Research

Quantitative Determination of Total Phenolic Compounds in Plant Extracts Using Supercritical Carbon Dioxide and Water-ethanol mixture

Bonin Luka[1,‡], Schofs Laureano[2,3,‡], Pečan Luka Irenej[4,5], Oblak Tine[6], Tavčar Gašper[6], Ponikvar-Svet Maja[6], Silverman Julian R[7], Sánchez Bruni Sergio[2,3], Jeran Marko[6,*]

1. University of Ljubljana, Biotechnical Faculty, Department of Biotechnology, Ljubljana, Slovenia
2. Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Buenos Aires, Argentina
3. Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET-UNCPBA-CICPBA, Tandil, Buenos Aires, Argentina
4. University of Trieste, Department of Life Sciences, Trieste, Italy
5. University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Chair for Agrarian Economics, Policy and Law, Ljubljana, Slovenia
6. "Jožef Stefan" Institute, Department of Inorganic Chemistry and Technology, Ljubljana, Slovenia
7. Fashion Institute of Technology, Department of Science and Math, New York, USA

‡ These authors contributed equally to this work

* Correspondence: Jeran Marko; marko.jeran@ijs.si

Citation: Bonin L, Schofs L, Pečan LI, Oblak T, Tavčar G, Ponikvar-Svet M, Silverman JR, Sánchez Bruni S, Jeran M. Quantitative Determination of Total Phenolic Compounds in Plant Extracts Using Supercritical Carbon Dioxide and Water-ethanol mixture. Proceedings of Socratic Lectures. **2025**, 12(II), 51-58. https://doi.org/10.55295/PSL.12.2025.II5

Abstract:
Natural products serve as an important source of bioactive compounds with significant applications in various fields of science. Among these, polyphenols are particularly noteworthy due to their antimicrobial and antioxidant properties and role in capturing free radicals. This study quantitatively determined the total phenolic content in plant extracts prepared using two distinct extraction methods: supercritical carbon dioxide (SC-CO_2) and water-ethanol maceration. The Folin-Ciocalteu method was employed for quantitative analysis, with results expressed as gallic acid equivalents per kilogram of plant material. The findings demonstrated that water-ethanol extracts consistently contained higher levels of phenolic compounds compared to SC-CO_2 extracts across all analyzed samples. For instance, rosemary extracts prepared with water-ethanol had the highest phenolic content, while thyme and marigold showed lower values. In contrast, SC-CO_2 extracts yielded significantly lower phenolic concentrations, likely due to the selective extraction of lipophilic compounds. Variations in phenolic content were attributed to factors such as sample polarity, plant type, and environmental conditions. These results highlight the importance of selecting appropriate extraction methods based on the desired application. Water-ethanol maceration proved superior for obtaining extracts rich in phenolic compounds, making it suitable for applications requiring high antioxidant activity. This study underscores the potential of plant-derived polyphenols as natural, "green" bioactive ingredients for diverse scientific and therapeutic purposes.

Keywords: Plant; Supercritical carbon dioxide; Water-ethanol, Extracts; Polyphenols, Biologically active compounds; Medicine

1. Introduction

Phenols and their derivatives, including polyphenols, are a prominent group of secondary metabolites found in plants (**Figure 1**) (Cowan, 1999; Daglia, 2012). Polyphenols are characterized by the presence of one or more hydroxyl groups attached to a benzene ring, and their production is closely linked to plant growth and responses to biotic or abiotic stress. These compounds exhibit a wide range of biological activities, such as anti-inflammatory, anti-infective, antiproliferative, antimicrobial, and antioxidant effects (Luna-Guevara et al., 2018; Othman et al., 2019).

Polyphenols are particularly known for their antioxidant properties, as they scavenge and neutralize free radicals, thereby reducing or preventing oxidative cell damage, including oxidative DNA degradation (Jeran et al., 2021; Nisca et al., 2021). Due to their diverse biological effects, polyphenols have found applications as potential active pharmaceutical ingredients and are widely used in the textile, food, and cosmetics industries (Albuquerque et al., 2021).

In addition, plant and its derived polyphenols, have long been used to treat infections due to their antimicrobial properties. The antimicrobial efficacy of polyphenols is directly influenced by their specific composition and the extraction method used. For instance, methanol extracts of rosemary have demonstrated stronger antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as yeasts, compared to water extracts (Moreno et al., 2006). Similarly, the antimicrobial effects of ginger extracts are attributed primarily to phenolic compounds such as eugenol, shogaols, zingerone, gingerdiols, and gingerols. Since these compounds are insoluble in water, organic ginger extracts tend to exhibit greater antimicrobial activity (Beristain-Bauza et al., 2019).

In thyme, the antibacterial activity of its extracts correlates with their total phenolic content, with key components such as thymol, carvacrol, and eugenol demonstrating antimicrobial efficacy in both planktonic and biofilm forms of pathogens (Mokhtari et al., 2023; Walsh et al., 2019). The choice of organic solvent also significantly impacts the extraction of antimicrobial compounds. For example, methanol extracts of *Calendula officinalis* L. showed larger zones of inhibition against several clinical bacterial pathogens compared to ethanol extracts. However, ethanol extracts demonstrated higher activity against specific Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* (Efstratiou et al., 2012).

Additionally, plant-derived polyphenols have shown synergistic effects when combined with conventional antimicrobial agents, leading to enhanced activity against clinical pathogens (Manso et al., 2021). For instance, phenolic compounds from *Mangifera indica* L. seeds significantly reduced the minimum inhibitory concentration (MIC) of penicillin G against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Jiamboonsri et al., 2011). Similarly, polyphenols from *Cuspidaria convoluta*, when combined with Ampicillin and Gentamicin, exhibited increased antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Torres et al., 2019).

In the context of rising antimicrobial resistance, these findings highlight the potential of plant-based polyphenols as promising agents for combating multidrug-resistant bacterial infections (Ahmed et al., 2024). Moving forward, plant-derived extracts could play an essential role in developing innovative antimicrobial therapies.

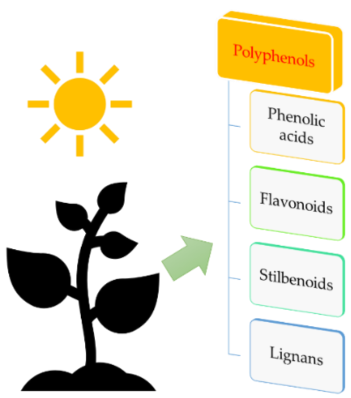


Figure 1. Types of polyphenols.

Due to the extraordinary properties of polyphenols and their versatile applications, especially in the fields of nutrition, pharmacy and medicine, their content in different samples of plant material was investigated in the present experimental work. The work was carried out with samples prepared using two different isolation techniques – water-ethanol extraction and supercritical carbon dioxide extraction. The quantitative determination of the total phenolic content was used to investigate which of the extraction techniques used has a greater yield in leaching the total phenolic compounds. These findings will thus contribute to the development and progress of this highly interdisciplinary field of science. Throughout the work we will use the following model extracts: ginger, calendula, thyme and rosemary.

2. Selected plants

Calendula, rosemary, common thyme and ginger are traditionally used as home remedies both internally and externally.

Calendula (*Calendula officinalis*), also known as pot marigold has been cultivated since ancient times and has a long tradition of use as a medicinal plant (Kemper, 1999). The most important active ingredients of marigold are terpenoids such as triterpenoids (faradiol esters), polyphenols (phenolic acids and flavonoids), carotenoids and polysaccharides. The European Medicines Agency (EMA) recognizes calendula as a traditional herbal medicinal product for the treatment of mild inflammation of the mouth and throat and mild inflammation of the skin as well as to support the healing of minor wounds (European Medicines Agency, 2018).

Rosemary (*Rosmarinus officinalis*) is an aromatic herbal plant known for its high essential oil content. It is rich in terpenes, phenolic compounds, terpenoids and other phytochemicals. These are bioactive substances with specific antibacterial and antioxidant effects (Shankar et al., 2024). It is traditionally used to treat dyspepsia and cramp-like gastrointestinal disorders. Externally, it is used in remedies to treat mild muscle and joint pain as well as mild circulatory disorders (European Medicines Agency, 2011).

Common thyme (*Thymus vulgaris*) main active ingredient is the essential oil, of which the thymol it contains is responsible for its antiseptic and antimicrobial effect. It also contains flavonoids, phenolic acids, triterpenes and other compounds (Lee et al., 2005). Thyme is traditionally used to treat respiratory diseases such as bronchitis, coughs or inflammation of the upper respiratory tract. The thymol contained in the extract has antiseptic and antimicrobial effects (Sakkas & Papadopoulou, 2017).

Ginger (*Zingiber officinale*) has long been used in traditional Chinese medicine for its versatile therapeutic properties, especially its strong anti-inflammatory effect. The main source of the characteristic ginger aroma is the essential oil and non-volatile pungent chemicals. The rhizome contains the main active components, namely gingerols, shogaols, zingerone and gingerdiol (Gupta et al., 2025). Ginger extracts are traditionally used in herbal remedies to prevent nausea and vomiting, mild stomach cramps, flatulence, coughs and sore throats (European Medicines Agency, 2012). The gingerols in ginger have antioxidant and antimicrobial effects. Studies have shown an effect against cardiovascular diseases (Mao et al., 2019). Ginger extract can also be used externally in massage preparations to improve blood circulation and thus help to relieve tension (Polasa & Nirmala, 2003).

3. Methods

3.1. Samples – Isolates from plant material

The isolates (**Table 1**) with supercritical carbon dioxide were prepared by Flavex Naturextrakte GmbH (Germany), the water-ethanol extracts by Epo S.r.l. (Italy).

Table 1. Selected plant material for the experimental work.

Plant	Method of isolation	Part of the plant	Origin (country)
Calendula	Supercritical carbon dioxid	Flowers	Egypt
	Water-ethanol extract	Flowers	Egypt
Rosemary	Supercritical carbon dioxide	Leaves	Spain
	Water-ethanol extract	Leaves	Italy
Thymus	Supercritical carbon dioxide	Leaves	Spain
	Water-ethanol extract	Leaves	Italy
Ginger	Supercritical carbon dioxide	Rhizome	Nigeria
	Water-ethanol extract	Rhizome	Italy

3.2. Determination of total phenolic content in plant samples

The content of total phenolic compounds in plant samples was determined according to the method of Jeran et al. (2023) and Zhang et al. (2010). The Folin-Ciocalteu reagent was used for the quantitative determination and the absorbance was measured by UV/Vis spectroscopy. Aliquots of the samples (2.5 µL) were mixed with 10-fold diluted Folin-Ciocalteu reagent (2 M, Sigma Aldrich, St. Louis, MO, USA) (12.5 µL) and 10 µL of 7.5% aqueous sodium carbonate solution (ACS reagent ≥99.5%, Sigma-Aldrich, Taufkirchen, Germany). The mixture was allowed to stand for 30 minutes in a dark place at room temperature. Then the absorbance at 760 nm was determined spectrophotometrically using Nanodrop One C (Thermo Scientific, Waltham, MA, USA). Ultra-pure water and 70 % ethanol solution served as a blank. The calibration curve (**Figure 2**) was generated with gallic acid standard solutions at concentrations of 2 – 90 µg/mL. The curve was treated as a linear function with the equation: $y = 0.0129x + 0.0586$, and the R^2 value of the line was 0.9959. The results were expressed as amount of gallic acid per plant weight. Each sample measurement was performed in triplicate and the average value was calculated, with the error bars presented in bar graphs.

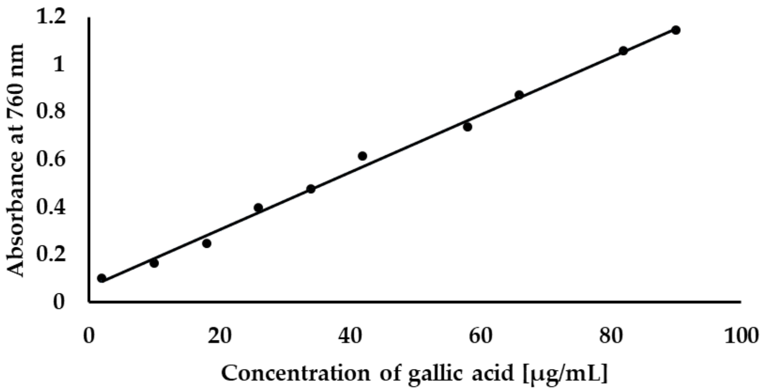
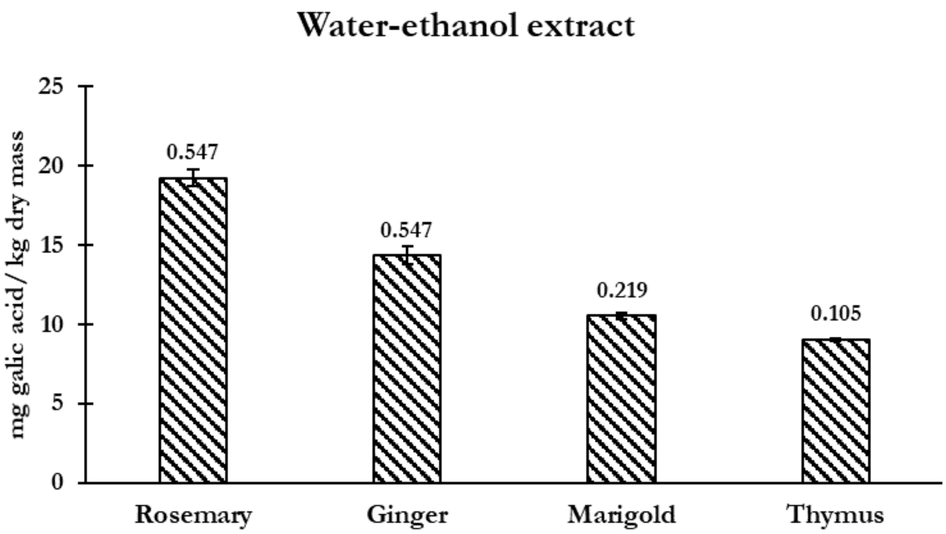


Figure 2. Calibration curve for determining the total phenolic content in plant material.

4. Results

The total phenolic content was analysed using the Folin-Ciocalteu reagent and by measuring the absorbance at 760 nm. The total phenolic content of the analyzed plant samples, comparing the two evaluated extraction methods, is presented in **Figure 3**.

(**a**)



(**b**)

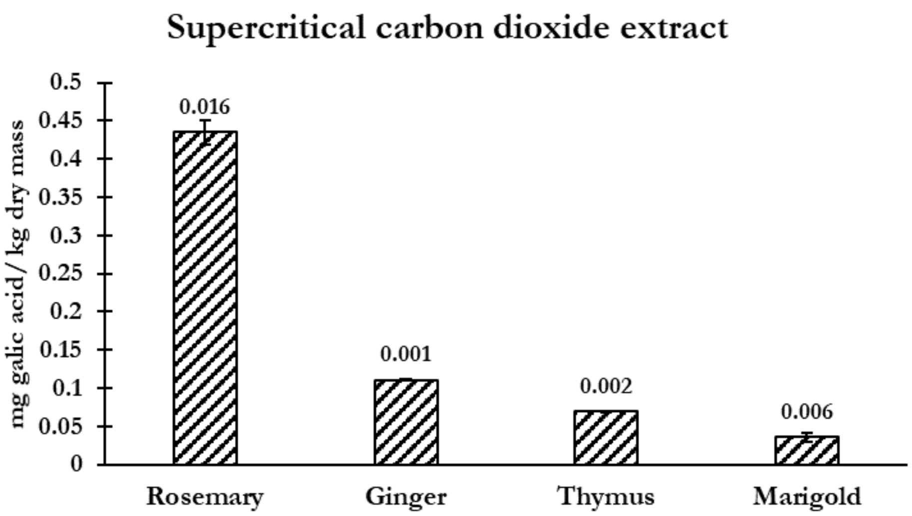


Figure 3. Total phenolic content of the (**a**): analyzed plant material in water – ethanol extract and (**b**): suprecritical carbon dioxide extract

The comparison of the two extraction methods—water-ethanol and supercritical carbon dioxide (SC-CO_2)—reveals important insights into the efficiency and selectivity of each method in extracting phenolic compounds from different plants.

For water-ethanol extracts, rosemary showed the highest total phenolic content, with a result of 19.25 ± 0.55 mg gallic acid/kg dry plant material. This high value indicates that rosemary contains a significant amount of phenolic compounds that are efficiently extracted using water-ethanol, which is known for its ability to solubilize polar compounds. Ginger followed with 14.34 ± 0.55 mg/kg, showing a slightly lower but still substantial phenolic content, with minimal variability, suggesting consistent extraction. Calendula and thyme, however, showed lower phenolic contents of 10.54 ± 0.22 mg/kg and 9.03 ± 0.11 mg/kg, respectively. While these values are lower, they still demonstrate that both plants contain polyphenols, though the concentration is inherently lower compared to rosemary and ginger. The relatively small errors in these values indicate that the method of analysis for these plants was precise, suggesting consistent results.

In contrast, the SC-CO_2 extracts yielded much lower total phenolic content across all plant samples. Rosemary still had the highest total phenolic content in the SC-CO_2 extracts, with a result of 0.44 ± 0.02 mg/kg, which is significantly lower than its water-ethanol extract. This dramatic reduction in phenolic extraction highlights that SC-CO_2 is less effective for polar compounds as it primarily extracts non-polar substances such as terpenes and essential oils. Ginger exhibited 0.11 ± 0.001 mg/kg, which is much lower than the water-ethanol extract, suggesting that SC-CO_2 cannot efficiently extract the polyphenolic compounds from ginger either. Similarly, thyme and marigold showed even lower values of 0.07 ± 0.001 mg/kg and 0.04 ± 0.006 mg/kg, respectively. These results suggest that both plants have significantly lower phenolic contents when extracted with SC-CO_2, further reinforcing the idea that this method is ineffective for extracting polar phenolic compounds.

In addition, the lower errors in the SC-CO_2 isolates indicate that the analytical determination was more consistent, but the phenolic content simply could not be effectively detected.

8. Discussion and Conclusion

Recent research has increasingly focused on natural, "green" active ingredients for a wide range of scientific and therapeutic applications because plants possess the ability to synthesize numerous bioactive compounds. Among these, polyphenols stand out as compounds with significant antioxidant potential due to their chemical structure, characterized by one or more hydroxyl groups attached to a benzene ring. This structure not only enhances their free radical scavenging ability but also imparts a stronger acidic character compared to other alcohol groups, underlying their antioxidant properties (Jeran et al., 2021). Furthermore, Nisca et al. (2021) demonstrated a strong correlation between the total polyphenolic content and antioxidant activity, with higher polyphenolic concentrations leading to stronger antioxidant effects.

The difference between the two methods arises from the contrasting solubility properties of the solvents used. Water-ethanol, a polar solvent system, is highly effective at extracting polar compounds like polyphenols, which are abundant in plant tissues. In contrast, SC-CO_2, a non-polar solvent, excels at extracting lipophilic compounds such as terpenes and oils but is less effective for polar compounds. Consequently, water-ethanol extraction is better suited for obtaining phenolic-rich extracts, making it the preferred method for applications requiring high phenolic content, such as antioxidant and antimicrobial activity or nutraceutical production.

For applications requiring samples rich in antioxidants or phenolic compounds, the findings clearly indicate that water-ethanol maceration is the preferred extraction method. These results further emphasize the value of optimizing extraction techniques to harness the full potential of plant-derived polyphenols for use in research, healthcare, and other applications.

Funding: This research was supported by Slovenian Research Agency through the core funding No. P1-0045.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ahmed SK, Hussein S, Qurbani K, Ibrahim RH, Fareeq A, Mahmood KA, Mohamed MG. Antimicrobial resistance: Impacts, challenges, and future prospects. Journal of Medicine, Surgery, and Public Health. 2024; 2: 100081. DOI: https://doi.org/10.1016/j.glmedi.2024.100081
2. Albuquerque BR, Heleno SA, Oliveira MBPP, Barros L, Ferreira ICFR. Phenolic compounds: Current industrial applications, limitations and future challenges. Food & Function. 2021; 12: 14-29. DOI: https://doi.org/10.1039/D0FO02324H
3. Beristain-Bauza S, Hernández-Carranza P, Cid-Pérez TS, Ávila-Sosa R, Ruiz-López II, Ochoa-Velasco CE. Antimicrobial activity of ginger (Zingiber Officinale) and its application in food products. Food Rev Int. 2019; 35 : 407-426. DOI: https://doi.org/10.1080/87559129.2019.1573829

4. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999; 12: 564-582. DOI: https://doi.org/10.1128/cmr.12.4.564
5. Daglia M. Polyphenols as antimicrobial agents. Curr Opin Biotechnol. 2012; 23: 174-181. DOI: https://doi.org/10.1016/j.copbio.2011.08.007
6. Efstratiou E, Hussain AI, Nigam PS, Moore JE, Ayub MA, Rao JR. Antimicrobial activity of Calendula officinalis petal extracts against fungi, as well as Gram-negative and Gram-positive clinical pathogens. Complement Ther Clin Pract. 2012; 18: 173-176. DOI: https://doi.org/10.1016/j.ctcp.2012.02.003
7. European Medicines Agency, 2011. "Committee on Herbal Medicinal Products (HMPC), Community herbalmonograph on Rosmarinus officinalis L., aetheroleum", EMA/HMPC/235453/2009. Available at: https://www.ema.europa.eu/en/medicines/herbal/rosmarini-aetheroleum
8. European Medicines Agency, 2012. "Final Community herbal monograph on Zingiber officinale Roscoe, rhizoma",EMA/HMPC/749154/2010.Available at: https://www.ema.europa.eu/en/medicines/herbal/zingiberis-rhizoma
9. European Medicines Agency, 2018. "European Union herbal monograph on Calendula officinalis L., flos", EMA/HMPC/437450/2017. Available at: https://www.ema.europa.eu/en/medicines/herbal/calendulae-flos
10. Gupta J, Sharma B, Sorout R, Gopal Singh R, Ittishree, Chandra Sharma M. Ginger (Zingiber officinale) in traditional Chinese medicine: A comprehensive review of its anti-inflammatory properties and clinical applications. Pharmacol Res - Mod Chin Med. 2025; 14: 100561. DOI: https://doi.org/10.1016/j.prmcm.2024.100561
11. Jeran M, Pečan LI, Barrios-Francisco R. Interdisciplinary insight on European spruce (Picea abies): Biologically active compounds and their usage. Proc Socr Lect. 2021; 6: 64-70. DOI: https://doi.org/10.55295/PSL.2021.D.009
12. Jeran M, Romolo A, Spasovski V, Hočevar M, Novak U, et al. Small cellular particles from European spruce needle homogenate. Int J Mol Sci. 2023; 24(5): 4349. DOI: https://doi.org/10.3390/ijms24054349
13. Jiamboonsri P, Pithayanukul P, Bavovada R, Chomnawang MT. The inhibitory potential of Thai mango seed kernel extract against methicillin-resistant Staphylococcus aureus. Molecules. 2011; 16: 6255-6270. DOI: https://doi.org/10.3390/molecules16086255
14. Kemper KJ. Calendula (Calendula officinalis). 1999; 1. Longwood Herbal Task Force.
15. Lee SJ, Umano K, Shibamoto T, Lee KG. Identification of volatile components in basil (Ocimum basilicum L.) and thyme leaves (Thymus vulgaris L.) and their antioxidant properties. Food Chem. 2005; 91: 131-137. DOI: https://doi.org/10.1016/j.foodchem.2004.05.056
16. Luna-Guevara Ma L, Luna-Guevara JJ, Hernández-Carranza P, Ruíz-Espinosa H, Ochoa-Velasco CE, Phenolic compounds: A good choice against chronic degenerative diseases. In: Atta-ur-Rahman FRS, editor. Studies in natural products chemistry. Amsterdam, The Netherlands, Elsevier. 2018; vol. 59, pp. 79-10. DOI: https://doi.org/10.1016/B978-0-444-64179-3.00003-7
17. Manso T, Lores M, de Miguel T. Antimicrobial activity of polyphenols and natural polyphenolic extracts on clinical isolates. Antibiotics. 2021; 11: 46. DOI: https://doi.org/10.3390/antibiotics11010046
18. Mao QQ, Xu XY, Cao SY, Gan RY, Corke H, Beta T, Li HB. Bioactive compounds and bioactivities of ginger (Zingiber officinale Roscoe). Foods. 2019; 8: 185. DOI: https://doi.org/10.3390/foods8060185
19. Mokhtari R, Kazemi Fard M, Rezaei M, Moftakharzadeh SA, Mohseni A. Antioxidant, antimicrobial activities, and characterization of phenolic compounds of thyme (Thymus vulgaris L.), sage (Salvia officinalis L.), and thyme–sage mixture extracts. J Food Qual. 2023; 2602454. DOI: https://doi.org/10.1155/2023/2602454
20. Moreno S, Scheyer T, Romano CS, Vojnov AA. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res. 2006; 40: 223-231. DOI: https://doi.org/10.1080/10715760500473834
21. Nisca A, Ștefănescu R, Stegăruș DI, Mare AD. Phytochemical profile and biological effects of spruce (Picea abies) bark subjected to ultrasound assisted and microwave-assisted extractions. Plants. 2021; 10: 870. DOI: https://doi.org/10.3390/plants10050870
22. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front Microbiol. 2019; 10: 911. DOI: https://doi.org/10.3389/fmicb.2019.00911
23. Polasa K, Nirmala K. Ginger: Its role in xenobiotic metabolism. ICMR Bulletin. 2003; 33(6). ISSN 0377-4910. Availabe from https://www.icmr.gov.in/icmrobject/custom_data/pdf/icmr-bulletins/BUJUNE03new.pdf
24. Sakkas H, Papadopoulou C. Antimicrobial activity of basil, oregano, and thyme essential oils. J Microbiol Biotechnol. 2017; 27: 429-438. DOI: https://doi.org/10.4014/jmb.1608.08024
25. Shankar A, Ali A, Muhammad Abdullah H, Balaji J, Kaure J, et al. Nutritional composition, phytochemical profile, therapeutic potentials, and food applications of rosemary: A comprehensive review. Journal of Food Composition and Analysis. 2024; 135: 106688. DOI: https://doi.org/10.1016/j.jfca.2024.106688
26. Torres CA, Sturla MA, Romero AM, Judis MA. Bioguided isolation of antimicrobial polyphenols from Cuspidaria convoluta leaves and their synergistic effect with antibiotics. Asian Pac J Trop Biomed. 2019; 9: 434-442. DOI: http://dx.doi.org/10.4103/2221-1691.269525

27. Walsh DJ, Livinghouse T, Goeres DM, Mettler M, Stewart PS. Antimicrobial activity of naturally occurring phenols and derivatives against biofilm and planktonic bacteria. Front Chem. 2019; 7: 653. DOI: https://doi.org/10.3389/fchem.2019.00653
28. Zhang L, Chen J, Wang Y, Wu D, Xu M. Phenolic extracts from Acacia mangium bark and their antioxidant activities. Molecules. 2010; 15:3567-3577. DOI: https://doi.org/10.3390/molecules15053567