emulsions reduces the perception of possible off-flavours or colours from the encapsulated material, facilitates storage, and extends shelf life without adverse influence on the physical, chemical or functional properties [18]. Coacervation, a widely used encapsulation technique for probiotics, involves the formation of a liquid rich in polymer phase in equilibrium with another liquid phase. This colloidal phenomenon can manifest in either a low or a highly dispersed state, offering promising encapsulation capabilities. There are various microencapsulation techniques employing coacervation, including spray drying, freeze drying, extrusion, spray cooling/chilling, fluidised bed drying, and more [6,19]. Extrusion is a widely used encapsulation technology for encapsulating different materials, microorganisms and phenols in hydrocolloid gel matrices [19,20].

Polymers such as chitosan and alginate, alongside dendrimers and others, have emerged as common choices for encapsulation matrices. These polymers offer distinct advantages in terms of biocompatibility, biodegradability, and functionality, making them well-suited for encapsulating a diverse array of bioactive compounds [21].

Sodium alginate, derived from brown seaweed, is a versatile anionic polymer renowned for its biocompatibility and low toxicity [22]. Its mild gelation upon exposure to divalent cations has spurred extensive exploration in biomedical fields. Alginate hydrogels, formed through diverse cross-linking techniques, mimic the structural properties of living tissue extracellular matrices, making them valuable for wound healing, bioactive agent delivery, and cell transplantation applications [23]. It also serves various beneficial roles in food products, such as gelling, thickening, and coating. Additionally, as a polyelectrolyte, it contributes to controlling solution rheology through external stimuli like pH and ionic strength. This property holds promise for manipulating emulsion rheology, showcasing practical applications in the field [24,25].

Chitosan-based particles are highly regarded as ideal drug carriers because of their biodegradable, biocompatible, and non-toxic nature. Additionally, the abundance of functional amino and hydroxyl groups in chitosan allows for the efficient immobilisation of active substances through chemical interactions [26]. The pH-responsive properties of chitosan, stemming from its amino groups and sodium alginate from its carboxyl groups, significantly enhance its suitability as a drug carrier. Leveraging these attributes, microencapsulation via extrusion techniques utilising both polymers proves to be a promising approach for drug delivery applications [27,28].

This research focuses on microencapsulation, which revolves around the careful selection of natural emulsifiers and polymers. Notably, the study emphasises the application of mustard extract, β-cyclodextrin, and polysorbate 80 to stabilise and solubilise bioactive compounds such as genistein and daidzein sourced from red clover extract. This combination of natural compounds represents a novel approach that enhances the encapsulation process, ensuring the stability and bioavailability of fragile bioactive molecules. This research addresses existing challenges in bioactive delivery and also paves the way for targeted delivery applications in pharmaceuticals, nutraceuticals, and functional foods from red clover extract. Therefore, the study aims to develop and optimise a microencapsulation delivery system, employing chitosan and alginate for stomach- and intestine-soluble microcapsules, respectively, using different emulsifiers and evaluating their stability, physical parameters and in vitro release profiles for genistein and daidzein in simulated gastrointestinal conditions.

2. Materials and Methods

2.1. Plant Material and Reagents

Trifolium pratense L. plant flower buds were collected in *Trifolium pratense* L. fields, Kupiškis district, Lithuania. The mustard powder was purchased at SALDVA. Ethanol (96%) used for extraction was purchased from Vilniaus degtine. (Vilnius, Lithuania). In this experiment, purified water was prepared with GFL2004 (GFL, Burgwedelis, Germany). Deionised water was prepared with Millipore, SimPak 1 (Merck, Darmstadt, Germany).

The following reagents were used as standards: genistein, daidzein, and biochanin A (Sigma-Aldrich, Steinheim, Germany). β-CDs were purchased from Sigma-Aldrich (Hamburg, Germany); Folin–Ciocalteu's phenol reagent (Merck, Darmstadt, Germany); shell material, alginic acid sodium salt from brown algae obtained from Sigma-Aldrich (Shanghai, China) was used. Calcium chloride (Farmalabor, Pozzillo, Italy) salt formulated microcapsules as a crosslinker. Medium molecular weight (MMW) chitosan, high molecular weight (HMW) 80/1000 chitosan, and high molecular weight (HMW) 80/3000 chitosan were purchased from Sigma-Aldrich (Steinheim, Germany). Emulsifiers Tween 80® were purchased from Sigma-Aldrich (Germany). Acetic acid and sodium hydroxide were obtained from Sigma-Aldrich (Germany).

2.1.1. Preparation of *Trifolium pratense* L. (Red Clover) Extract

The extract was prepared following the method outlined in a previous study [29]. The method used 1% of the excipient β-CD in a solution of 50% ethanol/water. Ultrasound-assisted extraction was conducted using a Grant Instruments™ XUB12 Digital ultrasound bath (Cambridge, UK) operating at 38 kHz for 10 min at a temperature of 40 ± 2 °C. Afterwards, the samples were refluxed for 1 h. The mixture was then cooled down, followed by centrifugation at 3382× *g* (5500 rpm) for 10 min, and the supernatant was concentrated in a porcelain dish.

2.1.2. Preparation of *Sinapis alba* (White Mustard) Extract

White mustard extraction was conducted using a Grant Instruments™ XUB12 Digital ultrasound bath (Cambridge, UK) operating at 38 kHz. 10.0 ± 0.001 g of dried and milled powder was macerated in 50 mL of water. Ultrasound processing was performed for 30 min at a temperature of 40 ± 2 °C. After processing, the samples were centrifuged at 3382× *g*

(5500 rpm) for 10 min, and the supernatant was decanted. The extract was then filtered through a paper filter for subsequent use in the research.

2.2. Emulsion Preparation

2.2.1. Alginate Solution Preparation

Initially, a 2% sodium alginate solution was prepared by dissolving alginic acid sodium salt in distilled water until complete solubilisation. This solution, serving as the shell material soluble in gut media, was utilised for emulsion preparation throughout the experiment. Two concentrations of alginate solution, namely 0.5% and 2%, were employed in the study.

2.2.2. Chitosan Solution Preparation

Various chitosan gels were prepared using different concentrations and molecular weights, including 2% MMW chitosan gel, 4% MMW chitosan gel, 2% HMW 80/1000 chitosan gel, and 2% HMW 80/3000 chitosan gel.

Chitosan powder was gradually added to acetic acid solution at concentrations ranging from 1% to 4% to form the gels. Stirring with an IKA EURO-STAR 200 digital stirrer (Staufen, Germany) ensued until a clear gel was achieved. The gel was centrifuged for 2 min at 447× *g* (2000 rpm) in 50 mL centrifuge tubes using a Sigma 3-18KS centrifuge (GmbH, Osterode am Harz, Germany) to remove air bubbles. Microcapsules were formed from the chitosan solution with ethanolic extract alone, employing HMW 80/1000 chitosan at a minimum concentration of 2% acetic acid and 1% nitrogen hydroxide.

2.2.3. Chitosan and Alginate Emulsions Preparation

To prepare the alginate emulsion with *Trifolium pratense* L. extract, a solution containing excipients (sweet almond oil, emulsifier-mustard extract, β-CD, or polysorbate 80-and xanthan) was combined with a sodium alginate solution. The mixture was stirred for 15 min using a magnetic stirrer (MSH-20A, Witeg, Wertheim, Germany), after which the extract was added. Formulations of the emulsions are detailed in Table 1.

Table 1. Quantities of the components used in emulsions formulations that were prepared using alginate.

Number of Emulsions	Alginate, %	Extract, %	Mustard Extract, %	β-CD Extract, %	Oil, %	Xanthan, %	Water, %
1 *	1	35	5	-	5	5	49
2 *	0.25	35	5	-	5	5	49.75
3 *	1	35	-	5	5	5	49
4 *	0.25	35	-	5	5	5	49.75
5	1	30	5	-	10	5	49
6	0.25	30	5	-	10	5	49.75
7	1	30	-	5	10	5	49
8	0.25	30	-	5	10	5	49.75
9	1	30	5	-	5	10	49
10	0.25	30	5	-	5	10	49.75
11	1	30	-	5	5	10	49
12	0.25	30	-	5	5	10	49.75
13	1	35	5	-	2.5	7.5	49
14	0.25	35	5	-	2.5	7.5	49.75
15	1	35	-	5	2.5	7.5	49
16	0.25	35	-	5	2.5	7.5	49.75

* Means that these formulations were the most stable.

Samples of chitosan and alginate emulsions were prepared using both ethanol and water extracts, but the ethanol extract is composed of more isoflavones and phenolic compounds, so only the ethanol extracts were used in further studies (Tables 1 and 2).

Table 2. Emulsions formulations that were prepared using chitosan.

Number of Emulsions	Chitosan, %	Extract, %	Polysorbate 80, %	β-CD, %	Oil, %	Acidified Water, %
1	0.7	40	1	-	3	34.3
2	0.7	40	-	1	3	34.3
3	0.92	50	1	-	3	45.08
4	0.92	50	-	1	3	45.08
5	0.9	51	1	-	3	44.1
6	0.9	51	-	1	3	44.1
7 *	2.00	10	1	-	3	84.00
8 *	2.00	10	-	1	3	84.00
9 *	2.00	20	1	-	3	74.48
10 *	2.00	20	-	1	3	74.48
11	2.00	30	1	-	3	64.68
12	2.00	30	-	1	3	64.68

* Means that these formulations were the most stable.

The following research used 8 of the most stable emulsions. For this article, we will use the codes provided in Table 3 for easier compression.

Table 3. Emulsions formulations and codes of chitosan and alginate samples.

Sample Code	Alginate, %	Chitosan, %	Extract, %	Mustard Extract, %	β-CD, %	Polysorbate 80, %	Oil, %	Xanthan Gum, %	Water, %	Acidified Water, %
A1M	1	-	35	5	-	-	5	5	49	-
A2M	0.25	-	35	5	-	-	5	5	49.75	-
A1B	1	-	35	-	5	-	5	5	49	-
A2B	0.25	-	35	-	5	-	5	5	49.75	-
C1P	-	2.00	10	-	-	1	3	-	-	84.00
C2P	-	2.00	20	-	-	1	3	-	-	74.00
C1B	-	2.00	10	-	1	-	3	-	-	84.00
C2B	-	2.00	20	-	1	-	3	-	-	74.00

A, C—alginate (A) or chitosan (C) was used as a shell material; 1; 2—the concentrations of the polymers; M, B, P-indicates emulsifiers used in formulation.

Before conducting any analysis on the emulsions (except the stability test), the samples are centrifuged at 112× *g* (1000 rpm) for 2 min. This step is to remove air bubbles, ensuring that the results are free from any interference caused by air.

2.3. Physical Parameters of Emulsions

2.3.1. Emulsions Stability Determination

Emulsion stability was evaluated using a Sigma 3-18KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The test, repeated three times, applied centrifugal forces from 1006× to 5478× *g* (3000 to 7000 rpm) for 5 min each. The test was conducted, and the percentage of non-layered emulsion, known as the centrifugation index (CI), was calculated using the following formula:

$$\mathrm{CI}\,(\%) = \frac{\mathrm{Ve}}{\mathrm{Vi}} \cdot 100\%$$

where V_e represents the volume of the remaining emulsion after centrifugation, and V_i is the volume of the initial emulsion. The test was repeated three times, and the average CI was determined. Test parameters included a centrifugal force of 3000 rpm, a temperature of 23 °C, and a duration of 5 min.

2.3.2. Particle Size and Distribution Measurements and Pictures

Oil particle size and distribution were analysed using the Mastersizer 3000 with a Hydro EV unit (Malvern Analytical Ltd., Malvern, UK). The emulsion was added dropwise to water until laser obscuration reached 9.5–10.5%, maintaining a constant pump speed of 2400 rpm. Refractive indices of 1.330 and 1.478 were used. Average particle size

distribution was calculated from five runs, with formulations characterised by percentile values (D10, D50, and D90).

For microscopic examination, samples were observed using a Nikon H550S microscope (Nikon, Chiyoda-ku, Japan) at 10× objective magnification. Images were captured with the NIS-Elements D3.2 program.

2.3.3. Dynamic Viscosity

Emulsion viscosity was assessed using a Fungilab ALPHA series viscometer (Fungilab, Sant Feliu de Llobregat, Barcelona, Spain). Each measurement involved adding 50 mL of emulsion to the viscometer's dedicated vessel and placing it on the apparatus's surface. The L3 head was used, and it was set to 100 rpm. The viscosity (mPa·S) was recorded at room temperature. Each sample was measured until the value of dynamic viscosity was stable.

2.4. Microcapsules' Formulation and Preparation

Alginate microcapsules were prepared by extrusion method. Medical 10 mL syringes were placed in an NE-1000 Programmable Single Syringe Pump (KF T technology SRL, Rozzano, Italy) and used to form microcapsules. The drops of emulsion were ejected from the needle into the crosslinker solution. The height from the needle to the solution surface was 20 cm, and the pumping speed was 0.5 mL/min. A solution of 3% calcium chloride was used as a crosslinker. Microcapsules were prepared by using an MSH-20A magnetic stirrer at 150 rpm. Particles in the crosslinker solution were stirred for 15 min, and then the microcapsules were filtered using filter paper and washed with distilled water. Manufactured capsules were left to dry at room temperature for 24 h. Dried and wet microcapsules were stored in sealed tubes until further tests.

Chitosan microcapsules were prepared similarly to alginate microcapsules. Medical 10 mL syringes were placed in an NE-1000 Programmable Single Syringe Pump (KF T technology SRL, Rozzano, Italy) and used to form microcapsules. A 1% sodium hydroxide solution was used, and the magnetic stirrer MSH-20A was set at 130 rpm. Chitosan gel was slowly dropped from the syringe (13 cm, 0.2 mL/min) to the 1% sodium hydroxide solution. Particles in the cross-linker solution were stirred for 20 min, and then the microcapsules were filtered using filter paper and washed with distilled water. Manufactured capsules were left to dry at room temperature for 24 h. Dried and wet microcapsules were stored in sealed tubes until further tests.

2.5. Physical Parameters of Microcapsules

2.5.1. Size and Shape of the Microcapsules

Microcapsule sizes were determined using a digital caliper (BGS Technic, Wermelskirchen, Germany). Thirty microcapsules were measured to calculate the mean and standard deviation of their diameters, both in dried and freshly prepared samples.

To examine morphological features, a light microscope Nikon H550S (Nikon, Chiyoda Ku, Japan) with an integrated monitor display was utilised. Emulsions and dried microcapsules were observed at 10× objective magnification using the NIS-Elements D3.2 program.

2.5.2. Firmness of the Microcapsules

Microcapsule firmness was evaluated with the TA.XT.plus texture analyser (Texture Technologies, Brewster, NY, USA). Using a P/100 platen probe, the force required to compress a 2 mm microcapsule was measured on freshly prepared samples. The maximum force was set at 6500 g. Each sample was analysed with five microcapsules, and the mean and standard deviation were calculated from five measurements.

2.5.3. Swelling Characteristic of Microcapsules

Dried microcapsules were swelled in water [30]. After 4 h, the microcapsules were weighed. Swollen microcapsules were then separated, filtered through a metal mesh, and

dried with a paper towel to remove excess fluid. The swelling index (SI) was calculated using the following formula:

$$SI\,(\%) = \frac{Ws - Wi}{Wi} \cdot 100\%$$

where W_s is the weight of swollen microcapsules at the time, and W_i is the dried microcapsules' weight.

2.6. Total Content of Active Compounds and In Vitro Release and Analysis of Microcapsules

2.6.1. In Vitro Release of Active Compounds

The test used the Sotax AT7 Smart Dissolution System (SOTAX AG, Aesch, Switzerland). The gastric medium was prepared according to the European Pharmacopoeia. To prepare gastric juice, 2.0 g of NaCl, 80 mL of 1 M HCl solution, and 3.2 g of pepsin were combined with distilled water to reach a volume of 1000 mL (pH = 1.2). Simulated intestinal juice was made using 6.8 g of KH_2PO_4, 77.0 mL of 0.2 M NaOH solution, 10 g of pancreas powder, and distilled water up to 1000 mL. Samples in the gastric medium were incubated for 30–90 min before transferring to the intestinal medium for another 30–90 min. Sampling for HPLC analysis occurred every half hour within the total in vitro release time of 0–270 min.

For HPLC analysis, samples were filtered and prepared to detect isoflavones daidzein, genistein, and biochanin A.

2.6.2. Total Phenolic and Flavanoid Content

The total phenolic content was determined as in our previous research [31]. Folin–Ciocalteu's phenol reagent (1:9 dilution in distilled water) was used. A 0.5 mL sample was combined with 2.5 mL of 7% sodium carbonate. After mixing, the absorbance was measured at 765 nm after 1 h using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Gallic acid served for the calibration curve, with results expressed as mg GA/g dw gallic acid equivalents.

Flavonoid content was measured by mixing 0.1 mL of extract with 1.0 mL of 96% ethanol, 0.05 mL of 33% acetic acid, 0.15 mL of 10% aluminium chloride, and 2 mL of 5% hexaethylenetetraamine. After a 30-min reaction at a 475 nm wavelength, spectrophotometric analysis was conducted. Results were presented as rutin equivalents in mg RE/g dw, using a calibration curve from rutin standards.

2.6.3. Isoflavones Determination Using High-Performance Liquid Chromatography

The release of daidzein, genistein and biochanin A were determined using high-performance liquid chromatography (HPLC) with diode array detectors (HPLC–PDA). The analysis was conducted using the Shimadzu Nexera X2 LC-30AD HPLC system (Shimadzu, Tokyo, Japan), equipped with an SPD-M20A diode array detector (DAD). The isoflavones' research conditions are described in the previous work of Kazlauskaite et al. [32].

2.7. Statistical Analysis

Data were analysed using SSPS version 20.0 (IBM Corporation, Armonk, NY, USA). Physical parameters of emulsions and microcapsules (except for size) and total phenolic and flavonoid content experiments were performed three times. The size of microcapsules was performed using 30 microcapsules. Data are expressed as mean ± standard deviation (S.D.). The comparisons between three different measurements were made using Friedman and Wilcoxon tests. In addition, comparisons between the two groups were made using the Mann–Whitney U test. The results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Determination of Chitosan and Alginate Emulsions Parameters

3.1.1. Emulsions Formation, Stability and Active Compounds Concentration

Various formulations of alginate emulsions were prepared, and their stability was assessed. In the case of alginate emulsions, variations in the concentration of additional water or extract did not significantly affect microcapsule formation. Consequently, our focus shifted towards investigating the impact of emulsifiers and alginate concentrations on both emulsions and microcapsules.

Conversely, when employing chitosan, the choice of extract proved to be influential in microcapsule formation. Notably, red clover extract altered the pH, hindering the formation of microcapsules at higher extract concentrations. Consequently, this investigation with chitosan prioritised assessing the effects of emulsifiers and the maximum extract concentration on both emulsion and microcapsule properties.

Optimal stability of alginitic samples was observed in formulations wherein extract concentrations remained below 35% (Table 1). The stability of these emulsions was systematically tested at three centrifugation speeds: 3000, 5000, and 7000 rpm. Following optimisation, eight preparations with alginate were identified as the most stable under centrifugation up to 5000 rpm. Formulations 3A and 4A notably exhibited stability, even at 7000 rpm.

Four samples were selected for further investigation into alginate emulsions: A1M, A2M, A1B, and A2B and their stability is provided in Figure 1. These emulsions featured varying concentrations of alginate solution (A1 and A2), specifically 0.5% (A2M, A2B) and 2% (A1M, A1B). The emulsifiers were mustard extract "M" and β-CD–"B" (Table 3). The stability of all alginate emulsion samples is provided in Figure 1.

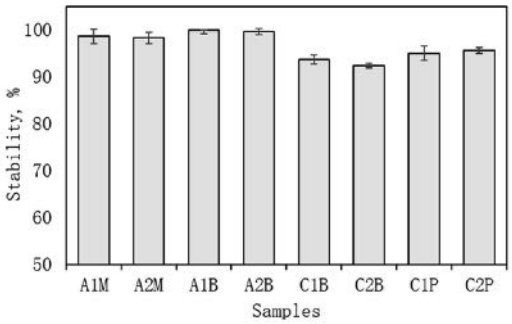

Figure 1. Stability at 7000 rpm on alginate and chitosan emulsions. The results are mean values (n = 3). Sample meanings are provided in Table 3.

In the study conducted by Wu et al., it was found that mustard extract outperformed gum arabic and citrus pectin in terms of both emulsion stability and surface activity among these three polysaccharides [33].

White mustard powder is rich in phytonutrients, particularly glucosinolates, and contains various minerals known for their anti-inflammatory properties, such as selenium and magnesium. Additionally, it serves as a good source of omega-3 fatty acids [34]. Mustard extract facilitates the formation of traditional emulsions and enhances the final product with supplementary advantages.

β-CD has extensive applications in the pharmaceutical industry. Its utilisation for preparing emulsions or enhancing their stability alongside conventional emulsifying agents has garnered increasing attention in research areas. Cyclodextrins' capacity to facilitate emulsion formation has been extensively explored in the food and cosmetic industries.

Studies comparing α-CD, β-CD, and γ-CD have revealed that β-CD exhibits the most pronounced emulsifying effect in the formation of Pickering emulsions [35,36].

In the alginate emulsions, xanthan gum was used as a co-emulsifier to enhance the stability of the system. In the literature, it was reported that higher xanthan gum concentrations ensure reaching the critical viscose concentration and complete retardation of creaming in emulsions [37].

In a parallel investigation, different concentrations and molecular weights of chitosan gels were prepared for emulsion formation, including 2% MMW chitosan gel, 4% MMW chitosan gel, 2% HMW 80/1000 chitosan gel, and 2% HMW 80/3000. Preliminary assessments determined that the chitosan solution could yield stable emulsions and sufficiently robust microcapsules. All chitosan gels were used with red clover ethanolic extract exclusively. Among them, 2% HMW 80/1000 was the sole chitosan solution that formed microcapsules at a minimum 2% acetic acid concentration. Consequently, all emulsions performed in this research were developed using this specific chitosan (HMW 80/1000).

Similarly to the alginate emulsions, stability evaluations were conducted at three centrifugation speeds: 3000, 5000, and 7000 rpm. While six formulations exhibited high stability, formulations 4C and 5C demonstrated remarkable stability even under centrifugation up to 7000 rpm, albeit not reaching 100% (Table 2). Four samples—C1P, C1B, C2P, and C2B—were chosen to investigate chitosan emulsions further. The chitosan concentration in samples was the same, but the amount of ethanolic red clover extract used differed between C1 (10%) and C2 (20%) (Table 3). Different emulsifiers in formulations were used where "P" signifies polysorbate 80 as the emulsifier (C1P and C2P) and "B" represents β-CD (C1B and C2B). The stability of all chitosan emulsion samples is provided in Figure 1.

In emulsions, chitosan acts not only as a shell material for later microcapsules but also as an emulsifier and emulsion stabiliser. It achieves this by forming a protective layer at the interfaces between oil and water, enhancing viscosity, and interacting with various surface-active agents such as surfactants, proteins, and polysaccharides [38]. Nevertheless, using higher concentrations of ethanolic red clover extract, such as in alginitic emulsions with chitosan, did not form stable emulsions or formed the emulsions, but was not enough to form robust microcapsules. Lowering chitosan concentration and increasing emulsifiers such as polysorbate 80 or β-CD did not yield the desired effect. Using xanthan gum to stabilise the emulsion system also did not help, and the emulsions were too thick for microcapsule formation. Therefore, the xanthan gum was removed from the formulation, and the chitosan concentration was increased.

Polysorbate 80 stabilisation mechanism resides in the surfactant's ability to reduce the interfacial tension by relatively short periods, and being a harmless surfactant contributes in more than one way to improve the efficacy of the drug in terms of increasing the solubility, bioavailability, and permeability of the drug [39,40].

Efforts have been dedicated to enhancing chitosan's capability to transport hydrophobic substances, primarily through the functionalisation of its structure with cyclodextrins. In certain studies, researchers have combined the advantages of both carriers to engineer more efficient carrier systems. These innovations offer enhanced mucoadhesive properties for the effective release of drugs in biomedical applications, as well as improved protein delivery, sustained release of insecticides, and removal of environmental contaminants. The results indicate that these carrier systems show promise as potential candidates for extending the longevity of sensitive or volatile compounds when exposed to environmental conditions [41,42].

In this research, after the preparation of stable emulsions, the total content of active compounds was determined in each sample (Table 4). Comparing the results of the emulsions with alginate, it is clear that the microcapsules with mustard would contain more bioactive ingredients. The mustard extract contains phenolic compounds, including flavonoids. The higher phenolic and flavonoid content was found in the A2M sample (Table 4). Nevertheless, the difference between the samples was statistically insignificant. The same situation was observed in samples A1B and A2B; even though sample A2B had

higher bioactive ingredient concentrations, the differences were negligible in emulsions (Table 4).

Table 4. The total content of active compounds in the emulsions. The results are mean values (n = 3). Sample meanings are provided in Table 3.

	Total Phenolic Content, mg GA/g	Total Flavonoid Content, mg RU/g	Daidzein, μg/g	Genistein, μg/g	Biochanin A, μg/g
Red clover extract (concentrated)	102.56 ± 2.15	29.43 ± 0.36	1066.97 ± 20.45	205.77 ± 12.89	139.73 ± 7.62
Mustard extract	22.43 ± 1.21	13.97 ± 0.39	-	-	-
A1M	66.62 ± 1.56	20.62 ± 0.52	642.54 ± 21.34	119.22 ± 3.46	82.69 ± 3.66
A2M	67.62 ± 1.68	20.95 ± 0.25	638.36 ± 32.41	126.32 ± 5.33	81.20 ± 6.87
A1B	60.62 ± 1.32	18.65 ± 0.81	664.45 ± 18.22	124.47 ± 6.97	84.92 ± 5.12
A2B	61.45 ± 1.45	18.82 ± 0.95	648.92 ± 24.81	126.12 ± 4.23	79.16 ± 5.73
C1P	10.11 ± 0.19	2.47 ± 0.06	99.68 ± 6.68	18.64 ± 1.92	11.91 ± 0.75
C2P	17.98 ± 0.54	5.62 ± 0.28	206.96 ± 12.87	34.99 ± 6.98	23.78 ± 2.64
C1B	9.98 ± 0.12	2.89 ± 0.19	94.79 ± 8.82	18.33 ± 3.14	12.15 ± 0.58
C2B	18.61 ± 0.68	5.78 ± 0.22	210.46 ± 11.56	36.21 ± 5.74	24.54 ± 1.01

In the formulations C1 and C2 of chitosan, the extract quantities used differ (C1—10% and C2—20%). Therefore, samples C2P and C2B had the highest bioactive compounds yields—C2B yielded 210.46 ± 11.56 μg/g daidzein, 36.21 ± 5.74 μg/g genistein and 24.54 ± 1.01 μg/g biochanin A; C2P consisted of 206.96 ± 12.87 μg/g daidzein 34.99 ± 6.98 μg/g genistein and 23.78 ± 2.64 μg/g biochanin A. The statistical difference of bioactive compounds between the formulations and emulsifiers was insignificant (Table 4).

The overall concentration of ethanolic red clover extracts in chitosan emulsions is lower compared to emulsions with alginate. Therefore, the results were lower in active compounds in all samples; the same concentrated ethanolic red clover extract was used.

The concentrated red clover extract was used in this research in order to have as high of an isoflavone concentration as possible. Red clover serves as the primary source for effectively isolating isoflavones. Utilising preparations abundant in isoflavones like genistein, daidzein, and biochanin A offers benefits for various conditions, including mood disorders, sedation, and alleviating hot flashes, among others.

3.1.2. Physical Emulsions Parameters

The dynamic viscosity values displayed variations among the samples. Alginate samples exhibited lower dynamic viscosity than chitosan samples (Table 5). Comparing the samples, it is noticeable that the used emulsifier influenced dynamic viscosity. Incorporating β-CD as an emulsifier, in contrast to mustard extract, increased viscosity significantly. Emulsions formulated with a 2% alginate solution demonstrated statistically significant higher viscosity (samples A1M and A1B—3460.6 ± 51.2 and 4425.8 ± 78.4 mPa·s, respectively) compared to those with a 0.5% alginate solution (A2M and A2B—2882.0 ± 87.8 and 3072.3 ± 47.1 mPa·s, respectively) (Table 5).

Table 5. Parameters of emulsions. The results are mean values (n = 3). Sample meanings are provided in Table 3.

Sample	Dynamic Viscosity, mPa·s	Dx(10)	Dx(50)	Dx(90)	Uniformity
A1M	3460.6 ± 51.2	0.449	0.561	0.931	0.365
A2M	2882.0 ± 87.8	0.469	0.589	0.944	0.370
A1B	4425.8 ± 78.4	0.387	0.525	0.934	0.399
A2B	3072.3 ± 47.1	0.402	0.550	0.981	0.396
C1P	5456.4 ± 63.6	1.39	2.19	3.81	0.346
C2P	4654.5 ± 81.9	1.31	2.00	3.82	0.346
C1B	5156.6 ± 58.4	1.58	2.64	3.91	0.319
C2B	4284.8 ± 65.3	1.52	2.50	3.94	0.357

Notably, the chitosan sample C1P, with a polysorbate 80 emulsifier, exhibited the highest viscosity at 5456.4 ± 63.6 mPa·s (Table 5). Polysorbate as an emulsifier is much denser than β-CD, so the samples prepared using this emulsion had thicker properties. Decreasing ethanolic red clover extract concentration in formulation resulted in more viscous emulsions, while increasing concentrations and the use of β-CD compared to polysorbate 80 yield more fluid emulsions.

Precise particle size control in emulsions is crucial for improving stability, bioavailability, and sensory qualities in the pharmaceutical and food industries, leading to better formulations and consumer experiences. Microemulsions, with their smaller droplet sizes, are particularly effective in enhancing drug delivery and improving the texture and appearance. Particle size varies based on the application. Macroemulsions have larger droplets the size of 1–100 µm, while microemulsions are 100 to 1000 nm [43–45]. In this study, oil droplet size was evaluated in the emulsions. The percentiles D10, D50, and D90 indicate the size for which 10, 50, and 90% of the particles are equal to or less, respectively (Table 5). Figures 2 and 3 show the particle size distribution for eighth emulsions in the microscope view and in a graph. The alginate emulsion droplets range from 0.3 to a maximum of 5.6 µm in size (Figure 3).

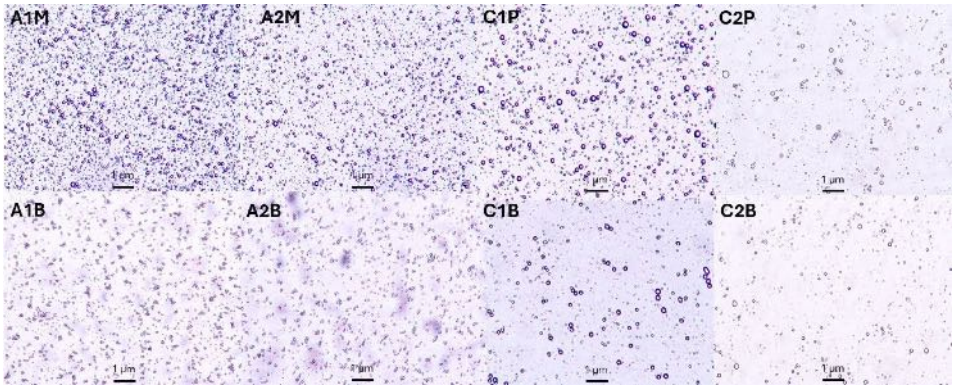

Figure 2. Microscopic pictures of emulsions (magnification 10×).

The emulsions' microscopic view also shows that the droplet sizes in alginate samples vary. The particle size where the cumulative distribution is 50% is the median droplet diameter. The emulsions with alginate had 50% of the particles under 0.589 µm, compared to chitosan emulsions, which was 2.64 µm, which was much higher. The chitosan emulsion droplets range from 0.8 to a maximum of 18.7 µm in size (Figure 3). A similar view can also be observed in Figure 2; alginate samples droplet sizes were smaller than chitosan. The chitosan emulsion droplet sizes were significantly higher compared to alginate emulsions (Figure 3).

In this study, the emulsions prepared with alginate, characterised by droplet sizes ranging from 0.3 to 5.6 µm, can be classified as microemulsions. It is essential to note that microemulsions are often defined not just by their particle size (100 to 1000 nm) but also by their thermodynamic stability. This stability arises from the spontaneous formation of droplets, minimising the system's free energy without requiring external energy for emulsification. In contrast, the chitosan-based emulsions, with droplet sizes from 0.8 to 18.7 µm, align more closely with macroemulsions, which are typically kinetically stable rather than thermodynamically stable. This distinction underscores the inherent

stability advantages of microemulsions, which may offer significant benefits in applications requiring long-term stability without phase separation [46].

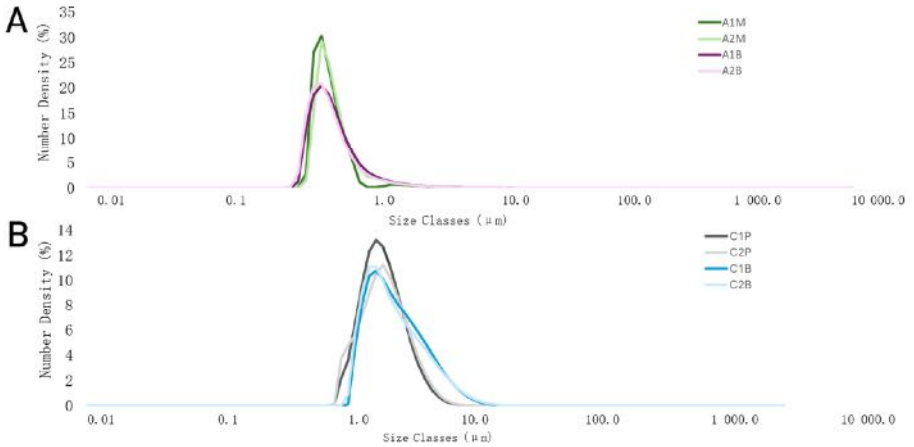

Figure 3. The graph of particle size distribution of (**A**)—alginate emulsions and (**B**)—chitosan emulsions. The results are mean values (n = 3). Sample meanings are provided in Table 3.

In this study, the oil was dispersed within a gel and water phase. Water has a refractive index of 1.33. This index is notably similar to those of alginate and chitosan solutions (ranging from 1.33 to 1.35) [47–49]. So, the water refractive index was used for the research to determine oil sizes. This close refractive alignment is crucial as it ensures the compatibility of the emulsion. Chitosan and alginate play integral yet distinct roles in the stabilization of emulsions. Chitosan, a cationic biopolymer, contributes to emulsion stability by forming a protective barrier around oil droplets, which prevents them from coalescing. Its positive charge effectively interacts with the negatively charged components of the oil, enhancing the emulsion's stability [38]. On the other hand, alginate, an anionic biopolymer, stabilizes emulsions by increasing the viscosity of the aqueous phase and forming a gel matrix that traps oil droplets. This prevents them from merging and settling, thereby maintaining the uniformity and consistency of the emulsion [50]. Each biopolymer's compatibility with the aqueous phase underpins its effectiveness in emulsion stabilization, highlighting the importance of selecting appropriate stabilizers based on their charge and interaction characteristics with the dispersed phase.

Uniformity in droplet size distribution is a critical factor contributing to the consistency and quality of emulsion-based products. Uniformity is a measure of absolute deviation from the median; comparing the uniformity of the same formulation samples but using different alginic concentrations, it is clear that sample uniformity was not the same but statistically insignificant ($p > 0.05$). Therefore, alginate concentration did not influence oil particle size. Using β-CD as an emulsifier, emulsions had higher uniformity but lower density than using mustard extract. In chitosan samples, the uniformity was almost the same. It was the same in both polysorbate samples. In the samples with β-CD, uniformity was similar. Uniform particle size can affect not just the aesthetic appeal of products but also the effectiveness of active ingredient delivery. If the uniformity is up 0.3, it might still be acceptable to consider an emulsion as having reasonably uniform droplet sizes, as can be said about all the samples in this research.

3.2. Microcapsules Formation

3.2.1. Physical Parameters of Microcapsules

Following the evaluation of emulsion stability, microcapsule formation using the emulsions was explored. The transition from stable emulsions to robust microcapsules marks a critical step towards achieving the goal of enhancing the delivery and efficacy of bioactive compounds. The microcapsules' physical parameters, including size, water absorption, morphology, and in vitro release profiles of phenols and isoflavones genistein daidzein and biochanin A are discussed below, illustrating the impact of our selected emulsifiers on improving bioactive compound encapsulation.

All microcapsules formed were spherical in shape and exhibited various shades of brown colouration. When placed in water, some appeared slightly yellowish (Figures 4 and 5).

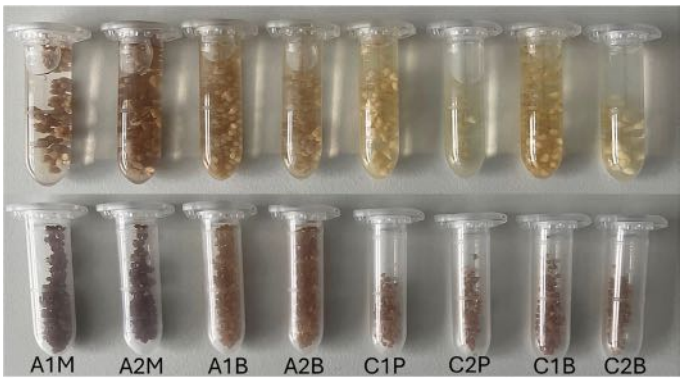

Figure 4. Microscopic pictures of microcapsules. On the top row are freshly prepared microcapsules in the preparation solution, and on the bottom are dried microcapsules. Sample meanings are provided in Table 3.

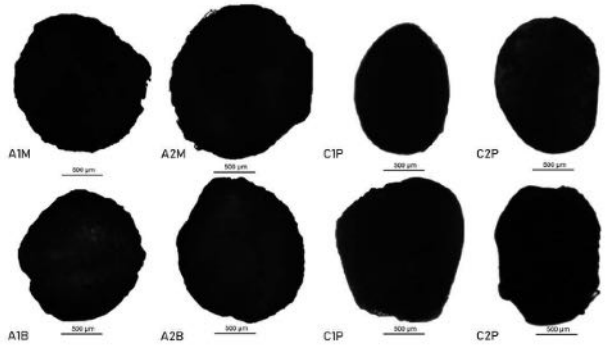

Figure 5. Dried microcapsule's microscopic view via light microscope (100×). Sample meanings are provided in Table 3.

Alginate microcapsules exhibited greater uniformity compared to those formed with chitosan. Microcapsules containing mustard extract appeared darker due to the extract's light brown colouration, accompanied by a subtle mustard aroma. Notably, when β-CD was employed as an emulsifier, microcapsules exhibited reduced aggregation and achieved

a more consistent morphology, contrasting with those formed using mustard extract as an emulsifier. When extruding alginate microcapsules, it was found that the optimal height from the needle to the solution surface was 20 cm, with minimal effect observed upon further increases. However, the pumping speed proved to be more critical, as exceeding 0.5 mL/min led to irregular capsule shapes.

When producing chitosan capsules at elevated heights, the formation of "tails" was observed, while higher emulsion ejection rates failed to yield uniform capsules. Due to the thicker consistency of chitosan emulsions, a lower pumping speed was necessary. Although various heights and speeds were tested, the methods outlined in Section 2.4 consistently yielded the most uniform microcapsules (Figure 5). Using polysorbate 80 as an emulsifier created more round microcapsules compared to β-CD.

When comparing chitosan and alginate capsules, it was observed that alginate microcapsules appeared rounder and exhibited a similar structure. During extrusion, chitosan microcapsules tended to form longer shapes, yet after complete water evaporation, they appeared smaller compared to alginate microcapsules (Figures 4 and 5).

The sizes of freshly prepared microcapsules varied depending on alginate concentration (Table 6). Microcapsules with lower alginate concentrations were bigger, but their swelling capacity was lower compared with microcapsules with higher alginate concentrations. Microcapsules with mustard extract were bigger compared to microcapsules with β-CD. β-CD, composed of seven glucose units, features a unique structure with a hydrophobic cavity and a hydrophilic exterior. This enables it to form inclusion complexes with hydrophobic compounds like oils or lipids, often found in emulsions [51]. By encapsulating these hydrophobic components, β-CD improves their stability and dispersibility in water-based systems, resulting in smaller microcapsules due to enhanced emulsion stabilisation. The capsules underwent drying and shrinkage. By the end of the day, their weight remained constant, indicating stability. The correlation between size and weight remained consistent. Notably, the largest capsules measured were A2M, prepared with alginate and mustard extract, measuring 1.74 ± 0.06 mm (Table 6).

Table 6. Microcapsules firmness and swelling parameters. The results are mean values (n = 3). Sample meanings are provided in Table 3.

	Firmness, g	**Microcapsules Diameter (mm)**		**Swelling, %**
		Wet	**Dry**	
A1M	4400.01 ± 159.66	2.58 ± 0.06	1.49 ± 0.10	135.37 ± 2.45
A2M	3138.10 ± 104.12	3.06 ± 0.03	1.74 ± 0.06	124.86 ± 1.56
A1B	3512.23 ± 51.97	2.18 ± 0.04	1.35 ± 0.07	146.11 ± 1.67
A2B	2609.90 ± 214.37	2.46 ± 0.08	1.46 ± 0.12	132.82 ± 1.11
C1P	1144.91 ± 110.29	1.79 ± 0.09	1.22 ± 0.08	268.19 ± 1.65
C2P	538.31 ± 20.32	1.97 ± 0.05	1.27 ± 0.06	310.85 ± 3.56
C1B	1266.78 ± 97.97	2.37 ± 0.05	1.32 ± 0.08	271.52 ± 2.64
C2B	960.40 ± 60.59	2.15 ± 0.02	1.26 ± 0.11	248.39 ± 1.84

In chitosan microcapsules, the use of polysorbate 80 yielded smaller microcapsules compared with β-CD. The study by P. Eslami (2017) et al. supported our findings, indicating that the use of β-CD significantly increased microcapsule size compared to polysorbate 80. Additionally, it was noted that adjusting the amount of β-CD polysorbate 80 in the formulation had a significant impact on both encapsulation efficiency and particle size [52]. In chitosan microcapsules with β-CD, the swelling capacity was opposite that of the alginate-based samples: larger microcapsules exhibited higher water retention. The same trend was observed in microcapsules with polysorbate 80 as well, where larger microcapsules demonstrated a higher swelling capacity. Polysorbate 80 creates a protective layer that stops water evaporation, whereas chitosan increases the viscosity and thickness of the microcapsule wall, thereby diminishing water retention [26,53]. Like alginate microcapsules, those made of chitosan also experienced shrinkage. Among them, the smallest was

the chitosan microcapsules C1P, incorporating polysorbate 80, measuring 1.22 ± 0.08 mm (Table 6).

The alginate and chitosan samples experienced significant shrinkage, reducing by more than 50% of their original sizes. Notably, chitosan microcapsules containing polysorbate 80 exhibited the least shrinkage due to their lower water content (C1P~53.70% and C2P~55.70%). Likewise, samples containing β-CD showed a comparable reduction of approximately 75%. Alginate samples prepared with mustard experienced shrinkage of around 61.56% and 68.25% for A1M and A2M, respectively (Table 6). When comparing alginate and chitosan microcapsules, a decrease in alginate concentration coupled with the use of β-CD leads to an increase in size. This is because fewer alginate molecules are available for gel formation, enabling greater expansion. In contrast, increasing extract concentration in chitosan microcapsules and incorporating β-CD resulted in smaller microcapsules. This is due to the reduced availability of chitosan for gel formation in ethanolic extract and additional phenols, leading to smaller microcapsules, an effect that is further enhanced by the presence of β-CD.

Alginate microcapsules were much stronger than chitosan. A1M sample had the highest firmness—4400.01 ± 159.66 g/The microcapsules prepared from emulsions with higher alginate concentrations were harder, and the use of mustard extract also influenced the firmness (Table 6). In the samples of alginate microcapsules, smaller microcapsules (A1M; A1B) showed higher firmness compared to larger ones (A2M; A2B). This was particularly noticeable in the larger capsules with lower alginate concentrations, leading to reduced water capacity (Table 6).

The firmness of the chitosan samples was significantly lower compared to that of the alginate samples. The stronger capsules were the ones with lower extract concentrations. Using emulsifiers in the C1 formulation has not considerably improved the firmness. However, using β-CD in formulation C2 (C2B) yielded higher firmness compared to polysorbate 80 sample (C2P).

3.2.2. In Vitro Release of Bioactive Compounds from Microcapsules

In this research, in vitro release from all the capsules was investigated, and total phenolic compounds (Table 7) and isoflavones—daidzein, genistein and biochanin A—were determined in the media (Figures 6–8). Chitosan microcapsules were fully solubilised in the stomach media; therefore, after 90 min, they were not continuously put through the intestine media. However, alginate microcapsules are soluble only in the alkaline media, so the active compounds were released in the gut media (Table 6). Nevertheless, after 270 min of in vitro release (150 min in gut media), not all alginate microcapsules entirely disintegrated. A1 formulation microcapsules were still intact, even though most of the capsules were dissolved. These samples had the thicker shells of the increased alginate concentration in the formulation.

Table 7. In vitro release of phenolic compounds from microcapsules. The results are mean values (n = 3). Sample meanings are provided in Table 3.

	30 min, mg GAE/g	60 min, mg GAE/g	90 min, mg GAE/g	120 min, mg GAE/g	150 min, mg GAE/g	180 min, mg GAE/g	210 min, mg GAE/g	270 min, mg GAE/g
A1M	1.99 ± 0.13	2.39 ± 0.13	2.82 ± 0.14	16.84 ± 0.16	18.32 ± 0.1	19.66 ± 0.12	20.32 ± 0.16	20.84 ± 0.29
A2M	2.31 ± 0.1	2.8 ± 0.2	3.23 ± 0.07	17.34 ± 0.05	18.26 ± 0.12	18.76 ± 0.1	22.71 ± 0.08	28.97 ± 0.14
A1B	1.79 ± 0.02	2.03 ± 0.08	3.04 ± 0.08	14.23 ± 0.1	14.88 ± 0.16	15.94 ± 0.18	17.4 ± 0.08	17.92 ± 0.06
A2B	1.6 ± 0.08	2.45 ± 0.02	3.05 ± 0.06	15.35 ± 0.12	16.98 ± 0.09	17.42 ± 0.12	18.33 ± 0.02	23.65 ± 0.09
C1P	9.2 ± 0.41	13.56 ± 0.46	14.83 ± 0.64	-	-	-	-	-
C2P	11.42 ± 0.56	16.5 ± 0.25	18.14 ± 0.19	-	-	-	-	-
C1B	7.98 ± 0.26	11.65 ± 0.2	14.52 ± 0.56	-	-	-	-	-
C2B	10.11 ± 0.18	14.65 ± 0.16	16.59 ± 0.6	-	-	-	-	-

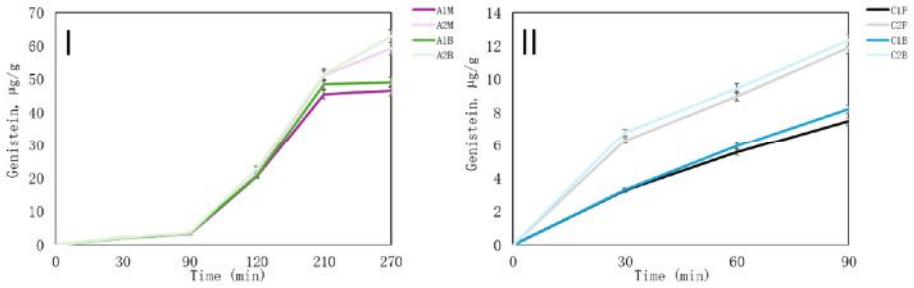

Figure 6. In vitro release of genistein from microcapsules. (**I**)—the release of microcapsules from alginate; (**II**)—the release of microcapsules from chitosan. The results are mean values (n = 3). Sample meanings are provided in Table 3.

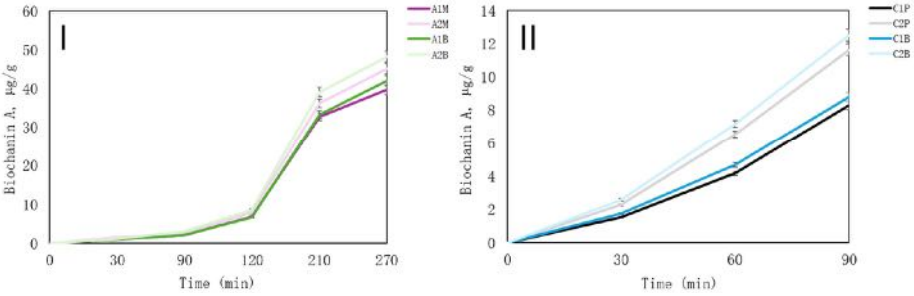

Figure 7. In vitro release of biochanin A from microcapsules. (**I**)—the release of microcapsules from alginate; (**II**)—the release of microcapsules from chitosan. The results are mean values (n = 3). Sample meanings are provided in Table 3.

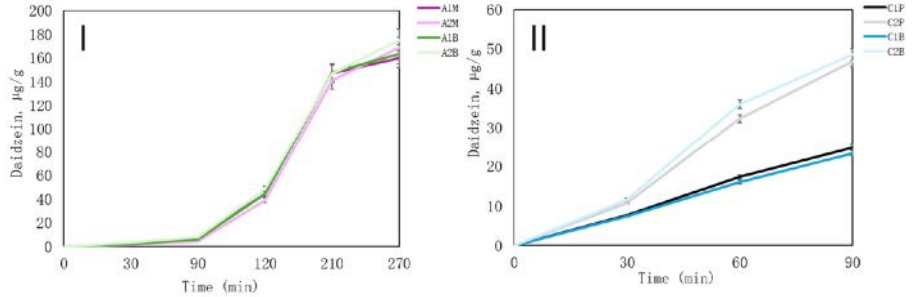

Figure 8. In vitro release of daidzein from microcapsules. (**I**)—the release of microcapsules from alginate; (**II**)—the release of microcapsules from chitosan. The results are mean values (n = 3). Sample meanings are provided in Table 3.

The bioactive compounds from microcapsules were released gradually. Most of the phenolic compounds were released from the microcapsules, but some extract losses occurred during the microencapsulation process. The highest phenolic content of alginate

microcapsules was released after 210 min. The formulation A2 (when the alginate concentration was 0.5%) solubilised faster than the A1 formulation with either β-CD or mustard extract (Table 7).

Chitosan microcapsules also gradually solubilised the C2 formulation, releasing almost all the active compounds. As in the alginate microcapsules, active compounds were lost because of the microencapsulation process and washing off of the extract. Nevertheless, by combining microcapsules of chitosan and alginate, it would be possible to obtain a product that can be soluble in the stomach and intestine for the best effect.

The release of three isoflavones—genistein, daidzein, and biochanin A—from microcapsules was assessed. Figure 6 illustrates the release of genistein from both alginate and chitosan microcapsules. Studies on portal vein plasma levels have indicated that genistein exhibits higher bioavailability in its aglycon form compared to its glycoside counterpart. Specifically, genistein is only partially absorbed in its glycosidic form [54]. Because of this reason, red clover extract was prepared by combining ultrasound processing and heat reflux. This extraction helps yield genistein instead of genistin. As mentioned before, after 270 min of in vitro release, only samples A1M and A1B did not completely dissolve, suggesting that not all of the active compounds were released. Most of the genistein was released in the sample A2B—62.85 ± 1.89 μg/g. The lowest concentration of genistein was released from sample A1M—46.57 ± 1.4 μg/g (Figure 6I). Genistein was released gradually, with formulation A2, particularly with β-CD, yielding superior results. Cyclodextrins (CDs) facilitate the delivery of poorly water-soluble and chemically unstable drugs to the body, thereby enhancing drug solubility when incorporated into microcapsules [55]. Genistein is mainly absorbed in the gastrointestinal tract, especially in the small intestine, where nutrient absorption primarily occurs. Its excellent bioavailability ensures efficient absorption throughout the gastrointestinal tract [56].

A similar trend was observed in chitosan microcapsules, where the incorporation of β-CD as an emulsifier resulted in improved genistein release. Specifically, formulation C2 exhibited higher isoflavone release, but within this formulation, the sample with β-CD (C2B) demonstrated greater genistein release compared to C2P with polysorbate 80 (Figure 6II).

A similar release trend was observed for daidzein and biochanin A, mirroring that of genistein. Biochanin A exhibits rapid absorption in the gut owing to its favourable permeability and lipophilic properties. Despite this, biochanin A is noted for its poor bioavailability [57]. The release of biochanin A is shown in Figure 7.

As alginate microcapsules are not soluble in acidic conditions, the release of isoflavones in stomach media is limited. The isoflavones that were released may have remained on the surface after microencapsulation. Most of the biochanin was released after 210 min in vitro (Figure 7I).

All chitosan samples were completely dissolved in stomach media. Among them, C2 formulations exhibited the highest release rates (Figure 7II). However, the concentrations of released biochanin A were relatively low. This may be attributed to its poor solubility, despite attempts to enhance it with the excipients used in the microcapsules.

Most of all, isoflavones daidzein was released in the highest concentrations in chitosan and alginate microcapsules (Figure 8I,II). Because of its chemical structure, daidzein as genistein and biochanin A exhibit low solubility in both water and lipids. Consequently, it is primarily absorbed in the intestinal tract following oral administration and readily undergoes metabolism, forming glucuronic acid or sulfuric acid conjugates [58].

Formulations A2 and C2, especially when formed with β-CD, demonstrate considerable potential for further refinement and advancement as proficient delivery systems for bioactive compounds. Moreover, incorporating mustard into alginate-based samples can enhance microcapsules by enriching them with additional flavonoids and phenolic compounds. Determining the in vitro release of isoflavones provides valuable insights into their behaviour in formulated products, aiding in the development of effective delivery systems and therapeutic strategies.

4. Conclusions

In conclusion, this study demonstrates the effectiveness of mustard extract and β-CD as emulsifiers in forming stable microcapsules, with alginate showing high stability. The choice of emulsifier significantly influences microcapsule morphology, emphasising the need for careful formulation design. Alginate microcapsules exhibit controlled release in both formulations, while chitosan microcapsules dissolve in stomach media, suggesting targeted delivery potential. The A2 and C2 formulations showed the most potential of all the samples with optimal swelling, release and firmness. Incorporating β-CD enhances bioactive compound release, offering opportunities for improving delivery systems in both gelificators samples, whereas mustard with alginate could additionally enrich the samples with phenols. Future research should focus on refining formulations for enhanced efficiency and kinetics. The use of alginate and chitosan microcapsules in a single pill holds promise for versatile delivery systems. These microcapsules, soluble in both stomach and intestines, containing phenols and isoflavones from red clover, could offer enhanced bioavailability and targeted delivery, benefiting various applications in health supplements, foods, pharmaceuticals, and drug delivery systems. Future investigations should focus on the clinical implications of these delivery systems, exploring their safety, efficacy, and potential benefits for human health.

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