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Polyphenols/poly(lactic acid) blends – structure, thermal and electret properties

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Poly(lactic acid) (PLA) based functional films incorporated with curcumin, rutin and quercetin were prepared using a solution casting method. The presence of the polyphenols in the polymer matrix was proven by X-ray diffraction analysis. Curcumin was uniformly dispersed in the PLA matrix and showed excellent compatibility with it, as evidenced by the SEM and DSC analysis. The addition of curcumin improved the thermal stability of the PLA film. In contrary, rutin and quercetin were present in micro-sized aggregates in the polymer matrix and showed poor compatibility. All incorporated polyphenols degraded the electret properties of the PLA films.

Keywords: Polyphenols, poly(lactic acid), rutin, curcumin, quercetin, structure, thermal properties

INTRODUCTION

The surge in plastic food packaging waste, largely driven by the fast-food industry, has prompted many countries to urge their packaging manufacturers to enhance food supply chain efficiency [1]. This aims to minimize food spoilage and waste. To tackle this issue, incorporating active agents like antimicrobial and antioxidant compounds into packaging materials has emerged as a promising solution [2]. These 'active' or 'smart' packaging systems can extend food shelf life and reduce food losses, ultimately boosting industry profitability. At the same time, since most plastics are non-biodegradable, their disposal without recycling poses significant environmental challenges [3]. Concerns over these issues, along with the depletion of petrochemical resources, have driven efforts to develop eco-friendly, renewable, and biodegradable biopolymeric materials [4]. Consequently, biodegradable and bioplastic packaging materials derived from renewable resources have garnered significant attention as plastics [5].

Poly(lactic acid) (PLA) is a biodegradable and biocompatible thermoplastic aliphatic polyester derived from renewable resources such as corn starch or sugar beet [6, 7]. It has gained significant attention as a sustainable alternative to traditional petroleum-based plastics due to its environmental friendliness and versatility. While widely used in medicine and some food packaging, PLA suffers from poor mechanical strength and lacks natural antimicrobial properties.

Polyphenols, a class of naturally occurring compounds found in plants, exhibit a wide range of beneficial properties, including antioxidant, anti-inflammatory, and antimicrobial activities. Curcumin, rutin, and quercetin are three prominent polyphenols with potent antioxidant properties [8, 9].

Blending PLA with polyphenols offers a promising approach to address the limitations of PLA while leveraging the advantageous properties of polyphenols. The incorporation of polyphenols into PLA can enhance its mechanical properties, thermal stability, UV resistance, and bioactivity. Additionally, the controlled release of polyphenols from the PLA matrix can provide sustained therapeutic benefits in various biomedical applications [10].

The present research aims to develop new PLA-based multicomponent films with incorporated polyphenols (curcumin, rutin and quercetin) and to examine their morphology, structure, thermal and electret properties.

MATERIALS AND METHODS

Materials

Poly(lactic acid) (PLA), curcumin, quercetin, rutin, and Tween® 20 were purchased from Sigma-Aldrich (Germany) and were used without further characterization or purification. All other chemicals were of analytical grade.

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Preparation of empty and polyphenol loaded PLA films

PLA-based films were prepared by the solvent casting method. First, 2 g of PLA was added to 100 ml of chloroform containing 1.5% Tween® 20, and dissolved for 6 h on a magnetic stirrer. Then 380 mg of polyphenol (curcumin, quercetin or rutin) was slowly added to the PLA solution and dissolved for 1 h at 1000 rpm, followed by homogenization at 12 000 rpm for 5 min. The film-forming solution was cast on levelled glass Petri dishes and dried at a temperature of 30 °C until complete chloroform evaporation. The dry films were stored in a desiccator at a temperature of 25 °C and relative humidity of 20 % for at least 72 h before use. For comparison, neat PLA film without polyphenols was prepared according to the above-described procedure.

The film thickness was measured ten times on each testing sample with a digital micrometer No. 293-5, Mitutoyo, Japan.

Morphology

Scanning electron microscopy (SEM) analysis (ZEISS EVO LS25—EDAX Trident) was applied to investigate the morphology of the materials. For this purpose, part of the sample was directly attached to SEM stubs and subsequently coated with carbon. SEM-EDX was performed at an accelerating voltage of 15 kV.

Powder X-ray diffraction

Powder X-ray diffraction analysis was conducted using an Empyrean Powder X-ray diffractometer (Malvern Panalytical, Almelo, The Netherlands) equipped with a copper X-ray source (λ = 1.5406 Å) and a PIXcel3D area detector. The diffraction patterns of the compounds PLA, curcumin, rutin, quercetin, PLA-curcumin, PLA-rutin, PLA-quercetin were collected in the 2–50° 2 Theta range using the following operation conditions: 40 kV/30 mA, step size of 0.013°, and rotation speed of 15 rpm. The powder patterns of the bulk microcrystalline products (curcumin, rutin, quercetin) were compared with the powder patterns of the composites, thus confirming the presence of desired/undesired crystalline/amorphous phases. Data Viewer ver. 1.9a software (Malvern Panalytical, Almelo, The Netherlands) was used for visualization of the powder patterns.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed on Discovery DSC 50 (TA instruments, New Castle, DE, USA). Samples weighing between 15 and 20 mg were heated in Al_2O_3 pans from 30 to 300 °C (heating rate of 10 °C.min^{-1}) in a dynamic ambient (air) atmosphere (flow rate of 40 mL.min^{-1}).

Differential scanning calorimetry

The melting phenomena and stability of PLA-based films were studied using differential scanning calorimetry (DSC). The DSC 204F1 Phoenix instrument (Netzsch Gerätebau GmbH, Germany) was employed for these investigations. The instrument was calibrated by an indium standard for both heat flow and temperature. The samples were carefully placed in aluminium pans and hermetically sealed, with an identical empty pan serving as a reference. The temperature profile for the DSC analysis included heating from 20 °C to 350 °C at a rate of 10 °C.min^{-1}. The acquired experimental data were analyzed using Netzsch Proteus—Thermal Analysis software (Version 6.1.0B, Germany).

Corona treatment and surface potential measurement

The created composite films were cut into 30 mm samples and charged under corona discharge in normal atmospheric conditions. The charging of the samples in a corona discharge was carried out by means of a conventional corona triode system consisting of a corona electrode (needle), a grounded plate electrode and a grid placed between them. For the charging process, positive 5 kV voltage was applied to the needle electrode and 1 kV of the same polarity was applied to the grid. All films were charged for 1 min. The electret surface potential of the charged samples was determined with the vibrating electrode method with compensation and the estimated error was less than 5%. The normalized surface potentials V/V_0 were calculated, as the value V_0 is the initial surface potential measured about 1 min just after charging the electret.

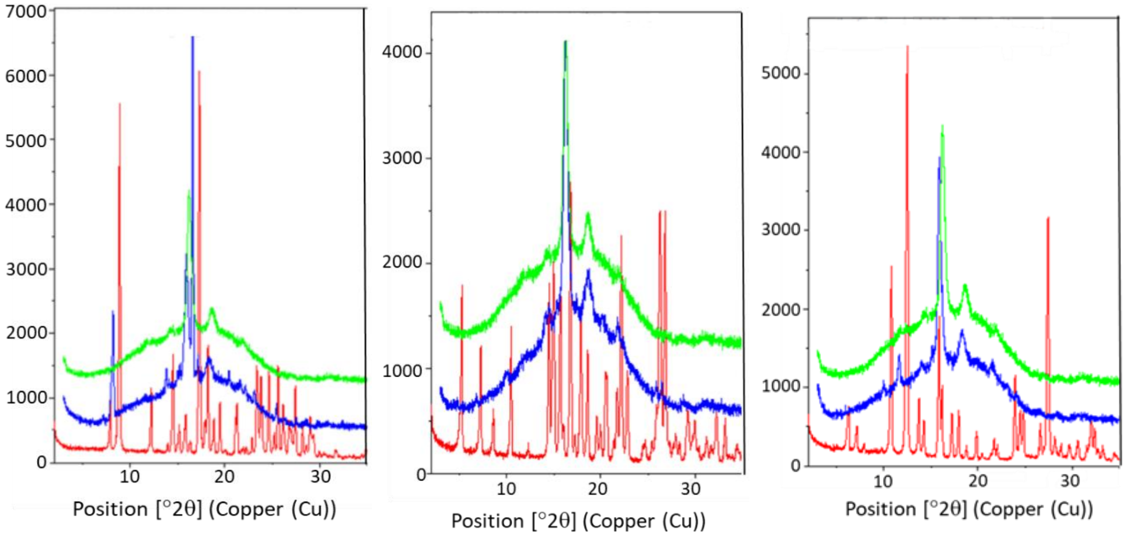
RESULTS AND DISCUSSION

The incorporation of the polyphenols in the PLA matrix and their phase state was confirmed by the X-ray diffraction method. The XRD patterns shown in Figure 1A demonstrate that curcumin was successfully loaded into the PLA matrices (blue graph).

In the case of PLA-curcumin multicomponent film, the PLA support remains basically unchanged as visualized by XRD and SEM (Figures 2a, 2b). The SEM images show the appearance of "roughness" on the surfaces of the films, though no separate crystallites related to curcumin are visible. This is not the case for rutin and quercetin, where, in addition to the surface “roughness”, aggregates can be observed on the surface (Figures 2c, 2d).

The XRD pattern for the PLA-rutin composite (Figure 1b) demonstrates similar features as the PLA-curcumin. However, the presence of rutin in the PLA matrix is not as clearly displayed as that of curcumin, as the two reflexes associated with the rutin phase possess lower intensity when compared to curcumin.

This may indicate inhomogeneous distribution of rutin as observed on the SEM images (Figure 2c). The situation is similar for the PLA-quercetin case (Figures 1c and 2d). The TGA data (Figure 3) reveal that the PLA matrix loses ~5% surficial water up to 100 °C (in two steps) then melts around 130 °C and decomposes above 250 °C. The thermal behavior of the composites seems to prevent the interaction of PLA with ambient moisture. The PLA-curcumin combination is very stable (only ~1% water losses) and starts to decompose after 275 °C.



A) Curcumin B) Rutin C) Quercetin

Figure 1. XRD patterns of PLA (green), polyphenol powder (red), and PLA-Polyphenol multicomponent film (blue)

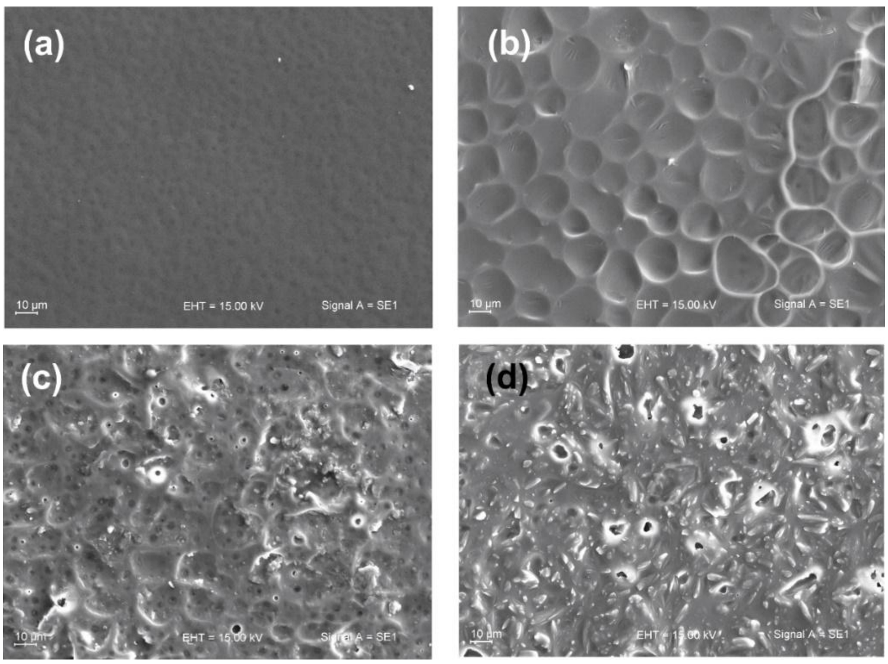


Figure 2. Scanning electron microscopy images of (a) PLA matrix, (b) PLA-curcumin film, (c) PLA-rutin film and (d) PLA-quercetin film.

A similar improvement in thermal stability was reported by Zia *et al.* for an LDPE-curcumin multicomponent film [11]. The thermal comportment of the PLA-rutin also follows this observation, although the losses are a little bit more pronounced (~2% up to 100 °C), and then decomposes above 260 °C. The TGA data for PLA-quercetin differ for the previous two sets. The losses are registered at higher temperatures (130 and 225 °C) and are more pronounced - ~4% in total. The decomposition/melting is registered at values similar to the PLA, rutin and curcumin blended films.

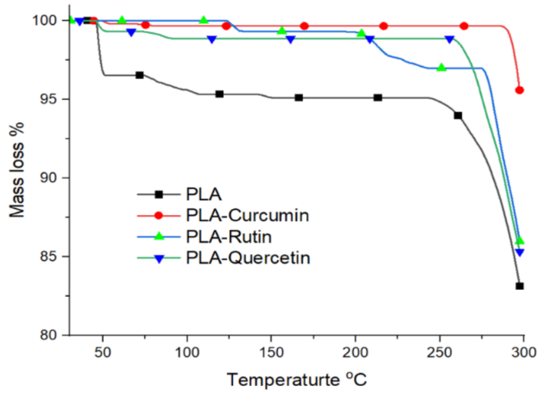


Figure 3. Thermogravimetric analysis (TGA) of PLA, PLA-curcumin film, PLA-rutin film, PLA-quercetin film.

The results from TGA are confirmed by the phase transitions in the PLA-based films, which are examined by differential scanning calorimetry (DSC). The DSC curves of neat PLA film and PLA films loaded with polyphenols, are presented in Figures 4 and 5, respectively. The thermogram of the neat PLA film is characterized by wide endothermic peak between 50 °C and 100 °C which is probably due to evaporation of some moisture content, which was also observed in the TGA curve. A second endothermic peak with a maximum at 155 °C corresponds to the melting phenomena. The increase of the DSC signal at 250 °C is related to thermal decomposition of the material. Similar results for the PLA thermal behavior were demonstrated by Chartarrayawadee and co-workers, who found that the melting temperature of pure PLA is 153.3 °C [12].

No water evaporation is observed in the curcumin-loaded PLA film, suggesting that its water content is very low. It is characterized by two endothermic processes at temperatures of 144.6 °C and 183.7 °C, which can be interpreted as melting of the PLA phase and curcumin crystals, respectively. These data are summarized in Table 1.

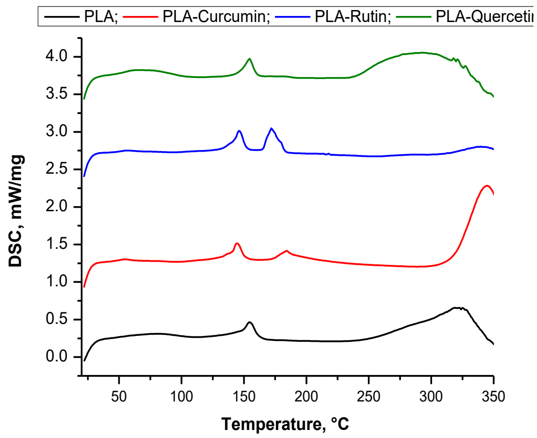


Figure 4. DSC curves of PLA-based films

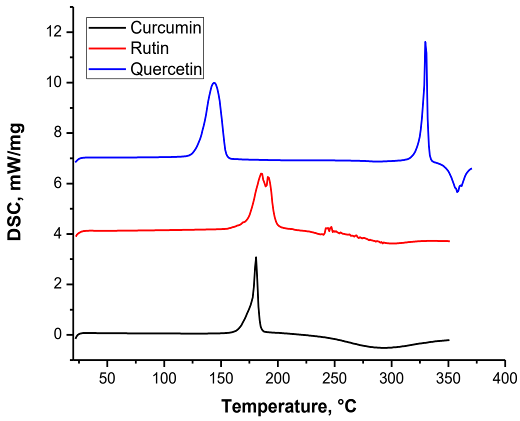


Figure 5. DSC curves of polyphenol powders

Table 1. Melting phenomena in PLA-based films, loaded with polyphenols

Film	Peak 1		Peak 2		
	Temperature, °C	Enthalpy, J/g	Temperature, °C	Enthalpy, J/g	Polyphenol crystallinity, %
PLA	154.6	16.23	–		
PLA - curcumin	144.6	9.829	183.7	6.264	30
PLA – rutin	146.2	11.3	171.8	17.48	42
PL-A - quercetin	154.4	13.6	–		

This film is stable until 300 °C. Taking into account the melting enthalpy of the non-immobilized curcumin and its content in the film, it can be estimated that about 30% of it recrystallized during the curing of the films. The thermal behavior of the PLA–rutin film is very similar. In it, the melting is realized at 146.2 °C and 171.8 °C and the degree of crystallinity of the loaded rutin is 42 %. The DSC data for PLA-quercetin film differ from the previous two sets. In this case no second peak is detected, which could mean that quercetin does not crystallize. The absence of a crystalline structure of the included quercetin is also confirmed by the absence of reflexes in the XRD pattern of the PLA-quercetin film (Figure 1c).

Analyzing the DSC curves of the multicomponent films, two general trends are observed: both the melting temperature of PLA and the enthalpy of the phase transition decrease. The decrease is most noticeable for the PLA-curcumin films, while almost no decrease is registered in the PLA-quercetin system. Such behavior may be related to the compatibility and homogeneity of the polyphenol distribution in the PLA matrix. The PLA–rutin film is the most homogeneous one, as confirmed by SEM analysis. Roy and Rhim also reported compatibility between PLA and curcumin, demonstrated by DSC analysis [13].

An important feature for food packaging are their electret properties [14]. They enable the material to retain a stable electrostatic charge, which can help prevent contamination by attracting and trapping dust, microorganisms, and other fine particles. This enhances the cleanliness and safety of the packaged food, extending shelf life and maintaining quality. Additionally, electret materials can offer antimicrobial benefits and improve barrier properties without relying on additives or external energy sources.

Time dependences of the normalized surface potential for positively charged PLA samples and PLA samples with different polyphenols were measured for 360 min. During the first half hour measurements were taken every 5 min due to the rapid decrease of the surface potential, after which measurements were taken less frequently, due to the decrease of the rate of decay. The measurement was continued until the determination of the steady state values (at 360 min) of each sample. Time dependences of the normalized surface potential of all investigated samples are presented in Figure 6. The data show the averaged value of 6 samples, with standard deviation less than 5% from the mean at 95 % confidence level.

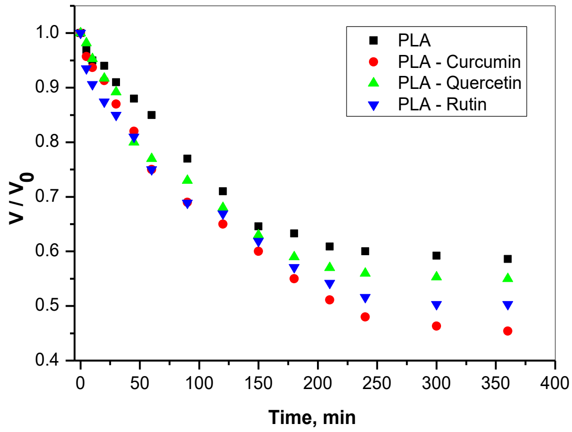


Figure 6. Time dependences of the normalized surface potential for PLA composite films charged in a positive corona.

The obtained experimental results demonstrate that the decay of the normalized surface potential for the first 120 min of the study is exponential, after which the normalized surface potential decreases and it is practically stabilized within 360 min. These results demonstrate the existence of different surface states that are localized on the surface of the sample and that contain entrapped charges within them. The initial exponential decay can be accredited to the release of weakly captured charges from the shallow energy states. After this period of time the normalized surface potential becomes stable at a set value, which can be attributed to the remaining tightly entrapped charges in deeper traps. Similar behavior has been observed earlier in [15, 16]. It was also observed that the normalized surface potential values decrease with the addition of different polyphenols. The results demonstrate that electrets created from PLA possess the highest values of the normalized surface potential when compared to all other types. This can be explained with the differing crystallinity degrees that were determined with the use of the DSC method (see DSC analyses) and the chemical structure of the polyphenols.

CONCLUSION

This study demonstrates the successful incorporation of three polyphenols – curcumin, rutin and quercetin in a poly(lactic acid) matrix. The PLA-curcumin film is characterized as homogeneous, while micro-sized aggregates are found in PLA-rutin and PLA-quercetin films. The appearance of inhomogeneities in the films containing rutin and quercetin is most likely caused by aggregation, which is due to the poor solubility of these two polyphenols in chloroform. To reduce the number and size of aggregates, it is necessary to increase the solubility, for example, by adding appropriate co-solutes. Loading of curcumin improves the thermal stability of the PLA matrix. In all studied multicomponent films, the presence of polyphenol reduces the surface potential and degrades the electret properties of the films. Considering the analyses performed and their results, it can be assumed that the most suitable polyphenol included in the polylactic acid matrix is curcumin. This type of film could be used for packaging of rapidly oxidizing food products.

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REFERENCES

1. H. Nasution, H. Harahap, E. Julianti, A. Safitri, M. Jaafar, *Polymers*, **15(20)**, 4103 (2023).
2. S. Ahmed, D. E. Sameen, R. Lu, R. Li, J. Dai, W. Qin, Y. Liu, *Crit. Rev. Food Sci. Nutr.*, **62**, 3088 (2022).
3. K. J. Groh, T. Backhaus, B. Carney-Almroth, B. Geueke, P. A. Inostroza, A. Lennquist, H. A. Leslie, M. Maffini, D. Slunge, L. Trasande, A. M. Warhurst, J. Muncke, *Sci. Total Environ.*, **651**, 3253 (2019).
4. N. Gontard, H. Angellier-Coussy, P. Chalier, E. Gastaldi, V. Guillard, C. Guillaume, S. Peyron, in: New Mater. Sustain. Film. Coatings, John Wiley & Sons Ltd, 2011, p. 211.
5. S. A. Miller, *ACS Macro Lett.*, **2,** 550 (2013).
6. I. S. M. A. Tawakkal, M. J. Cran, J. Miltz, S. W. Bigger, *J. Food Sci.*, **79**, R1477 (2014).
7. V. Juturu, J. C. Wu, *Crit. Rev. Biotechnol.*, **36**, 967 (2016).
8. K. Jakubczyk, A. Drużga, J. Katarzyna, K. Skonieczna-Żydecka, *Antioxidants*, **9(11)**, 1092 (2020).
9. A. O. Ademosun, G. Oboh, F. Bello, P. O. Ayeni, *Journal of evidence-based complementary & alternative medicine*, **21**(4), NP11 (2016).
10. M. A. Acquavia, J. J. Benítez, S. Guzmán-Puyol, J. M. Porras-Vázquez, J. Hierrezuelo, M. Grifé-Ruiz, J. A. Heredia-Guerrero, *Food Packaging and Shelf Life*, **46**, 101410 (2024).
11. J. Zia, U. C. Paul, J. A. Heredia-Guerrero, A. Athanassiou, D. Fragouli, *Polymer (Guildf)*, **175**, 137 (2019).
12. W. Chartarrayawadee, R. Molloy, A. Ratchawet, N. Janmee, M. Butsamran, K. Panpai, *Polym. Compos.*, **38**, 2272 (2017).
13. S. Roy, J. W. Rhim, *Int. J. Biol. Macromol.*, **162**, 1780 (2020).
14. M. Galikhanov, A. Guzhova, A. Borisova, *Bulg. Chem. Commun.*, **46(B)**, 142 (2014).
15. G. Sessler, *Journal of Electrostatics,* **51-52**, 137 (2001).
16. T. Yovcheva; A. Viraneva; M. Marudova; A. Grigorov, *IEEE Transactions on Dielectrics and Electrical Insulation*, **31(5)**, (2024

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Impact of the COVID-19 pandemic on antibiotic consumption in the Intensive Care Unit and Gynecology Ward at SHOGAT "Prof. Dr. D. Stamatov" Varna, Bulgaria

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One of the greatest threats to human health is bacteria's resistance to an increasing number of available antibiotics. This resistance can arise due to various factors like misuse and inappropriate antibiotic use in humans and animals. Our study aims to analyze the impact of the COVID-19 pandemic on antibiotic use in ICU and GW at the Specialized Hospital of Obstetrics and Gynecology for Active Treatment (SHOGAT) "Prof. Dr. D. Stamatov" Varna, Bulgaria. It is a retrospective analysis of data from 2017-2023 from two wards (ICU and GW) at SHOGAT. To calculate antibiotic consumption, we used the Anatomical Therapeutic Chemical/Defined Daily Dose (ATC/DDD) classification system which is an international standard for calculating antibiotic consumption in a unified technical unit - defined daily dose (DDD). The changes in antibiotic use in both wards of SHOGAT are related to COVID-19 and an adjustment in the hospital's antibiotic policy. A notable increase was observed in the use of two specific antibiotics: azithromycin and cefazolin. The high use of azithromycin in 2021 is explained by the fact that it was the main choice for patients with confirmed COVID-19. The increasing use of cefazolin is related to its application as the drug of choice for preoperative antibiotic prophylaxis. Strict adherence to antibiotic application protocols in the medical facility, along with the recommendations of EUCAST and the Bulgarian Association of Microbiologists, has limited the overuse of antimicrobial agents. This, in turn, leads to reduced treatment costs for patients and limits the development of resistant strains.

Keywords: antibiotic consumption, antibiotic stewardship, COVID-19, antibiotic guidelines

INTRODUCTION

Antibiotics are life-saving and cost-effective medicines, but the increasing resistance of pathogenic bacteria to available antibacterial therapy is one of the biggest threats to human health nowadays. In July 2022, Health Emergency Preparedness and Response (HERA) identified on an annual basis three specific high-impact health threats: pathogens with high pandemic potential; chemical, biological, radiological, and nuclear (CBRN) threats originating from accidental or deliberate release, and antimicrobial resistance [1]. The latest 2022 report from the European Centre for Disease Prevention and Control shows that the death rate from multidrug-resistant bacteria is comparable to that of HIV/AIDS, tuberculosis, and influenza combined. It is also estimated that approximately 70% of infections caused by antibiotic resistant bacteria are due to nosocomial infections [2].

Despite numerous programs to reduce usage and control prescription, resistance to clinically used antibiotics remains widespread, and the number of bacterial pathogens presenting multidrug resistance continues to rise [3]. The high consumption of antibiotics in our food chain and healthcare systems has drastically waned the effectiveness of antibacterial treatments, severely compromising our ability to manage infections. The development and introduction into clinical practice of new antibacterial therapies are inadequate to address the growing threat of antimicrobial resistance (AMR) [4]. According to the World Health Organisation (WHO) antibiotic pipeline data report, eleven new antibiotics have been approved since 2017. Only two of them represent a new class and have a new target of action (vaborbactam + meropenem and lefamulin) [5].

While not a novel paradigm, the One Health concept has seen a significant escalation in its importance in recent years, underscored by the advent of recent epidemics and pandemics, notably COVID-19. This has elucidated the complex interdependence between human, animal, plant, and environmental health, advocating against their isolated consideration. Concurrently, there has been a discernible surge in antimicrobial resistance (AMR) and the prevalence of zoonotic diseases

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(including COVID-19, avian influenza, and mpox) within the European region. Empirical evidence substantiates that identical pathogens proliferate amongst humans and animals, culminating in analogous pathologies primarily exacerbated by the homogeneous application of antibiotics across species. This homogeneity in treatment methodologies fosters an environment conducive to the amplification of AMR. In a preemptive measure, the European Union (EU) instituted a comprehensive prohibition on the utilization of antibiotics as feed additives for animals as early as 2006. Subsequently, in June 2017, the European Commission ratified the European One Health Action Plan against AMR [6]. An analytical review conducted in 2023 delineated the impediments to the efficacious enactment of AMR countermeasures within EU Member States, Norway, and Iceland [7].

According to Machowska and Lundborg [7], the drivers of the irrational use of antibiotics among healthcare providers in Europe are: knowledge, attitude, and perception of healthcare providers about antibiotic use and resistance; lack of adequate education for healthcare providers; pharmaceutical promotion; lack of rapid and sufficient diagnostic tests and local antibiotic susceptibility data; patient–doctor interaction; knowledge, attitude, and perception of pharmacists regarding antibiotic use and resistance [8]. In Europe, healthcare-associated infections (HAIs) have the highest public health burden compared to all other infectious diseases. Each year, 4.3 million patients in hospitals in the EU/EEA acquire at least one healthcare-associated infection during their stay in the hospital [9].

Antimicrobial stewardship (AMS) is a systematic approach to educate and support healthcare professionals to follow evidence-based guidelines for prescribing and administering antimicrobials [10]. AMS is the effort to measure and improve how antibiotics are prescribed by physicians and used by patients. Improving antibiotic prescribing and use is critical to effectively treat infections, protect patients from harms caused by unnecessary antibiotic use, and combat antibiotic resistance [11].

This work aimed to assess the impact of the COVID-19 pandemic on antibiotic usage in two wards: Intensive Care Unit (ICU) and Gynecology Ward (GW) at SHOGAT "Prof. Dr. D. Stamatov" Varna, Bulgaria. The analysis was conducted by reviewing antibiotic prescriptions and comparing annual antimicrobial consumption during the three years preceding the pandemic (2017–2019) with usage during various COVID-19 waves (2020–2023). Additionally, the study aimed to assess changes in antibiotic stewardship policies and their potential effects on antimicrobial use during this period.

EXPERIMENTAL

Study design

A retrospective investigation was conducted for seven years. The study was carried out in Varna, Bulgaria, at the Specialized Hospital of Obstetrics and Gynecology for Active Treatment "Prof. Dr. D. Stamatov" (SHOGAT). SHOGAT is a teaching hospital and has different wards, such as Intensive Care Units (ICU), Gynecology Ward (GW), Neonatal Ward (NW), Maternity Ward/ Labour Ward (MW/LW), High-Risk Pregnancy Ward (HRPW). The hospital has 140 beds [10 (7,14%) ICU, 10 (7,14%) NW intensive care beds, 40 (28,57%) NW and 80 (57,15%) medical. Of these 80 beds, 15 (10,72 %) are part of GW. All antibiotics for oral and parenteral use were set as inclusion criteria for the study. The limitation factor in the presented work is that antibiotic use was examined and analyzed only in two of the hospital wards (ICU and GW) because antibiotic use in them is higher.

Data collection

Data on antimicrobial use between January 2017 and December 2023 were extracted from the pharmacy information system. These data included the consumption of intravenous and oral antibiotics prescribed to inpatients. The collected data were validated by manually checking for errors. To calculate antibiotic consumption, we used the Anatomical Therapeutic Chemical/Defined Daily Dose (ATC/DDD) classification system which is an international standard for calculating antibiotic consumption in a unified technical unit defined daily dose (DDD) per 1000 inhabitants per day (IPD). When calculating antibiotic use in the separate wards, the number of occupied bed days in the ward is taken into account, while the total use is calculated based on occupied bed days in the hospital.

Classification of antibiotics

The used antibiotics were grouped according to their chemical structures into penicillins, cephalosporins, aminoglycosides, fluoroquinolones, tetracyclines, nitroimidazoles, and macrolides. The WHO AWaRe classification (Access, Watch, and Reserve) was used to categorise the antibiotics assessed.

Statistics and software

Antibiotic consumption was analysed using descriptive methods. The data are expressed as percentages and proportions. Data input, percentage calculation, and graphical visualization of the results were performed using Microsoft Excel.

RESULTS AND DISCUSSION

Calculated total antibiotic usage in SHOGAT "Prof. Dr. D. Stamatov" - Varna, Bulgaria by years, covering the period from 2017 to 2023, is presented in Table 1. The data in the table indicate that the total antibiotic usage in the hospital is considerably lower than the average reported for Europe. A 2022 EU/EEA report states that in the hospital sector, the population-weighted mean consumption of antibacterials for systemic use (ATC group J01) was 1,61 DDD per 1000 inhabitants per day (country range: 0.75–3.15), while in SHOGAT it is 0.40 DDD per 1000 inhabitants per day.

During the period 2013–2022, a statistically significant decrease in antibiotics use was observed at the EU/EEA level. Statistically significant decreasing trends were observed for six countries, and a statistically significant increasing trend was observed for two countries (Bulgaria and Croatia) [12]. This trend is also observed in the studied hospital, but despite this, the average values of antibiotic consumption (0.40 DDD/1000IPD) are lower compared to those in Bulgaria (1.72 DDD/1000IPD) and Europe (1.63 DDD/1000IPD) [12].

Table 1. Antibiotic consumption in SHOGAT hospital Varna, Bulgaria, DDD per 1000 inhabitants per day (IPD), from 2017 to 2023

	2017	2018	2019	2020	2021	2022	2023
Total antibiotic consumption *(DDD per 1000 IPD)*	0.339	0.294	0.236	0.419	0.521	0.518	0.445

Table 2. Antibiotics used in ICU and GW (DDD per 1000 IPD, 2017–2023)

ICU and GW antibiotics used *(DDD per 1000 inhabitants per day, from 2017 to 2023)*								
Ward	2017	2018	2019	2020	2021	2022	2023	Total
ICU	7.34	7.31	6.58	12.79	14.32	16. 7	12.46	77.67
GW	2.70	3.33	2.18	4.58	6.35	4.89	6.95	30.98
Total	10.04	10.64	8.76	17.37	20.67	21.76	19.41	108.65

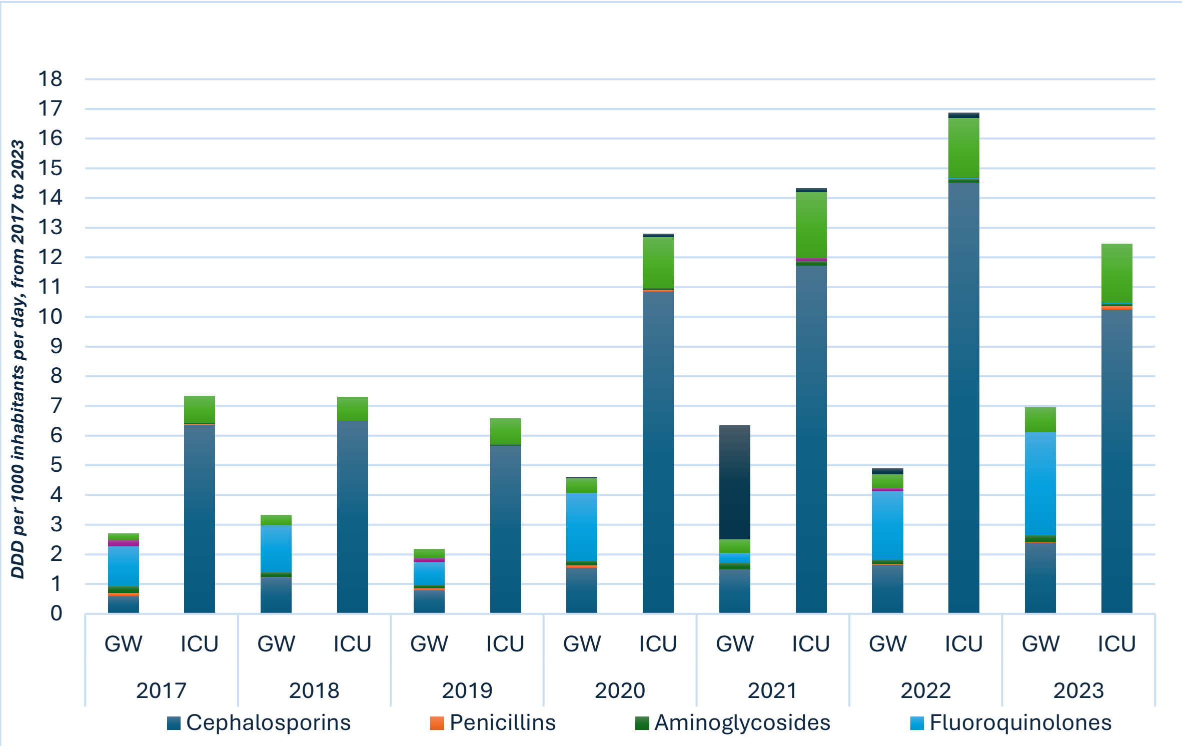


Figure 1. Antibiotics use in ICU and GW at SHOGAT "Prof. Dr. D. Stamatov", Varna, Bulgaria from 2017 – 2023 by chemical structure

These data can be explained by the fact that SHOGAT is a specialized hospital where patients with severe life-threatening infections are not treated. Table 2 presents data for antibiotic usage in two wards – ICU and GW. We chose these two wards because antibiotic consumption is higher than in other wards.

Table 2 shows a significant increase in antimicrobial use, particularly in intensive care units, with peaks in 2020 and 2021. The overall trend indicates that both ICU and GW have increased their use of antimicrobial agents, but the ICU demonstrates more significant variability and a higher average level of use.

A difference is observed between the groups of antibiotics used in the two wards (Fig. 1). In the ICU, the primary antimicrobial agents are cephalosporins, followed by nitroimidazoles (metronidazole). Quinolones (ciprofloxacin) are the preferred antimicrobial agents in the GW, followed by metronidazole as the secondary choice. Aminoglycosides are frequently used in the GW, while they are rarely used in the ICU. The use of penicillins is low in both wards.

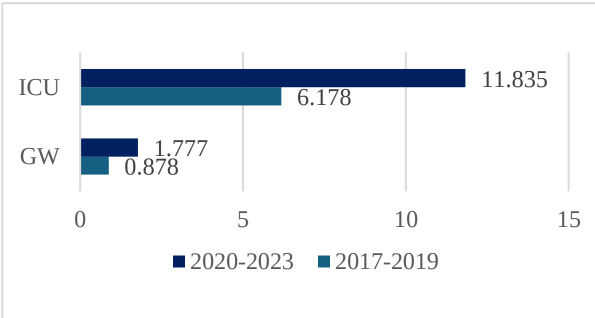
Figure 1 illustrates the impact of the COVID-19 pandemic on antimicrobial usage in the hospital. The use of macrolides (azithromycin) has been observed since 2020. The highest consumption of azithromycin was observed in 2021 in GW. The COVID-19 pandemic was associated with an increase in the consumption of azithromycin, notably during the first waves of the pandemic [13]. After a systematic review and meta-analysis, Khan *et al*. reported that antimicrobial consumption in patients with COVID-19 in high-income countries was lower compared with lower and middle-income countries (58% versus 85%) [14].

The significant reduction in the use of macrolides in 2022 is associated with the emergence of new studies and clinical data showing their limited effectiveness in the treatment of COVID-19 [15]. Data from Figure 1 indicate that macrolides were not used in 2023, which aligns with European and global trends. The measurable impact of the pandemic was a brief increase in macrolide consumption in 2021 compared to all other years.

The comparative analysis of cephalosporin use for the two considered periods (Fig. 2) shows a significant increase in both the GW ward (102.39%) and the ICU (91.57%) for the period 2020-2023.

Surgical site infections are an important complication of surgical procedures, and appropriate antibiotic prophylaxis can reduce the risk of infections during certain procedures [16]. The definition of surgical antibiotic prophylaxis is taken from the 2018 WHO publication "Prevention of infectious complications by administering an effective antimicrobial agent prior to exposure to contamination during surgery" [17]. To prevent infectious complications before exposure to contamination during surgery, WHO guideline recommended "Access group" antibiotics as the first-choice option in most cases. According to the recommendations of the Bulgarian Association of Microbiologists [18], a single dose of cefazolin 2g *i.v.* is applied for preoperative prophylaxis of caesarean section (or cefamandole, or cefuroxime), and as an alternative - metronidazole 500 mg (1000 mg) *i.v.*

The increased use of cephalosporins (cefazolin) after 2020 is associated with a change in the hospital's antibiotic policy. This policy is aligned with the recommendations of the Bulgarian Association of Microbiologists for preoperative prophylaxis in obstetrics and gynecology.



	ICU	GW
Cephalosporins use (2017-2019)	6.178	0.878
Cephalosporins use (2020-2023)	11.835	1.777
*Relative rate of change**	*1.92*	*2.02*

** Relative rate of change = DDD per 1000 IPD (2020-2023)/DDD per 1000 IPD (2017-2019)*

Figure 2. Cephalosporins consumption in two periods

Table 3 shows the detailed pattern of antibiotic prescriptions. The most prescribed antibiotics were cephalosporins (cefazolin, ceftriaxone, and ceftazidime), which account for nearly 70% of all antibiotics used in the hospital. The use of ceftriaxone (33.8%) and metronidazole (12.5%) in ICU and GW is similar to data from a study by Hodoșan *et al.*, conducted at the Emergency Clinical County Hospital of Oradea, Romania (26.46% and 13.05%, respectively) [19].

Table 3. Antibiotics used by WHO AWaRe category and consumption rates (DDD/1000IPD) from 2017 to 2023

Antimicrobials	WHO AWaRe category	DDD/1000IPD 2017-2023	DDD/1000 IPD% 2017-2023
Cefazolin	Access	38.414	35.5%
Amoxicillin	Access	0.321	0.%
Ceftriaxone	Watch	36.519	33.8%
Gentamycin	Access	1.724	1.6%
Ciprofloxacin	Watch	12.211	11.3%
Doxycycline	Access	0.5885	0.5%
Metronidazole	Access	13.5305	12.5%
Ampicillin	Access	0	0 %
Amikacin	Access	0.121	0.1%
Piperacillin/Tazobactam	Watch	0.2065	0.2%
Ampicillin/Sulbactam	Access	0.0635	0.1%
Azithromycin	Watch	4.3975	4.1%
Ceftazidime	Watch	0.005	0.005%
Meropenem	Watch	0.0105	0.01%
Vancomycin	Watch	0.0765	0.07%

Table 4. Antibiotics used according to WHO AWaRe category

	Access	Watch	Reserve
% of antibiotics used by WHO classification	60%	40%	0%

Table 5. Antimicrobial agents and their usage percentages by WHO AWaRe category

Antimicrobial agent	WHO AWaRe category	Percentage of usage (%)
Ceftriaxone	Watch	68.36
Ciprofloxacin	Watch	22.85
Azithromycin	Watch	8.23
Piperacillin/Tazobactam	Watch	0.39
Vancomycin	Watch	0.14
Meropenem	Watch	0.02
Ceftazidime	Watch	0.01

Table 4 presents a summary of the results of our study on total antibiotic use in both wards (ICU and GW) according to AWaRe classification. Notably, 60% of the used antimicrobial agents fall into the Access group, and 40% are from the Watch group. No use of antibiotics from the Reserve group was observed. In the annual report of the European Center for Disease Prevention and Control for 2022, it is stated that only 10 countries met or exceeded the EU target of at least 65% of antibiotic consumption being from the 'Access' group, as per WHO's AWaRe classification of antibiotics. The number of EU Member States at or above the 65% target has not changed since 2019 [12]. Even though the use of 'Access' group antibiotics in Bulgaria does not meet the ECDC's set target, SHOGAT nearly achieves these target values.

In Table 5, we showed the results for the use of antibiotics from the 'Watch' group in ICU and GW from 2017 to 2023. Ceftriaxone is the most used antimicrobial in this group, with a significant usage percentage of 68.36%. Ciprofloxacin follows with a usage percentage of 22.85%, indicating its common use but at a lower frequency than ceftriaxone, and then comes azithromycin with 8.23%. Piperacillin/ tazobactam, vancomycin, meropenem, and ceftazidime are used less frequently, with percentages of 0.39%, 0.14%, 0.02%, and 0.01%, respectively.

CONCLUSION

The antibiotic use changes in both SHOGAT wards are related to COVID-19 and an adjustment in the hospital's antibiotic policy. Despite the observed increase in antibiotic prescriptions during the period 2020-2023, the overall antibiotic use in the hospital remains below the levels for Bulgaria and Europe/EEA. Additional measures are needed to promote the rational use of antibiotics by implementing the WHO AWaRe classification and increasing the prescription of antibiotics from the 'Access' group. WHO recommends that antibiotics from this group should constitute 65% of all antibiotics used. A more extensive study will subsequently present the analyzed data on antibiotic use across all wards of SHOGAT.

Conflict of interest: *Authors declare no conflict of interest.*

REFERENCES

1. European Commission (EC). Public Health. available at: https://health.ec.europa.eu/document/download/18c127ce-da4b-4e4e-a27c-f7b93efb2980_en?filename=hera_factsheet_health-threat_mcm.pdf
2. Antibiotic resistance: a growing threat to human health. Available at https://antibiotic.ecdc.europa.eu/en/publications-data/antibiotic-resistance-growing-threat-human-health
3. T. Golkar, A.V. Bassenden, K. Maiti et al. Structural basis for plazomicin antibiotic action and resistance. *Commun Biol.* **4**, 729 (2021). https://doi.org/10.1038/s42003-021-02261-4
4. WHO Lack of innovation set to undermine antibiotic performance and health gains, 2022 available at: https://www.who.int/news/item/22-06-2022-22-06-2022-lack-of-innovation-set-to-undermine-antibiotic-performance-and-health-gains
5. J. Shrestha, F. Zahra, Jr, P. Cannady, Antimicrobial Stewardship. [Updated 2023 Jun 20], in: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK572068/
1. EU One Health Action Plan against AMR https://health.ec.europa.eu/system/files/2020-01/amr_2017_action-plan_0.pdf
6. European Commission, European Health and Digital Executive Agency, M. Dates, M. Davies A., Kobilsky, *et al*., Study on the barriers to effective development and implementation of national policies on antimicrobial resistance – Final report, Publications Office of the European Union, 2023, https://data.europa.eu/doi/10.2925/826400
7. A. Machowska, C. Stålsby Lundborg, *Int. J. Environ. Res. Public Health*, **16**(1), 27 (2018). Published 2018 Dec 23. doi:10.3390/ijerph16010027
8. European Centre for Disease Prevention and Control. Point prevalence survey of healthcareassociated infections and antimicrobial use in European acute care hospitals. Stockholm: ECDC; 2024. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/healthcare-associated-point-prevalence-survey-acute-care-hospitals-2022-2023.pdf
9. WHO Europe Promoting antimicrobial stewardship to tackle antimicrobial resistance, available at: https://www.who.int/europe/activities/promoting-antimicrobial-stewardship-to-tackle-antimicrobial-resistance
10. CDC's Core Elements of Antibiotic Stewardship, available at: https://www.cdc.gov/antibiotic-use/core-elements/index.html
11. European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA (ESAC-Net) - Annual Epidemiological Report 2022. Stockholm: ECDC; 2023, available at: https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-consumption-europe-2022
12. P. M. Roger, D. Lesselingue, A. Gérard, J. Roghi, P. Quint, S. Un, A. Chincholle, A. Assi, O. Bouchard, V. Javaudin, *et al.*, *Antibiotics*, **13**, 180 (2024). https://doi.org/10.3390/antibiotics13020180
13. S. Khan, S. S. Hasan, S. E. Bond, B. R. Conway, and M. A. Aldeyab. *Expert Review of Anti-Infective Therapy*, **20**(5), 749 (2021). ,Li, M. Zhang, X. Hu, L. Li, F. Meng, K. Li, Y. Lu, *Altern. Ther. Health Med.*, **29**(8), 722 (2023).
14. The WHO AWaRe (Access, Watch, Reserve) antibiotic book, 2022; ISBN 978-92-4-00623 8-2 (electronic version)
15. Global guidelines for the prevention of surgical site infection, second edition. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO
16. Bulgarian Association Of Microbiologists. Perioperative antibiotic prophylaxis in surgery, available at: https://w-=\[w.bam-bg.net/index.php/bg/2016-05-17-12-35-13/24-2016-06-03-12-34-54
17. V. Hodoșan, L. G. Daina, D. C. Zaha, P. Cotrău, A. Vladu, F. R. Dorobanțu, M. O. Negrău, E. E. Babeș, V. V. Babeș, C. M. Daina, *Healthcare*, **11**(9), 1302 (2023) https://doi.org/10.3390/healthcare1109130,2

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European and national regulations for the labeling of dairy products - Bulgarian market overview

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Dairy products, and white brine cheese in particular, are among those for which consumers often prefer farmer production. Product labeling is a primary tool for collaboration between the manufacturer, the food handler, and the consumer. In the present study, we observed and presented data on compliance with rules and norms in farm cheese labeling from cow`s milk in the commercial network, referring to national and European legislative requirements, through documentation and observation methods. We monitored the labeling of 22 commercial brands of farm products, all of which were white brine cow`s milk cheeses. The categories in the survey included the following: 1. Mandatory labeling information, 2. Additional mandatory data for specific types of food, 3. Optional (voluntary) food labeling information. Data analysis gives us a reason to conclude that all farm cheeses included in the study contain the mandatory information from the regulatory documents. A large part of them also contains additional (voluntary) information, which primarily aims to give greater clarity to the consumer about the organic origin of the product, further information about the farm producer, and compliance with national standards for the production of white brine cheese.

Keywords: farm cheese, white brine cheese, labeling, legislative requirements

INTRODUCTION

In recent years, Bulgarian farmers' markets have significantly expanded both their activities and the assortment they offer. Increasing number of consumers prefer to purchase directly from producers and selected traders instead of purchasing from large grocery chains. Farmers` markets and stores are associated with a number of benefits that make them an attractive choice for consumers. They offer fresh and natural products that are cultivated or produced by local farmers. This means that the products are seasonal, fresh and of good quality, they do not contain harmful additives and pesticides. Farmers` markets shopping supports local economic development, sustainable agriculture [1] and environmental protection [13].

Dairy products, and white brine cheese in particular, are among the products for which consumers often prefer the farmer production. Farm cheeses guarantee an organic composition and do not contain pesticides, hormones or traces of antibiotics [15]. Moreover, farm cheese can contain high amount of protein, calcium and vitamins.

"Bulgarian white brine cheese" has been recognized as a Protected Designation of Origin by the European Commission [2]. The term white brine cheese is used to define a semi-hard or soft cheese, matured and stored in a brine made from milk in addition with a starter culture (bacteria *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophiles*, as well as a leaven of bacteria *Lactoccocus lactis subsp. lactis* and *Lactobacillus casei*). It is curdled with a milk-coagulating enzyme, receiving the necessary technological processing, with a dry matter content in the final product of not less than 40 percent and a fat content in the dry matter of not less than 40 percent [16].

Labeling is the primary tool for collaboration between the producer, the food handler and the consumer. Consequently, it is an integral and important part of the marketing effort and also helps proper handling of the product. Most labeling regulations aim to provide objective and informative data to the consumer, assuming that the individual consumer is rational and capable of making informed choices [7]. Ensuring consumer access to

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complete and accurate information about the content and product composition is in the interest of both producers and consumers – in order to protect their health and interests [5, 6].

In Bulgaria, the requirements for providing information when labeling the white brine cheese are regulated in the Ordinance on the specific requirements for dairy products (from 04.11.2021), the Ordinance on the requirements for the labeling and food representation (from 13.12.2014), the Ordinance on the provision of food information to consumers (from 10.04.2021) and Regulation (EU) No. 1169/2011 of the European Parliament on the Provision of Food Information to Consumers and the Council from 25.10.2011. State control body of all industrial goods (with the exception of bottled natural mineral, spring and table waters) is carried out by the Bulgarian food safety agency (BFSA). In this regard, it exercises control over packaging, labeling, production, presentation and advertising of foods. The control of compliance with all labeling and consumer information requirements is subject to strict state control by the Commission of consumer protection. For effective government control, an active position on the part of consumers is also required. In this regard, the aim of the present study is to track the degree of compliance with all legislative requirements by farmers in the labeling of white brine cheese from cow`s milk.

MATERIALS AND METHODS

In the present study, we tracked and presented data on compliance with rules and norms in the labeling of farm brine white cheese from cow`s milk in the commercial network, referring to the national and European legislative requirements, by using both documentation and observation methods. After that, we proceeded to analyze the data we obtained.

The study was conducted in Varna city, Bulgaria during the period May-June 2024. The study aimed to collect data on the labeling of farmer products offered by producers by using some of the following commercial approaches:

- Online trade – 7 products;
- Online offer and subsequent delivery of the product to the buyer`s home – 9 products;
- Selling the products in small farmers` markets – 6 products.

We have monitored the labeling of 22 commercial brands of farmer products, all of which are white-brined cow`s milk cheeses. All dairy products were packed with a net weight between 400g. and 1000g. From the present information on the label, we selected the categories of interest to consumers.

We tracked the availability of data in three categories, regarding product labeling, based on the Ordinance on the specific requirements for dairy products (from 04.11.2021), the Ordinance on the requirements for the labeling and food representation (from 13.12.2014), the Ordinance on the provision of information to consumers about food (from 10.04.2021) and Regulation (EU) No. 1169/2011 of the European Parliament and the Council from 25 October 2011 on the provision of food information to consumers. The categories include the following:

- Mandatory labeling information including the name of the food, list of ingredients; ingredients causing allergies or intolerance; quantity of certain ingredients; net weight; expiry date; special storage conditions and/or conditions of use; the name or trade name and address of the business entity in the food industry; country or place of origin; terms of use; when in the absence of such terms, it would be difficult to use the food appropriately; nutrition contents; a batch number. The information provided has to be written in Bulgarian.
- Additional mandatory data for specific types of food: if labeling white brine cheese, it is also required to include in the label information the percentage content of dry matter and fat content in dry matter.
- Optional (voluntary) food labeling information. We analyzed the food labels and scored the data according to the selected categories of information. We have checked the presence or absence of information from different categories and the extent to which the specific requirements of national and European regulatory documents have been met.

RESULTS AND DISCUSSION

Food labeling is the first information tool that customers discover when shopping and it is informative regarding the ingredients, nutrition content, and presence of allergens in the selected product. However, food labeling is also a marketing tool and can influence the perception of food quality, and consumers` dietary choices. For this reason, there is increasing food labeling research, regulatory labeling control, and evaluation of the effects on consumers, food product operators, and the whole market [14]. This is supported by a wide range of manuscripts published in recent years whose results reveal that mandatory and voluntary information on food packaging influence food purchase and food consumption and in general, consumer`s behavior towards dietary nutrition [4, 8, 11].

Upon the review of the available scientific publications, we found a study [6] whose aim corresponded to that of the present report. The majority of the published studies investigate the influence of information included in product labels on the purchase [3, 9], food consumption [10], the consumer`s dietary nutrition [12]. According to Georgescu C et al., 2014, the data on milk labeling and milk product labeling need improvements in the direction of clearer and more complete information, as well as mentioning all the mandatory information on the product label.

For obtaining the study purposes, we purchased 22 commercial brands of farm white brine cheese for a one-month period. All products were purchased through online trading (16-products): through a courier delivery company (7 products) or direct home delivery (9 products) and from small shops and markets (6 products). The results of tracking the presence of mandatory and voluntary data on the label of white brine cheeses are represented in Table 1.

According to national and European regulatory documents, categories are defined as:

*-mandatory for the labeling of packaged food product;

**-mandatory for the labeling of white brine cheese;

***-optional (voluntary) when labeling food products.

The results of our research show that Bulgarian producers of farm cheeses have strictly complied with all state and European requirements for labeling their products. The labels included in the study contain the full set of mandatory data presented in an appropriate way to inform consumers.

Table 1. Tracking the presence of mandatory and voluntary data in the labeling of packaged dairy products for 22 analyzed samples of farm white brine cheese.

	*													**		***
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	∨	∨	∨	∨	∨	∨	∨	∨	–	–	∨	∨	∨	∨	∨	∨
2	∨	∨	∨	∨	∨	∨	∨	∨	∨	–	∨	∨	∨	∨	∨	∨
3	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
4	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	–
5	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	∨
6	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	–
7	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
8	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	∨
9	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	∨
10	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
11	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	–
12	∨	∨	∨	∨	∨	∨	∨	∨	–	–	∨	∨	∨	∨	∨	–
13	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
14	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	∨
15	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	–
16	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
17	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
18	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	–
19	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	∨
20	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	∨
21	∨	∨	∨	∨	∨	∨	–	∨	–	–	∨	∨	∨	∨	∨	∨
22	∨	∨	∨	∨	∨	∨	–	∨	∨	–	∨	∨	∨	∨	∨	∨

Legend: ∨ - the product contains the indicated information; – - the product does not contain the indicated information (vertical columns: 1=food name, 2=ingredients content, 3=ingredients causing allergies or intolerance, 4=amount of certain ingredients, 5=net weight, 6=expiry date, 7=special storage conditions and/or conditions of use, 8= name or trade name and address of the business entity in the food industry, 9=country of origin or place of origin, 10=rules for use, if in the absence of such rules, it would be difficult to use the food appropriately, 11=nutrition content, 12=Bulgarian language, 13= a batch number, 14=percentage of dry matter, 15=oiliness in dry matter, 16= voluntary additional information).

The majority of the products examined by us (Table 1) lack two of the categories of mandatory data - special storage conditions and/or conditions of use and instructions for use. However, the European Regulation mentions that these categories are included in the label by the manufacturer, when in the absence of such instructions it would be difficult to use the food in an appropriate way.

We should to pay attention to the fact that a large part of the products lack another mandatory category of data – country of origin or place of origin. The National Regulation and the European Regulation No. 1169/2011 stipulates that indicating the country of origin or place of origin is mandatory. The lack of indication may mislead the consumer according to the real country or place of food origin and when the country or place of origin is indicated but it is not the same as that of the main ingredient.

The European Regulation stipulates that all ingredients or additional substances listed in an appendix of this document, causing allergies or intolerance must be present on the product label. In the case of white brine cheeses, the main ingredient belongs to the allergen category "milk and milk products (including lactose)" and in all analyzed labels the ingredient "milk" or "cow`s milk" is in bold or enlarged font size text and most of the labels contain underlined text.

Regarding the mandatory information when labeling white brine cheese - percentage of dry matter and oiliness in dry matter, all farm cheeses contained these data.

Nearly 50% of farmer white brine cheese labels observed contained additional information. We expected such results because farm products are usually of higher price that guarantees the consumer an organic composition, quality raw materials, and following good production practices during the production process. All this is information that the consumers seek and ask for confirmation when purchasing products that they believe are better quality than others on the market. The main additional information that farmer white brine cheeses contained described the duration of ripening of the diary product; the BNS standard according to which the characteristics of the product correspond; size for products with a larger net weight than usually offered by the manufacturer; precise location of the farmer production with a geographical map, as well as the calculated price per kilogram of the product.

CONCLUSION

The analysis of the data presented for labeling 22 farmer white brine cheeses gives us a reason to conclude that all the examined products contain the mandatory information required in the regulatory documents. A large part of them also include additional (voluntary) information, which primarily aims to give greater clarity to the consumer about the organic origin of the product, further details about the farmer`s production and compliance with the national standards for the production of white brine cheese.

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REFERENCES

1. P. Branzova, Development of the farmers' markets in Bulgaria. Proceedings of the International scientific and practical conference "Bulgaria of regions'2018". University of Agribusiness and Rural Development, 223-**30** (2018).
2. Commission Implementing Regulation (EU) 2023/1571 of 24 July 2023 entering a name in the register of protected designations of origin and protected geographical indications ('Българско бяло саламурено сирене/Bulgarsko byalo salamureno sirene' (PDO)). C/2023/5115Document 32023R1571. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32023R1571
3. P. Czine, Á. Török, K. Pető, P. Horváth, P. Balogh, *Nutrients*, **12**:1768 (2020). doi: 10.3390/nu12061768.
4. S.P. de Morais, L.A. Mais, N. Khandpur, M.D. Ulian, A.P. Bortoletto Martins, M.T. Garcia et al. *PLoS ONE.*; **14** e0218813 (2019). doi: 10.1371/journal.pone.0218813.
5. C. Ene Securitatea alimentara – coordonate si implicatii, Ploiesti, Romania: Editura Universitatii Petrol-Gaze, 2009.
6. C. Georgescu, C.F. Ognean, O. Tita. *Acta Universitatis Cibiniensis Series E: Food Technology*. **18** (2), 85 (2014).
7. C. Heggum. Labeling of dairy products, John W. Fuquay (ed.), Encyclopedia of Dairy Sciences (Second edn.), Academic Press, p. 1 (2011), ISBN 9780123744074, https://doi.org/10.1016/B978-0-12-374407-4.00248-X.
8. D. Martini, D. Menozz, *Nutrients,* **13**(1), 268 (2021). doi: 10.3390/nu13010268.
9. D. Menozzi, T.T. Nguyen, G. Sogari, D. Taskov, S. Lucas, J.L.S. Castro-Rial, C. Mora, *Nutrients*. **12**, 2650 (2020). doi: 10.3390/nu12092650.
10. B.D. Merillat, C. González-Vallejo. *Nutrients*. **12**, 394 (2020). doi: 10.3390/nu12020394.
11. C. Mora-García, L. Tobar, J. Young. *Nutrients*. **11**, 49 1(2019). doi: 10.3390/nu11030491.
12. J. Potthoff, A. La Face, A. Schienle. *Nutrients*. **12**, 312 (2020). doi: 10.3390/nu12020312.

13. Y. Qiao, N. Halberg, S. Vaheesan, S. Scott. *Renewable Agriculture and Food Systems*. **31**(3), 246 (2016). doi:10.1017/S1742170515000162
14. S. Shangguan, A. Afshin, M. Shulkin, W. Ma, D. Marsden, J. Smith, M. Saheb-Kashaf, P. Shi, R. Micha, Imamura F et al. *Am, J Prev, Med*., **56**, 300 (2019). doi: 10.1016/j.amepre.2018.09.024.
15. B.B. Soares, E.C. Alves, J.A. de Almeida Neto, L.B. Rodrigues. Chapter 8 - Environmental impact of cheese production. Charis M. Galanakis (ed.), Environmental Impact of Agro-Food Industry and Food Consumption, Academic Press, 2021, p. 169. ISBN 9780128213636, https://doi.org/10.1016/B978-0-12-821363-6.00009-6.
16. Ordinance on the specific requirements for dairy products in force from 04.11.2021 https://www.mzh.government.bg/odz-haskovo/Libraries/%D0%9D%D0%B0%D1%80%D0 %B5 %D0%B4%D0%B1%D0%B8/naredba_za_spetsifichnite_iziskvaniia_km_mlechnite_produkti.sflb.ashx

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Green synthesis and photothermal application of superparamagnetic iron oxide nanoparticles for cancer hyperthermia

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Cancer remains a major global health challenge, with traditional treatments like chemotherapy and radiation often yielding limited long-term success. Hyperthermia, one of the oldest cancer therapies, is promising due to the disorganized blood supply in tumors, making them more sensitive to heat compared to healthy tissues. Nanomedicine, particularly nanoparticle-mediated hyperthermia, is emerging as an innovative treatment strategy. Iron oxide nanoparticles (IONPs) are of interest due to their ability to convert near-infrared (NIR) laser radiation into localized heat while functioning as contrast agents for imaging. In this study, we investigated the synthesis of IONPs using *Ganoderma lingzhi* (Reishi) extract (IONP@GL) by green synthesis method. The synthesized IONPs were characterized by their size, morphology, magnetic properties, and photothermal efficiency. IONP@GL exhibited good heat generation under 808 nm laser irradiation. These findings highlight the potential of green-synthesized IONPs for application in photothermal cancer therapy.

Keywords: green synthesis, photothermal therapy, hyperthermia, iron oxide nanoparticles, nanomedicine

INTRODUCTION

Cancer continues to be a significant global health challenge, with traditional therapies such as chemotherapy and radiation often resulting in limited long-term success [1]. As the incidence of cancer continues to rise, there is an increasing demand for more effective and innovative treatment strategies. Hyperthermia is one of the oldest cancer therapies. Due to their disorganized blood supply, tumors have a reduced ability to dissipate heat, making them more vulnerable to thermal stress compared to well-vascularized healthy tissue [2]. Additionally, hyperthermia has been shown to improve the efficacy of other therapies, including radiation and chemotherapy [3], which has renewed interest in its role in modern cancer treatment.

Nanotechnology presents a promising approach to achieving localized hyperthermia. Appropriately designed nanoparticles (NPs) can overcome biological barriers [4] and selectively accumulate in pathological sites. Accumulated in tumor tissue, the NPs could convert various external physical stimuli, such as magnetic fields or laser radiation, into localized heat [5]. For example, *Nanotherm*® iron oxide NPs have been approved for magnetic hyperthermia in the treatment of glioblastoma and prostate cancer [6], while *AuroShell* are experimental NPs used for laser-mediated (photothermal) ablation of solid tumors [7]. Additionally, *Thermodox*®, a thermally-activated drug carrier in human clinical trials, releases its payload at hyperthermic sites, allowing localized chemotherapy [8].

Among the various methods of inducing hyperthermia, nanoparticle-mediated laser hyperthermia has gained popularity due to its minimal invasiveness, spatiotemporal selectivity, and the availability of cost-effective laser equipment. Laser radiation can penetrate to depths of up to 1 cm under the skin, making photothermal therapy particularly suitable for treating superficial tumors or those located in body cavities. Near-infrared (NIR) lasers, which offer maximum tissue penetration, are typically used in these treatments. At wavelengths in the NIR range, the safety limit (maximum permissible exposure) is 0.33 W/cm^2 for 808 nm lasers and 1.0 W/cm^2 for 1064 nm lasers [9]. Iron oxide NPs (IONPs) are particularly attractive for laser hyperthermia because in addition to being good at converting NIR radiation into heat, they also function as contrast agents and can mediate multiple therapies [10]. Superparamagnetic IONPs which exhibit magnetic properties only in the presence of an external magnetic field, find applications as magnetic targeting agents in drug delivery [11] and as contrast agents in magnetic resonance imaging

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(MRI) [12].

Among the various methods for synthesis of IONPs, green synthesis offers an environmentally friendly and non-toxic approach [13]. This method uses plant extracts as reducing agents, making it a cost-effective alternative of other methods. An additional advantage of green synthesis is that biologically active molecules from the plant extracts can be adsorbed onto the surface of the nanoparticles, potentially enhancing their therapeutic properties.

The aim of the present study was to synthesize coated IONPs using *Ganoderma lingzhi* (Reishi) extract (IONP@GL), and demonstrate their potential in photothermal therapy. The green-synthesized NPs were compared with uncoated IONPs and characterized in terms of size, shape, magnetic properties, zeta potential, and photothermal efficiency. The results show that green synthesis can successfully produce superparamagnetic IONPs with efficient infrared-to-heat conversion properties, highlighting their potential for use in photothermal cancer therapy.

MATERIALS AND METHODS

Materials

Iron(III) chloride hexahydrate, iron(II) chloride tetrahydrate and NaOH were purchased from Merck KgaA (Darmstadt, Germany). *Ganoderma lingzhi* mushroom was purchased from a local pharmacy.

Preparation of Ganoderma lingzhi aqueous extract

For preparation of the aqueous extract of *Ganoderma lingzhi*, 1 g of mushroom powder was added to 100 mL of distilled water. The mixture was heated at 80°C for 60 min under continuous magnetic stirring. After cooling to room temperature, the extract was filtered using a vacuum filtration system (0.45 µm cellulose acetate filter) to remove any particulate matter. The final extract was used immediately for nanoparticles synthesis.

Synthesis of IONP@GL using Ganoderma lingzhi extract

IONPs were synthesized using an aqueous solution of $FeCl_3$ and $FeCl_2$ in a molar ratio of 2:1. The iron salts were dissolved in 200 mL of distilled water under constant stirring at 80°C for 30 min. Then 50 mL of the *Ganoderma lingzhi* extract was added to the solution. The pH of the reaction was raised to above 6 by dropwise addition of 0.1 M NaOH, and the mixture was stirred for another 30 min at 80°C. The resulting nanoparticles were separated using a magnet and washed three times with distilled water. The synthesis of the NPs was carried out under nitrogen atmosphere to prevent oxidation, and ultrasound was applied to reduce the formation of NP clusters.

Synthesis of uncoated IONPs

$FeCl_3$ and $FeCl_2$ in a molar ratio of 2:1 were dissolved in 200 mL of distilled water and stirred at 80°C for 30 min. After that, 0.1 M NaOH (25 mL) was added, and the mixture was stirred again for 30 min at 80°C under nitrogen atmosphere, and application of ultrasound. The resulting nanoparticles were washed three times with distilled water.

Characterization of IONPs

Transmission electron microscopy, TEM, (Talos F200X, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the morphology and size of the NPs. Elemental composition was determined using energy-dispersive X-ray spectroscopy, EDX, (Prisma E SEM, Thermo Scientific, Waltham, MA, USA). Magnetic characteristics and phase composition of the IONPs were determined by Mössbauer spectroscopy (WissEl-Wissenschaftliche Elektronik GmbH, Germany). Dynamic light scattering, DLS, (Microtrac, York, PA, USA) was used for particle size, size distribution and zeta potential measurements.

Photothermal performance evaluation

The photothermal performance of the synthesized IONPs was demonstrated by irradiating the samples with an 808 nm laser at a power density of 0.330 W/cm² for 15 min. NPs suspensions were prepared at concentrations of 0.025 mg/mL, and 0.05 mg/mL in distilled water. The temperature of the suspensions was monitored by infrared camera FLIR (FLIR Systems Inc., Boston, MA, USA).

RESULTS AND DISCUSSION

TEM showed that the synthesized IONP@GL had a predominantly spherical morphology with an average particle size of approximately 20 nm (Fig. 1 C). It is well known that IONPs with diameters of 20 nm or less are superparamagnetic [14]. The magnetic properties of the produced IONPs were evident when a static magnetic field was applied, allowing them to overcome gravitational forces, thus demonstrating their superparamagnetic behavior (Fig. 1 A and B).

EDX analysis showed that the uncoated IONPs exhibited peaks only for Fe and O (Fig. 1D), indicating their pure inorganic composition. In contrast, IONP@GL displayed an additional peak for C (Fig. 1E), confirming the adsorption of organic molecules from the plant extract onto the surface of the NPs.

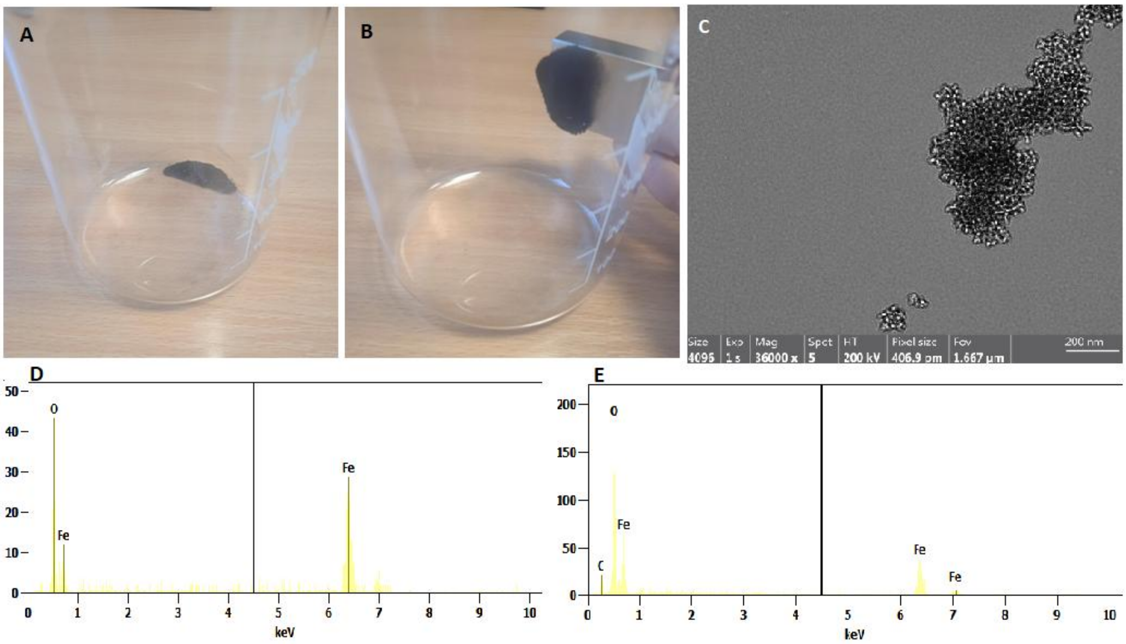

Fig. 1. Demonstration of superparamagnetic properties of IONPs (A and B); TEM micrographs of IONP@GL (C); EDX of uncoated IONPs (D) and IONP@GL (E).

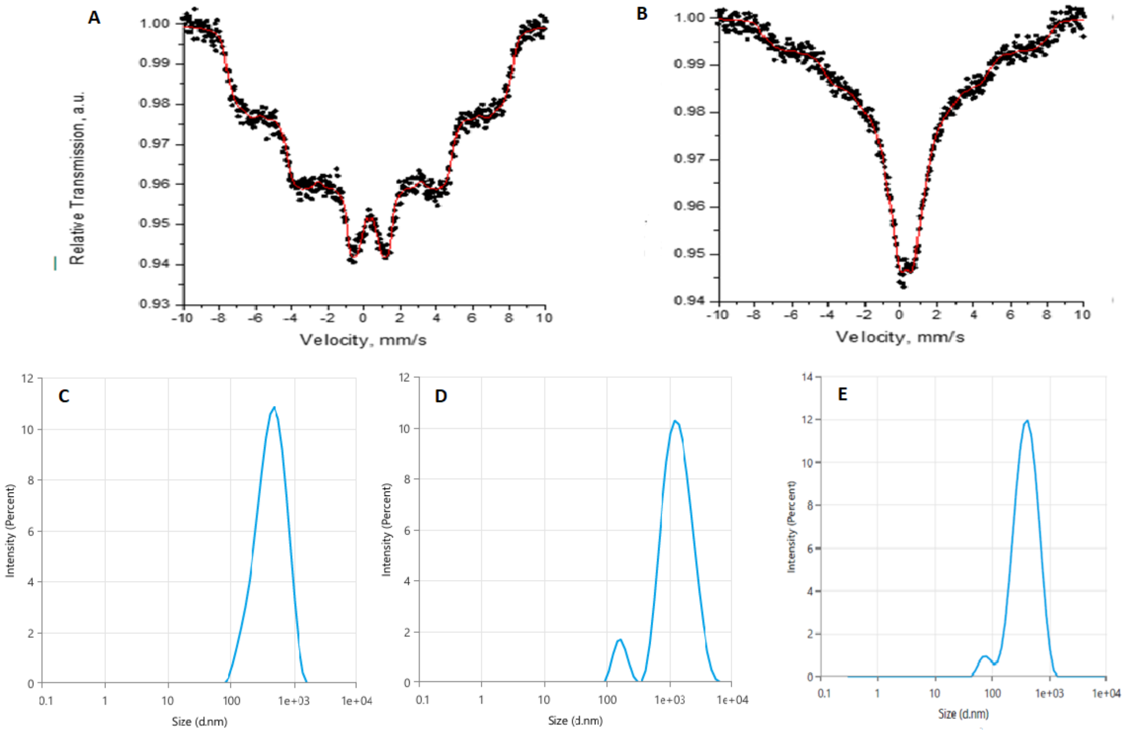

Fig. 2. Mössbauer spectra of uncoated IONPs (A) and IONP@GL (B); hydrodynamic size of uncoated IONPs (C); hydrodynamic size of IONP@GL before (D) and after additional ultrasound application (E).

The Mössbauer spectra of the samples (Fig. 2 A and B) resemble those of nanosized maghemite (γ-Fe_2O_3), with an average diameter larger than 10 nm [15]. The average magnetic field was determined to be 26.8 T for uncoated IONPs, and 19.4 T for IONP@GL.

DLS measurements show a significantly larger hydrodynamic size of NPs, around 160 nm for IONP@GL, suggesting the formation of clusters of NPs in suspension. To reduce aggregation, ultrasound was applied immediately after the synthesis for 3 h. This resulted in a reduction of the hydrodynamic size below 90 nm, and a noticeable decrease in the extent of cluster formation. The estimated polydispersity index was 0.216 for uncoated IONPs and 0.245 for IONP@GL. Zeta potential analysis showed a change from 41.3 mV for uncovered IONPs to -27.24 mV for IONP@GL. The change in polarity suggests adsorption of biomolecules from the plant extract onto the NPs surface.

During the irradiation of the samples, an initial temperature rise was observed at the point of penetration of the infrared laser light. Over time, this temperature rise deepened in the sample, indicating heat diffusion under prolonged laser exposure (Fig. 3A). Interestingly, IONP@GL exhibited a better photothermal effect at both concentrations. The IONP@GL samples demonstrated a 2°C increase in temperature compared to the uncoated IONPs under the same conditions (Fig. 3 B, C). A plausible explanation for the enhanced photothermal effect could be the presence of biomolecules from *Ganoderma lingzhi* on NPs, which reduce their agglomeration, in addition to application of ultrasound after NPs synthesis. This stabilization allows a larger number of NPs to remain dispersed, increasing the surface area available for interaction with infrared photons and for heat transfer to the surrounding medium [16]. The temperature increase in all samples was sufficient for mild hyperthermia.

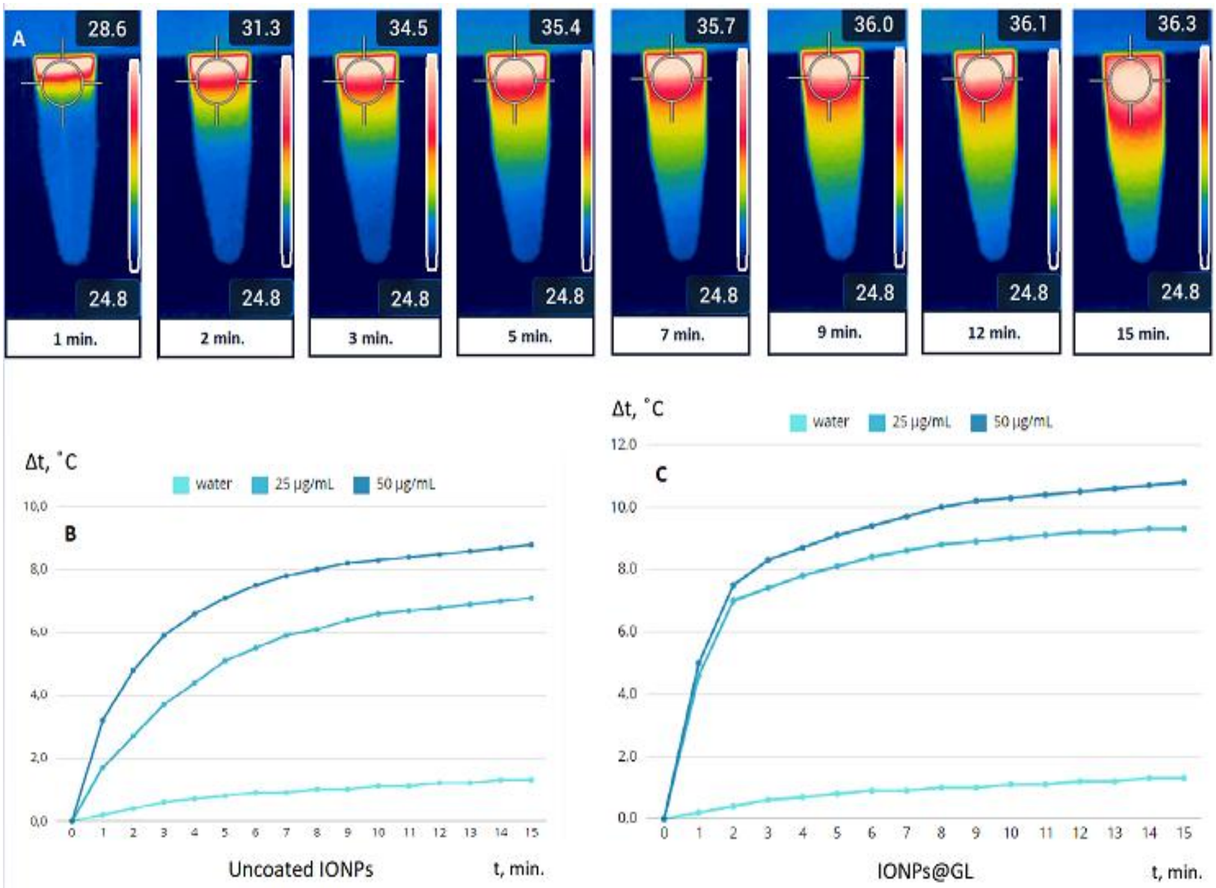

Fig. 3. Temperature changes with depth in a 50 µg/ml IONP@GL sample during 808 nm-laser irradiation (A); Temperature changes in uncoated IONPs at 25 µg/mL and 50 µg/mL concentrations during 808 nm-laser irradiation (B); Temperature changes in IONP@GL samples at 25 µg/mL and 50 µg/mL concentrations during 808 nm-laser irradiation (C).

CONCLUSION

This study successfully demonstrated the green synthesis of superparamagnetic iron oxide nanoparticles (IONP@GL) using *Ganoderma lingzhi* extract, and their promising photothermal performance. The IONP@GL samples exhibited a temperature increase that was by 2°C higher than that of uncoated IONPs under identical conditions. Both IONP@GL and uncoated IONPs at concentrations of 25 µg/mL and 50 µg/mL achieved sufficient temperature increase to induce mild hyperthermia, highlighting their potential for photothermal cancer therapy.

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REFERENCES

1. R. Kaur, A. Bhardwaj, S. Gupta, *Molecular Biology Reports*, **50**(11), 9663 (2023).
2. P. K. Deb, B. Al-Jaidi, R. R. Akkinepalli, A. Al-Aboudi, R. K. Tekade, in: Biomaterials and Bionanotechnology, Academic Press, 2019, p. 375.
3. M. Dunne, M. Regenold, C. Allen, *Advanced Drug Delivery Reviews*, **163**, 98 (2020).
4. E. Blanco, H. Shen, M. Ferrari, *Nature Biotechnology*, **33**(9), 941 (2015).
5. S. H. Pham, Y. Choi, J. Choi, *Pharmaceutics*, **12**(7), 630 (2020).
6. F. Rodríguez, P. Caruana, N. De la Fuente, P. Español, M. Gámez, J. Balart, M. V. Céspedes, *Biomolecules*, **12**(6), 784 (2022).
7. R. Zhang, F. Kiessling, T. Lammers, R. M. Pallares, *Drug Delivery and Translational Research*, **13**(2), 378 (2023).
8. N. Borys, M. W. Dewhirst, *Advanced Drug Delivery Reviews*, **178**, 113985 (2021).
9. C. Bastiancich, A. Da Silva, M. A. Estève, *Frontiers in Oncology*, **10**, 610356 (2021).
10. E. Alphandéry, *Drug Discovery Today*, **25**(1), 141 (2020).
11. Z. Zhuo, J. Wang, Y. Luo, R. Zeng, C. Zhang, W. Zhou, H. Chen, *Acta Biomaterialia*, **134**, 13 (2021).
12. S. M. Dadfar, D. Camozzi, M. Darguzyte, K. Roemhild, P. Varvarà, J. Metselaar, T. Lammers, *Journal of Nanobiotechnology*, **18**, 1 (2020).
13. J. A. Kumar, T. Krithiga, S. Manigandan, S. Sathish, A. A. Renita, P. Prakash, S. Crispin, *Journal of Cleaner Production*, **324**, 129198 (2021).
14. J. Dulińska-Litewka, A. Łazarczyk, P. Hałubiec, O. Szafrański, K. Karnas, A. Karewicz, *Materials*, **12**(4), 617 (2019).
15. L. Rebbouh, R. Hermann, F. Grandjean, T. Hyeon, K. An, A. Amato, G. Long, *Phys. Rev.* B, **76**, 174422 (2007).
16. X. Cui, Q. Ruan, X. Zhuo, X. Xia, J. Hu, R. Fu, ... H. Xu, *Chemical Reviews*, **123**(11), 6891 (2023).

Wild umbellifers traditionally used for food in Sicily and Bulgaria and their health benefits

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Numerous studies demonstrate that plant-based foods provide a large spectrum of bioactive compounds with lots of health benefits. The wide spectrum of consumed plants improves the nutritional adequacy of the diet. Ethnobotanical studies provide valuable data about plants used for food for centuries. The aim of this study is 1) to identify wild plants from the family *Apiaceae* traditionally used for food in Bulgaria and in Sicily and 2) to reveal their bioactive compounds and health benefits. As a result of the ethnobotanical review the folowing taxa are listed: *Aegopodium podagraria* L., *Anthriscus cerefolium* (L.) Hoffm., *Anthriscus sylvestris* (L.) Hoffm., *Helosciadium nodiflorum* (L.) W. D. J. Koch, *Carum carvi* L., *Chaerophyllum bulbosum* L., *Daucus carota L.* subsp. *carota, Eryngium campestre* L., *Foeniculum vulgare* Mill. subsp. *vulgare*, *Kundmannia sicula* (L.) DC., *Smyrnium perfoliatum* L., and *S perfoliatum* subsp. *rotundifolium* (Mill.) Bonnier & Layens (synonym *S. rotundifolium* Mill.). As members of the family *Apiaceae* some of them are popular spices due to the essential oils characterized by some interesting components such as germacrene D, D-limonene, limonene, eucalyptol, 1-nonene, pentadecane, estragole, β-(*E*)-, β-(*Z*)-ocimene, terpinolene, sapthulenol, pcymene-8-ol, p-cymene, myrtenol, (E)-anethole, α-pinene, estragole, p-cymene, etc. Additionally, they contain flavonoids (rutin, cynaroside, apigetrin, luteolin, vanillin, myricetin, quercetin, luteolin, kaempferol and chrysin apigenin and its glycosides); phenolic acids (caffeic, p-coumaric, ferulic, hydroxybenzoic, 3.5-*O*-dicaffeoylquinic acid, protocatehuic, phydroxybenzoic, chlorogenic, p-coumaric, ferulic, rosmarinic acids), carotenoids (β-carotene, α-carotene, lutein, lycopene), polyacetylenes (falcarinol, falcarindiol, falcarindiol-3-acetate) and others. The pharmacological activities and the health benefits are discussed. The sustainable use will include protection of their native habitats and cultivation of some of them.

Keywords: wild umbellifers, bioactive compounds

INTRODUCTION

Numerous studies demonstrate that plant-based foods provide a large spectrum of bioactive compounds with lots of health benefits [1, 2]. The wide spectrum of consumed plants improves the nutritional adequacy of the diet. The traditional Mediterranean diet is known for its health benefits [3, 4], Additionally it is sustainable with interrelated components such as biodiversity, traditions and nutrition [5]. Many umbellifers take important place in the Mediterranean diet [6]. They are domesticated long ago and cultivated for food due to their valuable secondary metabolites [7, 8]. Ethnobotanical studies provide valuable data about plants traditionally used for food for centuries [9]. They can help identifying new candidates for cultivation and domestication that possess powerful health benefits. Some of the umbellifers, which are interesting form this point of view, have restricted distribution either to the Mediterranean or to Sicily [10].

The aim of this study is 1) to identify wild plants from family *Apiaceae* traditionally used for food in Bulgaria and in Sicily and 2) to reveal their bioactive compounds and health benefits.

MATERIAL AND METHODS

We accessed Google Scholar, Web of Science, and PubMed to identify publications using the search string: “Sicily”, “Bulgaria”, “traditionally” “wild”, “food”, “plants”, “ethnobotany”, “compounds”, “metabolites”, “pharmacological”, “health”, etc.

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Table 1. Wild umbellifers traditionally used for food in Bulgaria and Sicily.

Plant	Used part	Country
Aegopodium podagraria L.	Sprouts, salad raw, soup,	Bulgaria
Anthriscus cerefolium (L.) Hoffm.	Leaves, shoots, salad raw, soup, spice	Bulgaria
Anthriscus sylvestris (L.) Hoffm.	Roots, leaves, shoots, salad raw, soup, pastry	Bulgaria
Helosciadium nodiflorum (L.) W. D. J. Koch (synonym *Apium nodiflorum* (L.) Lag. subsp. *Nodiflorum)*.	Tender leaves and stems	Sicily
Carum carvi L.	Fruits, spice	Bulgaria
Chaerophyllum bulbosum L.	Young shoots, corms, salad raw and stew	Bulgaria
Daucus carota L. subsp. *carota*	Tender basal leaves and roots	Sicily
Eryngium campestre L.	Sprouts	Sicily
Foeniculum vulgare Mill. subsp. *vulgare*	Leaves, mericarps, spice	Bulgaria and Sicily
Kundmannia sicula (L.) DC.	Basal leaves	Sicily
Smyrnium perfoliatum L.	Tender leaves and stems	Bulgaria and Sicily
Smyrnium perfoliatum subsp. *rotundifolium* (Mill.) Bonnier & Layens (synonym *S. rotundifolium* Mill.)	Tender leaves and stems	Sicily

RESULTS AND DISCUSSION

Ethnobotanical data

As a result of the ethnobotanical review 12 taxa are listed of which 5 taxa are used for food only in Bulgaria [9, 11-15], 5 taxa only in Sicily [16-19] and 2 taxa in both regions (Table 1).

Bioactive compounds and pharmacological effects

The wild umbellifers consumed for food are source of various bioactive compounds. The essential oils are characterized by some interesting components such as germacrene D, D-limonene, limonene, eucalyptol, 1-nonene, pentadecane, estragole, β-(*E*)-, β-(*Z*)-ocimene, terpinolene, sapthulenol, p-cymene-8-ol, p-cymene, myrtenol, (E)-anethole, α-pinene, estragole, p-cymene, etc. The contents of unique or highly abundant components are pointed as markers to distinguish certain species, e.g., selinene is typical of *Apium graveolens* while *Helosciadium nodiflorum* (*A. nodiflorum*) is rich of limonene [7]. Coumarins and furanocoumarins are found in many of the species listed above. Moreover, the wild members of *Apiaceae* consumed for food contain flavonoids such as rutin, cynaroside, apigetrin, luteolin, vanillin, myricetin, quercetin, luteolin, kaempferol, chrysin apigenin and their derivates. The phenolic acids reported in the listed umbellifers are caffeic, p-coumaric, ferulic, hydroxybenzoic, 3.5-*O*-dicaffeoylquinic acid, protocatehuic, p-hydroxybenzoic, chlorogenic, p-coumaric, ferulic, rosmarinic acids. The terpenoids obtained from these plants are carotenoids (β-carotene, α-carotene, lutein, lycopene), polyacetylenes (falcarinol, falcarindiol, falcarindiol-3-acetate) and others.

Aegopodium podagraria L. is a perennial plant (Fig. 1) widespread in shady habitats of the Temperate Eurasia [20]. The composition of the essential oil varies depending on the geographical and ecological factors with main components α-pinene, β-pinene, myrcene, α-thujone, dehydro-p-cymene, and β-phellandrene, limonene, germacrene D, spatulenol, (E)-β-caryophyllene, γ-terpinene. The plant also contains phenolic acids such as hydroxycinnamic acid, and flavonoids such as quercetin, kaempferol and derivatives of quercetin (hyperoside, isoquercetin), derivatives of kaempferol (trifoline). Coumarins (angelicin) and furanocoumarins, as well as carotenoids (β-carotene, xanthophyll neoxanthin) are identified in this plant [21]. *A. podagraria* is known for its calming, diuretic, anti-inflammatory and antimicrobial properties, cytotoxic and antioxidant activities [21, 22]. The dry extract obtained from goutweed (standardized on hydroxycinnamic acids) has a low toxicity level, normalizes metabolic processes and protects the liver and the kidneys [23].

Figure 1. First row *Aegopodium podagraria* (left), *Anthriscus cerefolium* (middle left) *A. sylvestris* (middle right) and *Carum carvi* (right); Second row *Helosciadium nodiflorum* (left), *Foeniculum vulgare* subsp. *vulgare* (middle) and *Daucus carota* (right); Third row *Chaerophyllum bulbosum* (left), *Eryngium campestre* (middle left), *Kundmannia sicula* (middle right) and *Smyrnium perfoliatum* (right)

Anthriscus cerefolium (L.) Hoffm. is an annual or biennial plant (Fig. 1) native to the temperate biome of Central and Eastern Europe to Northern Iran [20]. The plant contains essential oil rich of D-limonene, eucalyptol, 1-nonene, pentadecane, estragole, and germacrene D, flavonoids such as rutin, cynaroside, apigetrin, luteolin, apigenin and its glycosides, as well as phenolic acid (caffeic, p-coumaric, ferulic, hydroxybenzoic, 3.5-*O*-dicaffeoylquinic acid); [24, 25]. *A. cerefolium* has antimicrobial, antioxidant, and anticancer activities [24]. The extract of *A. cerefolium* aerial parts decrease proliferation rate of glioblastoma cells while being non-toxic to the control cell line [9]. Neither acute nor subacute toxicity is reported.

Anthriscus sylvestris (L.) Hoffm. is a biennial or perennial (Fig. 1) which grows in Temperate Eurasia to Tropical African Mountains [20]. It is related and alike to *A. cerefolium.* The umbels of *A. sylvestris* have mostly 6-15 primary branches, ultimate segments of leaf blades mostly 15-50 mm long, and bracteoles of umbellets narrow-ovate while *A. cerefolium* has umbels with 2-6 primary branches, ultimate segments of leaf blades mostly 5-10 mm long, and bracteoles of umbellets linear) (Fig. 1). *A. sylvestris* is the most common species of the genus *Anthriscus* [26]. One of the main compounds of *A. sylvestris is* deoxypodophyllotoxin. It has wide-ranging effects, including antitumor, antiproliferative, antiplatelet aggregation, antiviral, anti-inflammatory, and insecticidal properties. Additionally, it serves as a pivotal precursor to epipodophyllotoxin, crucial in the semisynthesis of cytostatic agents like etoposide and teniposide. The cytotoxic effect of *Anthriscus* extracts is due to podophyllotoxin-related lignans, which are currently of interest due to the high availability of these ruderal species [26].

Helosciadium nodiflorum (L.) W. D. J. Koch (synonim *Apium nodiflorum* (L.) Lag.) is a perennial plant (Fig. 1) which grows in the Mediterranean [20]. The typical subspecies is consumed in Sicily [16-19]. The essential oil of *H. nodiflorum* is rich of germacrene D, limonene, β-(Z)-ocimene, terpinolene and phenylpropanoids (dillapiol, myristicin) identified in this plant [27, 28]. The plant contains phenolic acids such as 3,4-diccaffeoylquinic and chlorogenic acids as well as flavonoids such as quercetin-3-O-glucoside which are also reported [28]. The plant is not reported toxic, on the contrary, it has antitoxic effect and reduces aflatoxins production by *Aspergillus parasiticus* [29].

Carum carvi L. is a perennial plant (Fig. 1) which grows in meadows. It is native to Temperate Eurasia [20]. The main components of the "seed" essential oil (yields 0.6% - 5.4%) are carvone limonene (with negative correlation between these two), β-myrcene, *E*-dihydrocarvone, and *E*-carveole, α-pinene, sabinene, n-octanal, *E*-β-ocimene, γ-terpinene, linalool, *Z*- and *E*-limonene oxide, *Z*-dihydrocarvone, *Z*-carveol, perillaldehyde, E-anethole, and *E*-β-caryophyllene [30, 31]. The plant substance (mericarps or fruits, called often seeds) is known for its carminative effect and is cultivated as a spice [32] but the essential oil has also antibacterial, antifungal and anticancer activity [31] and lower level of blood glucose [33]. The plant substance contains flavonoids [31, 33]. It is not reported toxic.

Chaerophyllum bulbosum L. is a biennial plant (Fig. 1) native to Europe, Northern Türkiye and Central Asia [20]. Phenolic acids (protocatehuic, p-hydroxybenzoic, chlorogenic, p-coumaric, ferulic, rosmarinic acids); flavonoids (vanillin, myricetin, quercetin, luteolin, kaempferol and chrysin); essential oils (with main components β-(*E*)-, β-(*Z*)-ocimene, terpinolene, sapthulenol, p-cymene-8-ol, p-cymene, myrtenol) and sugars are reported for this species, as well as antioxidant, antidiabetic anticholinesterase, anti-urease, anti-tyrosinase, activities [34]. Toxicity is not reported but possibly not studied enough.

Daucus carota L. subsp. carota is a biennial plant (Fig. 1). The wild carrot is a polymorphic taxon widespread in the temperate zone but native to Macaronesia, Europe and Western Asia. *D. carota* has 13 subspecies, with one being cultivated (*D. carota* L. ssp. subsp. *sativus* (Hoffm.) Arcang.) [35]. Even though the cultivated carrot is famous for its high content of carotenoids (β-carotene, α-carotene, lutein, lycopene), the wild one is also a source of such compounds, but in much less quantity [35-37], polyacetylenes (falcarinol, falcarindiol, falcarindiol-3-acetate), phenolic acids (chlorogenic acid, p-hydroxybenzoic acid, caffeic acid) [38]. The essential oil is rich of β-caryophyllene, geranyl acetate, bicyclogermacrene, β-bisabolene, E-α-bisabolene, δ-cadinene, isoelemicin, carotol, daucol, α-asarone [39]. The wild carrot subspecies possess antioxidant, anticancer, antipyretic, analgesic, antibacterial, antifungal, hypolipidemic, and hepato- and gastroprotective properties [35]. The plant is not reported toxic in the published research papers.

Eryngium campestre L. is a wide spread perennial plant (Fig. 1) native to Europe to Caucasus and Northwestern Africa [20]. It is a perennial plant and grows primarily in the temperate biome. The plant is rich of flavonoids (quercetin-3-O-rutinoside (rutin), kaempferol 3,7-di-O-α-L-rhamnopyranoside, kaempferol 7-O-α-L-rhamnopyranoside, isoquercitrin, luteolin 7-O-β-D-glucopyranoside, quercitrin, astragalin, naringenine 7-O-α-L-rhamnopyranosyl-(1→2)-O-β-D-glucopyranoside, tiliroside, 3'-O-methyl quercetin 3-rutinoside, 4'-methoxy-3-(β-D-glucopyranosyl) kaempferol) and phenolic acids (chlorogenic acid, caffeic acid, rutoside, p-coumaric acid, ferulic acid, R-(+)-3'-O-β-D-glucopyranosyl rosmarinic acid, rosmarinic acid) [40]. It is used for various urinary tract conditions including kidney and bladder stones, kidney pain and swelling, and difficult urination. *E. campestre* has antioxidant and anti-inflammatory activity and protective effect against mycotoxins [41]. *Eryngium* spp. are considered a prospective ally for treating metabolic syndrome and diabetes [42]. Toxicity is not reported.

Foeniculum vulgare Mill. subsp. *vulgare* is a perennial plant (Fig. 1) native from Mediterranean to Ethiopia and Western Nepal [20]. It contains fatty acids, phenolic acids (rosmarinic, neochlorogenic, chlorogenic, caffeoylquinic, feruloylquinic); flavonoids (rutin, kaempferol, isorhamnetin, quercetin, apigenin); essential oil (with main components E-anethole, α-pinene, limonene, estragole, p-cymene). The fennel (in particular its essential oil) is known for its anti-inflammatory, anticancer, antioxidant, cardioprotective, antidiabetic, antithrombotic, antidiabetic activities [43-45] but most of all expectorant (due to the anethole) and carminative effects [46, 47]. It is also used as a galactagogue agent for lactating mothers [44]. *In vitro* study shows that *F. vulgare* juice is toxic to mice (hepatocytic degeneration and necrosis, congestion with perivascular mononuclear cell infiltrations and peribiliary mononuclear cell aggregations) when administered in a dose of 9.772 mg/kg/BW but harmless in doses less than 0.98

ml/kg BW. Also, estragole (methylchavicol) is associated with malignant tumors *in vitro* [45].

Kundmannia sicula (L.) DC. is a perennial plant (Fig. 1) which grows primarily in the subtropical biome. The native range of this plant is the Mediterranean coastal area where it occurs in arid places [10, 20]. It contains hexadecanoic acid and essential oil rich of isocurcumenol, spathulenol, 10-epi-γ-eudesmol, α-cubebene E-dihydro occidentalol [48]. Also, the plant is a rich source of germacrene D [49]. Germacrene D shows antibacterial and antifungal activities, the mechanism of antimicrobial action of it may be related to permeabilization of the cells and disruption of the membrane integrity [50]. *K. sicula* is reported non-toxic [51].

Smyrnium perfoliatum L. is a biennial plant (Fig. 1) which grows in shady places. It is native to Eastern and Central Europe, Mediterranean and Caucasus. The plant contains flavonoids (quercetin 3-β-D glucoside, kaempferol, kaempferol 3-β-D-glucoside, kaempferol 3,4'-β-D-diglucoside) [52]; The main components of the essential oils are arornadendrene, neryl isovalerate, γ-muurolene, α-santalene [53]. The toxicity and pharmacological activities are poorly studied and only broad spectrum of antimicrobial effects is confirmed [54]. *Smyrnium rotundifolium* Mill. is now accepted as a subspecies of *S. perfoliatum* [20, 55]. Its native distribution is narrower, namely Western Central Mediterranean to Western Türkiye and Cyprus. The bioactive compounds and the pharmacological effects of this plant are poorly studied but it is known for the content of the flavonoids kaempferol, kaempferol 3-β-D-galactoside, kaempferol 3-methyl ether 7-β-D-glucoside, kaempferol 3-diglycoside [52] and germacrane derivatives [56]. The essential oil is dominated by furanosequiterpenes, curzerene, and isofuranodiene [57].

Pharmacological effects

Wild umbellifers used for food in Bulgaria and Sicily have anti-inflammatory and antioxidant activity, neuroprotective effect and other valuable activities, as shown above. They can be attributed to particular compounds found in the listed umbellifers. For example, luteolin has significant anti-inflammatory, antioxidant, and neuroprotective activity [58] Kaempferol exhibits anti-oxidative stress, anti-inflammatory properties and alleviates neurodegenerative disorders [59, 60]. Ferulic acid is a powerful antioxidant and anti-inflammatory agent that is non-steroidal in nature [61, 62]. It also has anti-thrombosis, and anti-cancer activities and protects against coronary disease, lowers cholesterol and increases sperm viability [63]. P-coumaric acid also demonstrates anti-inflammatory activity [64-67]. Falcarinol, and falcarinol-type polyacetylenes are potent proliferation inhibitors of human glioblastoma cancer cell line DBTRG-05MG [68].

Toxicity

Family *Apiaceae* is popular with spices such as dill, celery and parsley. However, it is also known for some poisonous members such as *Aethusa cynapium* L., *Cicuta virosa* L., and *Conium maculatum* L. [69]. Therefore, caution is needed when used for food. As mentioned above, some species from the family such as *Anthriscus cerefolium, Foeniculum vulgare* subsp. *vulgare, Daucus carota, etc.,* contain furanocoumarins. These can cause a phototoxic reaction when they come in contact with skin which is exposed to UV-A light [70]. However, this is not a toxicity related to the use for food. Additionally, fennel seed oil rich of estragole (considered cancerogenic) *in vitro* is proven to not cause any primary or fixed DNA damage or any sign of toxicity in human hepatoma cells [71]. Some of these plants deserve further research regarding the subacute toxicity, for example *Chaerophyllum bulbosum, Smyrnium perfoliatum* and *Eryngium campestre.*

CONCLUSION

The listed wild umbellifers are prospective candidates for more extended consumption with health benefits. They are sources of valuable bioactive compounds. Their consumption is beneficial for the human health. The sustainable use will include protection of their native habitats and cultivation of some of them.

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REFERENCES

1. M. Samtiya, R.E. Aluko, T. Dhewa, J. Moreno-Rojas, *Foods,* **10,** 839 (2021).
2. J. Shen, J. Shan, L. Zhong, B. Liang, D. Zhang, M. Li, H. Tang, *Plant Foods Hum. Nutr.*, **77**, 12 (2022).
3. J. Salas-Salvadó, N. Becerra-Tomás, J.F. García-Gavilán, M. Bullo, L. Barrubes, *Prog. Cardiovasc. Dis.*, **61**(1), 62 (2018).
4. V. Rosato, N. J. Temple, C. La Vecchia, G. Castellan, A. Tavani, V. Guercio, *Eur. J. Nutr.*, **58**, 173 (2019).

5. B. Burlingame, S. Dernini, *Public Health Nutr.*, **14**, 2285 (2011).
6. A. Stefanaki, T. van Andel, in: Aromatic Herbs in Food, Academic Press, 2021, p. 93.
7. U. Plitmann, E. Plammer, L. Hanus, O. Cohen, *Bocconea,* **23**, 145 (2009).
8. F. Pollastro, S. Gaeta, in: Carrots and related Apiaceae crops, Wallingford, UK, Cabi. 2020, p. 35.
9. T. Ivanova, A. Marchev, M. Chervenkov, Y. Bosseva, M. Georgiev, E. Kozuharova, D. Dimitrova, *Diversity,* **15**(3), 435 (2023).
10. F.M. Raimondo, G. Domina, V. Spadaro, *Quad. Bot. amb. appl*, ***21***, 189 (2010).
11. N. Stoyanov, B. Kitanov, Wild useful plants. Bulgarian Academy of Sciences Publ. House, Sofia, 1960, p. 480.
12. I. Cheshmedzhiev, S. Genchev, D. Dimitrova, K. Varbanova, Wild plants on our table. Zemizdat, Sofia, 1999, p. 147.
13. A. Nedelcheva, *J. Biosci.* **7**, 77 (2013).
14. Y. Dogan, A. Nedelcheva, Ł. Łuczaj, C. Drăgulescu, G. Stefkov, A. Maglajlić, J. Ferrier, N. Papp, A. Hajdari, B. Mustafa, Z. Dajić-Stevanović, A. A. Pieroni, *J. Ethnobiol. Ethnomed.* **11**(1), 1 (2015).
15. T. Ivanova, M. Chervenkov, E. Kozuharova, *Diversity* **14**(6), 416 (2012).
16. F. M. Raimondo, F. Lentini, *Naturalista Siciliano*, s.**, 4**, 14 (3-4): 77 (1990).
17. G. Giardina, F. M. Raimondo, V. Spadaro, *Bocconea* **20**, 5 (2007).
18. R. Schicchi, A. Geraci, Verdure Spontanee per L'alimentazione e la Salute; Palermo University Press, Palermo, Italy, 2021.
19. P. Mazzola, Le piante selvatiche nella tradizione alimentare dei Monti Nebrodi (Sicilia). Università di Palermo e Parco dei Nebrodi, 2020, p. 174.
20. POWO (2024). Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; https://powo.science.kew.org/ Retrieved 03 September 2024.
21. K. Jakubczyk, K. Janda, D. Styburski, A. Łukomska, *Adv. Hyg. Exp. Med.*, **74**, 28 (2020).
22. S. Y. Sarıaltın, D. C. Polat, C. Ö. Yalçın, *Food Biosci.*, **52**, 102359 (2023).
23. O. V. Tovchiga, O. O. Koyro, S. I. Stepanova, S. Y. Shtrygol, V. V. Evlash, V. G. Gorban T. K. Yudkevich, Goutweed (*Aegopodium podagraria* L.) biological activity and the possibilities of its use for the correction of the lipid metabolism disorders, 2017.
24. S. Fejes, É. Lemberkovics, A. Balázs, P. Apáti, T.S. Kristó, É. Szoke, Á. Kéry, A. Blázovics,. *Acta Hortic.* **597**, 191 (2004).
25. R. Chizzola, *Nat. Prod. Commun.* **6**, 1147 (2011).
26. S. Berežni, N. Mimica-Dukić, G. Domina, F. M. Raimondo, D. Orčić, *Plants* **13**, 1087 (2024).
27. C. Guijarro-Real, A. Rodríguez-Burruezo, J. Prohens, M.D. Raigón, A. Fita, *Food Res. Int.* **121**, 765 (2019).
28. F. Maggi, C. Giuliani, G. Fico, M. Ricciutelli, M. Bramucci, L. Quassinti, ... S. Dall'Acqua, *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, **153**(2), 325 (2019).
29. F. Noorbakhsh, E. Lotfali, A. Ghajari, S. Ansari, R. Mohammadi, Z. Arab-Mazar, *Herb. Med. J.*, 60 (2017).
30. A. Raal, E. Arak, A. Orav, *J. Essent. Oil Res.*, **24**(1), 53 (2012).
31. A. Aly, R. Maraei, A. Rezk, A. Diab, *Int. J. Radiat. Biol.*, **99**(2), 318 (2023).
32. B. Galambosi, *J. Essent. Oil Res*, **8**(4), 389 (1996).
33. H. Erjaee, H. Rajaian, S. Nazifi, M. Chahardahcherik, *Comp. Clin. Pathol.*, **24**, 1197 (2015).
34. Z. Molo, G. Tel-Çayan, E. Deveci, M. Öztürk, M. E. Duru, *Food Biosci.*, **42**, 101201 (2021).
35. J. Ismail, W. N. Shebaby, J. Daher, J. C. Boulos, R. Taleb, C. F. Daher, M. Mroueh, *Plants,* **13**, 93, (2024).
36. V. H. Heywood, *Isr. J. Plant Sci.*, **32**(2), 51 (1983).
37. M. Iorizzo, D.A. Senalik, S. L. Ellison, D. Grzebelus, P. F. Cavagnaro, C. Allender, ... P. W. Simon, *Am. J. Bot.*, **100**(5), 930 (2013).
38. T. Ahmad, M. Cawood, Q. Iqbal, A. Ariño, A. Batool, R. M. S. Tariq, ... S. Akhtar, *Foods*, **8**(9), 424, (2019).
39. H. Mohammedi, S. Mecherara-Idjeri, Y. Foudil-Cherif, A. Hassani, *J. Essent. Oil Bear. Plants*, **18**(4), 873 (2015).
40. B. Soumia, *Polyphenols: Mech. Action Hum. Health Dis.*, 69 (2018).
41. N. Hymery, X. Dauvergne, H. Boussaden, S. Cérantola, D. Faugère, C. Magné, *Toxins*, **13**(5), 312 (2021).
42. E. P. Pérez-Muñoz, M. Antunes-Ricardo, M. Martínez-Ávila, & D. Guajardo-Flores, *Front. Nutr.*, **9**, 878306 (2022).
43. F. Senatore, F. Oliviero, E. Scandolera, O. Taglialatela-Scafati, G. Roscigno, M. Zaccardelli, E. De Falco, *Fitoterapia,* **90**, 214 (2013)
44. S. B. Badgujar, V. V. Patel, A. H. Bandivdekar, *BioMed. Res. Int.,* **(1)**, 842674 (2014).
45. M. A. Rather, B. A. Dar, S. N. Sofi, B. A. Bhat, & M. A. Qurishi, *Arabian J. Chem.*, **9**, S1574 (2016).
46. S. J. Virshette, M.K. Patil, J.R. Shaikh, *J. Pharmacogn. Phytochem.* **9**(3), 142 (2020).
47. S. Zafar, M.K. Khan, S. Perveen, M. Iqbal, A.A. AL-Huqail, Fennel, in: Essentials of Medicinal and Aromatic Crops, Cham: Springer International Publishing, 2023, p. 483.
48. K. Faidi, R. El Mokni, R.K. Joshi, S. Hammami, M.F. M'Henni, Z. Mighri, *Natural Product Research,* **28**(20), 1680 (2014).
49. S. Casiglia, M. Bruno, M. Bramucci, L. Quassinti, G. Lupidi, D. Fiorini, F. Maggi, *Journal of Essential Oil Research*, **29**(6), 437 (2017).
50. N. Badalamenti, M. Bruno, C. Formisano, D. Rigano, *Natural Product Communications*, **17**(4), 1934578X221096963 (2022).

51. H. J. De Boer, A. Ouarghidi, G. Martin, A. Abbad, A. Kool, *PLoS One*, **9**(1), e84291 (2014).
52. A. Ulubelen, H. Abdolmaleky, T. J. Mabry, *J. Nat. Prod.*, **45**(4), 507 (1982).
53. B. B. Tirillini, A. M. A. Stoppini, R. R. Pellegrino, *J. Essent. Oil Res.*, **8**(6), 611 (1996).
54. E. Minareci, F. Kalyoncu, *AJPP*, **6**(13), 968 (2012).
55. R. Hand, Apiaceae. Euro+Med-Plantbase — The Information Resource for Euro-Mediterranean Plant Diversity. 2011. Available online: https://europlusmed.org/cdm_dataportal/taxon/205266ca-b449-419c-acfa-98aa3b2d8450 (accessed on 10 September 2024)
56. N. Gören, A. Ulubelen, J. Jakupovic, F. Bohlmann, M. Grenz, *Phytochemistry*, **23**(10), 2281 (1984).
57. F. Papa, M. Ricciutelli, K. Cianfaglione, F. Maggi, *Nat. Prod Res.*, **30**(3), 345 (2016).
58. D. K. Jayawickreme, C. Ekwosi, A. Anand, M. Andres-Mach, P. Wlaź, K. Socała, *Pharmacol. Rep.*, **76**, 644 (2024).
59. M. Jazvinšćak Jembrek, N. Oršolić, L. Mandić, A. Sadžak, S. Šegota, *Antioxidants*, **10**(10), 1628 (2021).
60. H. R. Nejabati, L. Roshangar, *J. Food Biochem.*, **46**(12), e14375 (2022).
61. C. Mancuso, R. Santangelo, *Food Chem. Toxicol.* **65**, 185 (2014).
62. H. J. Hwang, S. R. Lee, J. G.Yoon, H. R. Moon, J. Zhang, E. Park, ... J. A. Cho, *Antioxidants*, **11**(8), 1448 (2022).
63. S. Ou, K.C. Kwok, *J. Sci. Food Agric.*, **84**(11), 1261 (2004).
64. S. J. Pragasam, V. Venkatesan, M. Rasool, *Inflammation*, **36**, 169 (2013).
65. K. Pei, J. Ou, J. Huang, S. Ou, *J. Sci. Food Agric.*, **96**(9), 2952 (2016).
66. Y. Shen, X. Song, L. Li, J. Sun, Y. Jaiswal, J. Huang, ... Y. Guan, *Biomed. Pharmacother.*, **111**, 579 (2019).
67. S. M. Godarzi, A. V. Gorji, B. Gholizadeh, S. A. Mard, E. Mansouri, *Nefrologia*, **40**(3), 311 (2020).
68. S. S. Cheung, D. Khelifi, Z. S. Yang, D. Hasman, L. J. Dai, J. X. Xin, ... J. Tai, *Pharmacol. Res.-Modern Chinese Medicine*, **8**, 100271 (2023).
69. J. E. Jang, E. S. Kang, J. S. Kim, Y. O. Yoon, H. Y. Gil, D. C. Son, *BioInvasions Records*, **13**(1), 27 (2024).
70. K. Munjal, S. A. Amin, S. R, Mir, V. K. Gauttam, S. Gupta, Furanocoumarins and lectins as food toxins, in: Analysis of naturally occurring food toxins of plant origin, CRC Press, 2022, p. 67.
71. S. Levorato, L. Dominici, C. Fatigoni, C. Zadra, R. Pagiotti, M. Moretti, M. Villarini, *Food Chem. Toxicol.* **111**, 616 (2018).

Bibliometric analysis of scientific publications about food supplements

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Nowadays, food supplements are becoming increasingly popular based on the assumption of their safety and ability to supply the body with the necessary substances for the optimal course of all physiological processes. This has led to a strong increase in the production and trade of nutritional supplements worldwide, and as a result, scientific knowledge in the field of food supplements is also growing.

This article examines the publishability of articles in the scientific database Scopus concerning the use of dietary supplements. Bibliometric analysis has recently become a popular and rigorous technique for exploring and analyzing the literature. Bibliometrics is a field that uses mathematical and statistical methods to study and quantify the processes of written communications or apply mathematical techniques to study books and other written communication media. The bibliometric method was originally used to review the subjects, authors, scientific research institutions, journals, and research area of literature. Scientific publications on food supplements from 1990 to 2023 were searched in the Scopus database. Data was downloaded from Scopus and analyzed with Python packages. From the perspective of time distribution, the number of relevant studies published on food supplements showed an overall growth trend from 1990 to 2023. The growth rate was relatively fast after 2008, and there was a surge in the number of papers in 2021. This might be related to the COVID-19 pandemic and searching for alternative treatments.

Keywords: bibliometric analysis, food supplements, COVID-19 pandemic, Python

INTRODUCTION

Food supplements differ from conventional foods and medicines. They are defined as substances intended to supplement the regular diet and are "substances containing concentrated nutrients or other elements with a nutritional or physiological effect, alone or in combination" [1]. These substances are available in diverse drug forms — capsules, dragees, tablets, powders, solutions, and others — and are intended to be taken orally.

Food supplements contain vitamins and minerals, plant-based substances - plant parts or concentrated extracts, and others (e.g., amino acids or probiotics). All nutritional supplements are emphasized as not being a substitute for a varied diet. Their task is to supplement and enrich the menu, filling the lack of certain trace elements, vitamins, and minerals. That is why they are called "supplements." The primary purpose of nutritional supplements is to correct dietary deficiencies, maintain adequate intake of certain substances, or support specific physiological processes. They are not medicines and, as such, cannot exert a pharmacological, immunological, or metabolic effect. Therefore, their use is unrelated to treating or preventing diseases [2].

The burgeoning nutritional supplements market is underpinned by the diverse dietary habits and lifestyles of modern individuals living hectic live. These dietary patterns are shaped by anthropogenic factors, including cultural, economic, and religious, resulting in a wide array of food habits worldwide. Food habits that exclude the intake of a specific group of nutrients or products should be considered with particular attention. Furthermore, the growing consumer demand for products designed to alleviate or prevent symptoms of chronic diseases has been a significant driver behind the recent expansion of the nutritional supplement market.

Therefore, understanding the relationship between nutritional supplements containing vitamins and minerals and diet quality is essential in determining their optimal use. Consumers must be well informed about dietary supplements' health risks and potential benefits to make informed decisions about their use [3].

Specific socio-demographic characteristics and lifestyles characterize users of nutritional supplements containing vitamins and minerals. Supplements use generally increases with age, income, and education. In addition, women are more likely to use nutritional supplements than men.

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However, health status was not a predictor of dietary supplement use [4].

Bibliometric analysis has become a crucial tool for measuring the scientific outputs of various items such as papers, authors, keywords, journals, institutions, and countries in any research field. It helps examine how the relevant field's intellectual, social, and conceptual structure has evolved based on the relationships and interactions between these items. Researchers primarily use this analysis to recognize, evaluate, and comprehend the literature within a specific research field [5, 6].

Bibliometric analysis involves examining scientific publications in a specific field or academic journals using numerical analyses and statistics to gather various outputs. This includes indicators such as number of articles per year, popular topics, universities with the most publications, top journals prolific authors, citation counts, and keywords. This technique is used to map the intellectual structure of a research field or discipline and to understand the field's evolution and the relationships between authors, topics, and papers [7, 8].

EXPERIMENTAL

Scientific publications focused on food supplements from 1990 to 2023 were searched in the Scopus database. The Scopus database was utilized to gather relevant journal articles for the investigation. It was chosen because it covers many top-notch peer-reviewed publications from reputable publishers and is widely used in similar systematic reviews. After collecting the relevant data from Scopus, we analyzed it using various Python packages.

To track trends and innovations in the study of dietary supplements, we conducted a bibliometric analysis of publications containing "food supplement" in their title. We expanded the analysis with a keyword search focusing on the most common food supplements containing probiotics, magnesium, and zinc. Additionally, we reviewed materials on nutritional supplements and their association with COVID-19.

RESULTS

As the first step in article identification, a preliminary search was conducted using the string "food supplement" in the articles' titles to broadly cover the subject and build a solid string for subsequent queries.

Following this, we conducted a search using keywords to find relevant documents on probiotics, zinc, magnesium, and COVID-19 to achieve the study's objectives.

Analysis of publication trend about food supplements

We searched the literature using the Scopus database and yielded 2633 publications for analysis; we searched for papers published between 1990 and 2023 containing the words “food supplement” in the title.

Food supplements consumption has significantly increased over the last two decades. The dietary supplements market is growing in sales and, more importantly, in terms of products available. This inevitably affects scientific knowledge in the field of nutritional supplements. The continuous marketing of new forms and ingredients results in an increase in scientific knowledge in the field. This is visible on Fig. 1, which shows that the number of scientific publications has substantially increased for twenty years, rising from just 9 in 1990 to 161 in 2010. This upward trend continued in the subsequent years, culminating in a peak in 2021 when 186 articles were published. This exponential growth in scientific output is a testament to the expanding interests and capabilities within the research community, and it underscores the increasing pace of scientific discovery and dissemination of knowledge about food supplements.

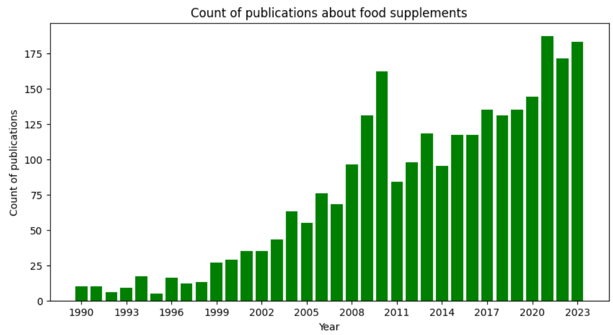

Figure 1. Count of publications including food supplements in the title

The most cited article about food supplements is "Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements," published in 2005. As of 2023, this article has amassed an impressive 4230 citations, making it one of the most widely referenced pieces of research in dietary supplements. This level of attention speaks to the significance and impact of the research and its influence on subsequent studies.

The article was published in the Journal of Agricultural and Food Chemistry, which is far from the top of the journals that publish the most articles on food supplements. In first place with the most significant number of articles is the EFSA Journal, which is an open-access, free-of-charge online scientific journal that publishes the scientific outputs of the European Food Safety Authority (EFSA), which is the keystone of the European Union (EU) risk assessment regarding food and feed safety.

When analyzing papers by journal, the EFSA Journal published the most papers, establishing itself as a prominent source of research. After that, they formulated the Risk Assessment of Phytochemicals in Food: Novel Approaches and Nutrients. The British Journal of Sports Medicine follows closely behind, contributing significantly to the body of knowledge in this area. Additionally, the journal Food Chemistry and Deutsche Apotheker Zeitung made noteworthy contributions, further enriching the literature on this subject. This demonstrates the diverse range of sources engaged in the topic of nutritional supplements. The indicated data are presented in Fig. 2.

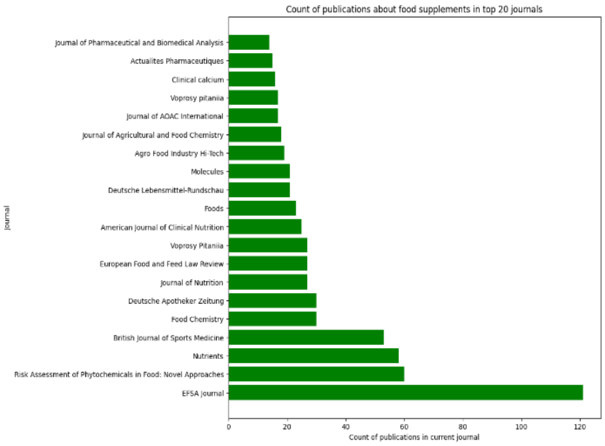

Figure 2. Analyzing publications about food supplements by journal

A peak in scientific publications on food supplements was reported in 2020-2021, when 186 publications were recorded, marking a significant increase in research and interest in this area. This surge in attention can be attributed to the global COVID-19 pandemic, which led to an increased search for alternative therapeutic approaches and strategies for disease prevention.

Analysis of publication trend about food supplements and COVID-19

The public health crisis brought on by the COVID-19 pandemic prompted a heightened interest in utilizing dietary supplements as a potential means of bolstering the immune system, minimizing the risk of inflammation, and potentially offering protection against the emerging disease. Additionally, concerns and hesitations regarding the availability and efficacy of COVID-19 vaccines in various regions of the world have further spurred interest in exploring dietary supplements as an alternative or complementary approach to promoting health and potentially mitigating the impact of the virus.

Global economic and health-related issues have arisen since the declaration of the COVID-19 pandemic in March 2020. The pandemic has led to a surge in demand for dietary supplements, including vitamins and minerals, as potential additions to the treatment of COVID-19. These DS are believed to have the ability to boost the immune system and reduce disease severity. Despite the development of several COVID-19 vaccines and the availability of numerous pharmacological therapies, the demand for food supplements has significantly increased worldwide. This increased interest in dietary supplements showcases a growing interest in alternative or complementary approaches to managing and supporting overall health, especially in the context of the pandemic. As individuals seek ways to protect themselves and support their well-being during these challenging times, the market for dietary supplements has experienced notable growth. Exploring the potential benefits of dietary supplements in managing COVID-19 represents a significant area of interest for researchers and healthcare professionals alike.

The confluence of these factors has driven a significant uptick in scientific inquiry and publications focused on food supplements as researchers and health professionals seek to understand the potential role of dietary supplements in supporting immune function and overall health during the global health crisis. Consequently, the intersection of the COVID-19 pandemic and the heightened demand for strategies to enhance immune function and mitigate health risks has catalyzed a notable surge in interest and research activity related to dietary supplements.

Therefore, we conducted an additional keyword search to find scientific publications specifically addressing food supplements and the COVID-19 pandemic. The data obtained are presented in Fig. 3.

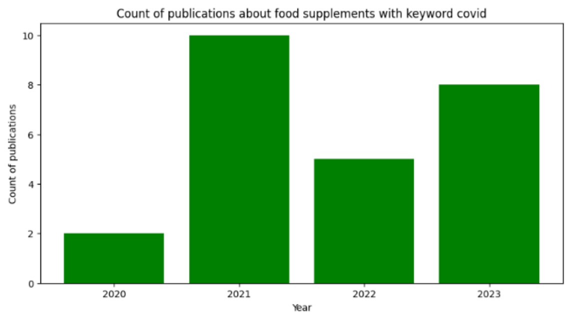

Figure 3. Publications about food supplements with the keyword “COVID”

In 2020, only 2 scientific publications with food supplements in the title included the keyword COVID-19 were found. However, in 2021, this number significantly increased, reaching 10 publications. This sharp rise in scientific interest and attention toward COVID-19 underscores the impact and relevance of this global health crisis. The increased research and publications reflect the urgency and importance of understanding and addressing the challenges posed by the COVID-19 pandemic.

When we searched using the keyword “COVID”, we discovered 25 scientific publications specifically addressing food supplements in relation to the pandemic. Among these publications, the most cited one is titled "Immune-Boosting, Antioxidant, and Anti-inflammatory Food Supplements Targeting Pathogenesis of COVID-19," which has garnered 213 citations in scientific databases up to 2023. It is worth noting that all articles about food supplements and their impact on the COVID-19 pandemic are authored in English. Furthermore, most of these articles are published in Nutrients, an international, peer-reviewed open-access journal.

In contrast, the journal ranks third in publication when discussing scientific articles on food supplements. In this case, the EFSA journal is the most preferred. These data are presented in Fig. 4.

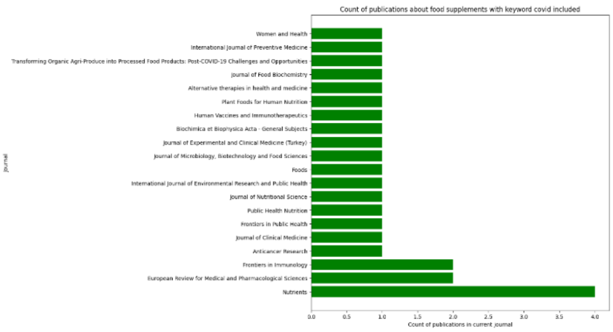

Figure 4. Analyzing publications about food supplements with the keyword “COVID” by journal

Fig. 5 shows a word cloud generated from keywords with the highest frequency included in publications about food supplements and COVID-19, visually representing vital thematic elements in the dataset. These terms strongly emphasize topics related to the immune system and SARS. Other prevalent terms, including “dietary supplements”, “vitamin”, “antioxidant”, and “minerals”, contribute to a rich thematic landscape. The prominence of these terms in the word cloud indicates their recurrent presence in the dataset, offering researchers valuable insights into the focal points of research contributions and potential areas of emphasis within the analyzed period. By identifying the most frequently occurring terms in the dataset, researchers can gain a deeper understanding of the prevalent topics and subjects that have been the research focus during the analyzed period. This analysis provides a foundation for further exploration and investigation into specific topics that garnered significant attention within the research community. Furthermore, it allows for identifying emerging trends and areas of interest, which can inform future research directions and contribute to advancing knowledge within the field.

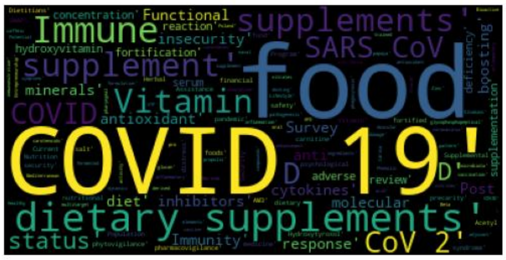

Figure 5. Word cloud about publications for food supplements with “COVID” included as a keyword

Analysis of publication trend about food supplements and probiotics

In addition to reviewing the scientific publications on dietary supplements related to COVID-19, we also reviewed materials concerning the fast-growing probiotics segment.

Probiotics are food supplements containing live microorganisms intended to maintain or improve the "good" bacteria (normal microflora) in the human body.

Modern science is continually discovering more possibilities for using these products as an adjunct to the primary therapy for various diseases, ranging from gastrointestinal tract (GIT) disorders to stress and migraine. The increasing popularity of these products is also evident in scientific databases [9].

The effects of probiotic supplementation on metabolic issues such as hyperglycemia, hypertension, and hyperlipidemia have been extensively studied [10].

Numerous studies are being conducted on probiotics' behavior in the body and their potential effect on health. Although many possible positive effects from the use of probiotics exist, there is still no consensus on how probiotics can support the therapy of various diseases. This inevitably results in an increasing number of scientific articles on probiotics. The results show that 112 articles were published on probiotics between 1990 and 2023, representing a significant increase. Fig. 6 displays data related to scientific publications with “probiotics” as a keyword.

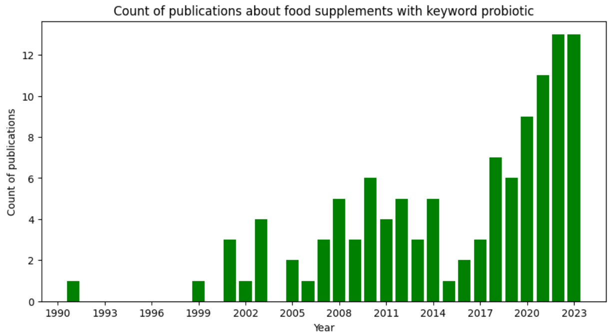

Figure 6. Data related to scientific publications with “probiotics” as a keyword.

Probiotics became popular in scientific publications after 2000 when the number of articles published in the world's databases increased. This is probably due to increasing scientific knowledge and successful marketing strategies to push probiotics into the pharmaceutical market. The significant majority of these articles are published in Nutrients, followed by Frontier in Microbiology and Pharmazeutische Zeitung (Fig. 7).

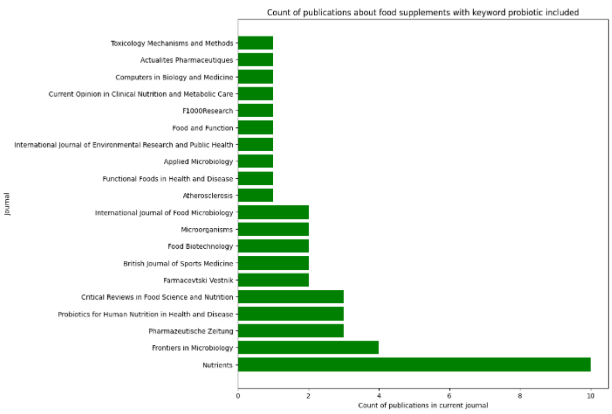

Figure 7. Analyzing publications about food supplements with keyword “probiotics” by journal

The most cited article about probiotics is “The role of functional foods, nutraceuticals, and food supplements in intestinal health”.

Word cloud, visually representing vital thematic elements in the dataset, is presented in Fig. 8.

Figure 8. Word cloud about publications for food supplements with “probiotics” included as a keyword

These terms suggest a strong emphasis on topics related to gut microbiota and metabolic health in the human body. Other prevalent terms, including “food supplements”, “inflammation”, “health”, “dietary”, and “probiotics”, contribute to a rich thematic landscape.

Analysis of publication trend about food supplements and microelements zinc and magnesium

According to our thorough analysis, we included the keywords zinc and magnesium in the current research. These essential components are widely present in numerous nutritional supplements and are gaining popularity among consumers. Zinc and magnesium are utilized to rectify deficiencies and uphold and enhance the body's overall tone and condition, playing a crucial role in supporting various bodily functions. Their utilization extends beyond addressing deficiencies, as they are also instrumental in promoting overall health and well-being. Therefore, we add the keywords zinc and magnesium to the search.

The role of zinc-containing dietary supplements has been known for a long time, but in recent decades, considerable data have emerged regarding its importance in immune modulation. The obtained results are presented in Fig. 9.

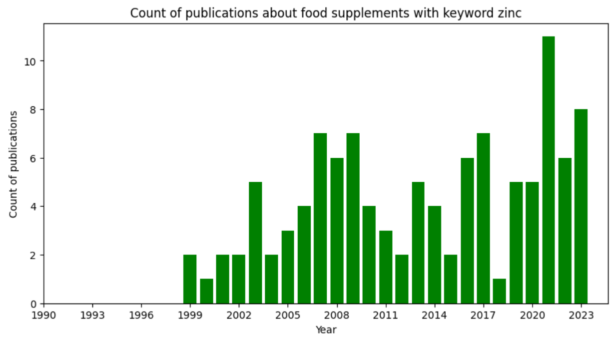

Figure 9. Number of publications including food supplements in the title and “zinc” as a keyword

Magnesium is the fourth most abundant cation in the body. It has several functions in the human body, including its role as a cofactor for more than 300 enzymatic reactions. Many studies have reported that reduced magnesium levels are associated with many chronic diseases. Magnesium can play important therapeutic and preventive roles in several conditions, such as diabetes, osteoporosis, bronchial asthma, preeclampsia, migraine, and cardiovascular diseases [11].

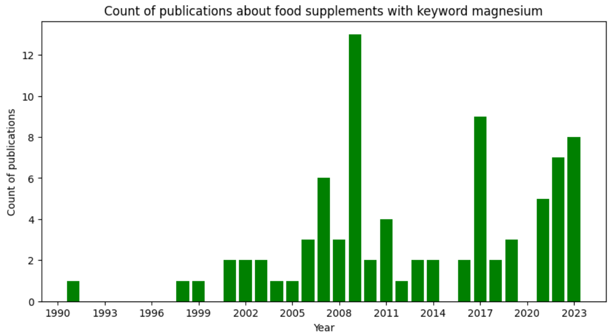

Figure 10. Number of publications including food supplements in the title and “magnesium” as a keyword

Based on the previous data, it is clear that the number of scientific publications about nutritional supplements, particularly zinc and magnesium, has increased in recent years. Notably, the pandemic has impacted this field of study. While food supplements are not included in the guidelines for treating the disease, a growing number of scientific publications have suggested the potential benefits of zinc, vitamin D, vitamin C, and magnesium [12].

The rise in research on nutritional supplements can be attributed to the growing interest in exploring alternative and complementary approaches to health. As people become more health-conscious, there is a greater focus on the potential role of nutritional supplements in supporting overall well-being, immune function, and disease prevention.

The EFSA journal published the most articles on food supplements with the additional keywords zinc and magnesium, followed by Nutrients. When searching with the keyword zinc, both journals displayed an equal number of published scientific articles for the specified period, which is 12.

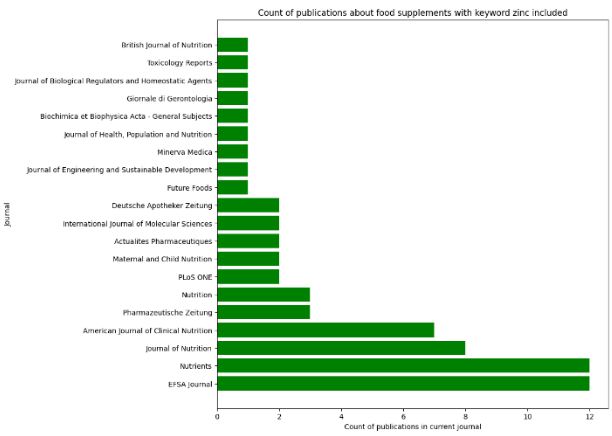

Figure 11. Analyzing publications about food supplements with keyword “zinc” by journal

The most cited article including zinc and magnesium as a keyword is “Foods, Fortificants, and Supplements: Where do Americans get their nutrients?” it has 392 citations.

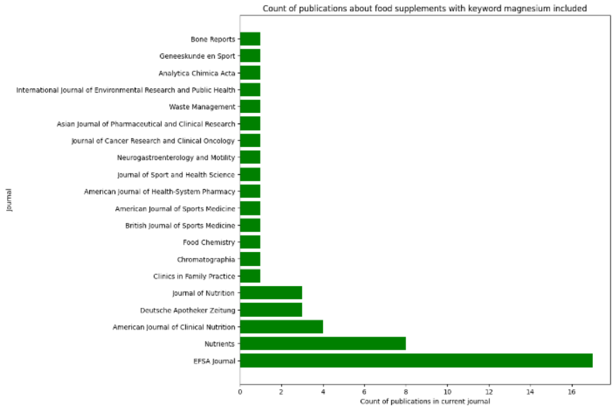

Figure 12. Analyzing publications about food supplements with keyword “magnesium” by journal

The following figure presents a word cloud visually representing vital thematic elements in the dataset.

Figure 13. Word cloud about publications for food supplements with “zinc” included as a keyword

The relationship of zinc with other minerals, such as magnesium and calcium, is visible (Fig. 13). Apart from them, the term "food supplements" most clearly stands out. The situation is similar to the word cloud generated from keywords with the highest frequency when we search with the keyword magnesium. Apart from its relationship with other minerals such as potassium, zinc, and calcium, the appearance of amino acids as a term is also noticeable (Fig. 14). This points out a complex network of crucial interrelated components when examining dietary supplements.

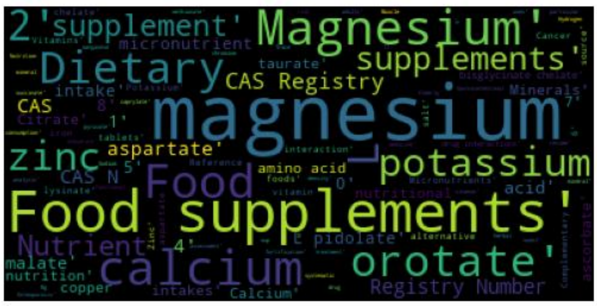

Figure 14. Word cloud about publications for food supplements with “magnesium” included as a keyword

CONCLUSIONS

Bibliometric analysis is a method for quantitatively analyzing academic publications, including research articles, journals, and other scholarly publications. It involves evaluating patterns, citations, and other bibliographic data to gain insights into the impact and influence of research within a specific field or discipline. Bibliometric analysis can provide valuable information about trends, collaborations, and the overall landscape of scholarly work, making it an essential tool for researchers, academic institutions, and funding agencies. Bibliometric analysis is increasingly used to review trends and progress in different fields and research areas.

Regarding time distribution, the number of relevant studies published on food supplements showed an overall growth trend from 1990 to 2023. The growth rate was relatively fast after 2008, and there was a surge in the number of papers in 2021. This increase may be related to the COVID-19 pandemic and the search for alternative treatments.

The COVID pandemic has led to significant changes in the pharmaceutical sector, including the food supplements segment. The market has witnessed a surge in demand for products that support immune and digestive health. Selling supplements such as probiotics, vitamins, omega-3 fatty acids, and others has grown immensely. These factors are having a positive impact on the market and are leading to an increased scientific interest in the field.

Moreover, the global health crisis brought about by the pandemic has heightened interest in identifying ways to boost immune health and resilience. This has increased emphasis on the potential benefits of specific nutrients such as probiotics, zinc, vitamin D, vitamin C, and magnesium in supporting human health.

It is important to note that while the scientific community continues to explore the potential benefits of nutritional supplements, further research is needed to fully understand their effectiveness and safety, particularly in specific health conditions and individual needs. Therefore, ongoing research and evidence-based analysis will be essential in determining the role of food supplements in promoting health.

REFERENCES

1. A. L. Young, I. S. Bass, *Act. Food Drug Law J.,* **50,** 285 (1995).
2. O. Djaoudene, A. Romano, Y.D, Bradai, F. Zebiri, A. Ouchene, Y. Yousfi, M. Amrane-Abider, Y. Sahraoui-Remini, K. Madani, *Nutrients,* **15**, 3320 (2023), https:// doi.org/10.3390/nu15153320
3. W. Hu, Y. Xu, *Front. Immunol*. **13,** 1065724 (2022). doi: 10.3389/fimmu.2022.1065724
4. N. Donthu, S. Kumar, D. Mukherjee, N. Pandey, W. Marc Lim *Journal of Business Research*, **133**, 285 (2021), ISSN 0148-2963,https://doi.org/10.1016/j.jbusres.2021.04.070.
5. E.D. Kantor, C.D. Rehm, M. Du, E. White, E.L. Giovannucci, *JAMA Intern. Med.,* **316**, 1464 *(*2016) doi: 10.1001/jama.2016.14403.
6. O .Öztürk, R. Kocaman, D. K. Kanbach, *Science*, (2024), https://doi.org/10.1007/s11846-024-00738-0
7. O. Ellegaard, J.A Wallin, *Scientometrics*, **105(3),** 1809 (2015). doi: 10.1007/s11192-015-1645-z. Epub 2015 Jul 28. PMID: 26594073; PMCID: PMC4643120.
8. M. Fernandez-Alles, A. Ramos-Rodríguez, *Journal of the American Society for Information Science and Technology* (2008), https://doi.org/10.1002/asi.20947
9. H. F. Dale, S. H. Rasmussen, Ö. Ö. Asiller, G. A. Lied, *Nutrients*. **11** (9), 2048 (2019). doi:10.3390/nu11092048. ISSN 2072-6643. PMC 6769995
10. G.T. Rijkers, W.M. de Vos, R.J. Brummer, L. Morelli, G. Corthier, P. Marteau, *The British Journal of Nutrition*. **106** (9), 1291 (2011) doi:10.1017/S000711451100287X
11. A.M. Al Alawi, S.W. Majoni, H. Falhammar, *Int. J. Endocrinol*., **16,** 9041694 (2018). doi: 10.1155/2018/9041694
12. M. Jamilian, M. Maktabi, Z. Asemi, *Arch. Iranian Med.,* **20,** 640 (2017).

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Evaluation of antioxidant activity of tobacco extracts by CUPRAC and H_2O_2 assay

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Tobacco (*Nicotiana tobacco* L.) is a plant containing biologically active substances (secondary metabolites) like phenolic acids, flavonoids, coumarins, saponins, carotenoids, etc. These compounds exhibit antioxidant activity. The aim of the study is to investigate the antioxidant activity of tobacco extracts by using cupric ion reducing antioxidant capacity (CUPRAC assay) and hydrogen peroxide scavenging assay (H_2O_2 assay). For the research purpose Bulgarian varieties of tobacco from Basmi variety group, grown conventionally or by organic production, and from conventionally grown Kabakulak and Virginia variety groups were used. 60 % methanolic extracts were obtained and the extracts were investigated for antioxidant activity by CUPRAC assay and H_2O_2 assay. The antioxidant activity determined by CUPRAC assay varied between 327.31±22.89 mM TE/g DM (variety Hanski 277, II cl.) and 751.45± 52.6 mM TE/g DM (variety Nevrokop, II cl.), while by H_2O_2 assay – between 226.14±14.46 mM TE/g DM (variety Hanski 277, II cl.) and 409.01±26.16 mM TE/g DM (variety Virginia 0842, II cl.). The obtained results indicated that the extracts from Basmi variety group grown conventionally or by organic production and the extracts obtained from tobaccos of the Virginia variety group, conventionally grown, have higher antioxidant activity compared to the tobacco extracts obtained from the Kabakulak variety group in both investigated methods. The results of the present study showed that 60 % methanolic tobacco extracts, obtained by Basmi, Virginia and Kabakilak variety groups, have significant antioxidant activity, determined by CUPRAC method and H_2O_2 assay.

Keywords: tobacco, tobacco extracts, antioxidant activity, CUPRAC assay, H_2O_2 assay

INTRODUCTION

The use of tobacco leaves for therapeutic purposes dates back years and increases in practice [1]. Tobacco as a medicinal plant synthesizes secondary metabolites (antioxidants) - polyphenols, coumarins, saponins, carotenoids, etc., which exhibit biological activity. The presence of biologically active substances in tobacco is a prerequisite for obtaining extracts with high biological (antioxidant) activity [2, 3].

Reactive oxygen species are unstable oxygen species with unpaired electron. They include radicals - superoxide radical ions ($O_2^{-\bullet}$), hydroxyl radicals ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen radicals ($O_2^{\bullet}$) and non-radical species - hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) which are continuously generated under the physiological processes in the body. These unpaired electrons are called free radicals that can interact with important biomolecules like DNA, lipids, proteins which can destabilize molecules and damage the cell membrane by acquiring free electrons from a stable lipid membrane [4-6].

On the other hand, antioxidant molecules which are diverse in their nature, can prevent the oxidizing effect of free radicals by reducing them [5, 7].

According to the mechanism of action antioxidant assays may be classified into electron transfer (ET) based assays and hydrogen atom transfer (HAT) [4, 8, 9]. ET-based assays include FRAP and CUPRAC assays. Method for oxygen adsorption capacity of free electrons (ORAC method) and method for scavenging H_2O_2 radicals (H_2O_2 assay) belong to hydrogen atom transfer (HAT) methods. Finally, ABTS and DPPH methods are considered as borderline between ET- and HAT based assays) [4, 8, 9].

In each of the listed methods, only the antioxidant activity is examined in relation to the specific reaction for the respective method, and not the overall activity. For this reason no single assay is sufficient for reliable determination of antioxidant activity. The use of several different methods based on different types of reaction or the use of different radicals is recommended [4, 8-11]. Because of this, for a more complete characterization of tobacco extracts, it is necessary to apply different methods for determining their biological activity.

The aim of the study is to investigate the antioxidant activity of tobacco extracts by using cupric ion reducing antioxidant capacity (CUPRAC assay) and scavenging H_2O_2 radicals (H_2O_2 assay).

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EXPERIMENTAL

Material

Dry leaves of Bulgarian tobacco varieties from Basmi group (variety Krumovgrad 58, and variety Nevrokop - grown conventionally or under organic production), Kabakulak group (variety Han Tervel 39, and Hanski 277, conventionally grown) and Virginia group (variety Virginia 514 and Virginia 0842, conventionally grown – II cl.) were used as a material. The cultivars are from the collection of the Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria.

Chemicals

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA).

Instrument

Spectrophotometer "Spectroquant Pharo 300", UV/Vis (Merck, USA)

Preparation of plant extract

Dry tobacco powder (0.2 g) was extracted with 60 % methanol (10 ml), for 30 min on a mechanical shaker. The extracts were filtered by a syringe filter and were used for further analysis.

In vitro antioxidant activity

- *CUPRAC assay*. The reducing power of the cupric ions (Cu^{2+}) of 60 % methanolic tobacco extracts was determined according to Apak *et al.* [12] with slight modifications: 1 ml $CuCl_2$ solution (10 mM dissolved in water), 1 ml neocuproine alcoholic solution (7.5 mM dissolved in ethanol), 1 ml ammonium acetate buffer solution (1 M, pH=7), 0.1 ml tobacco extract and 1 ml water are successively added and well mixed. The absorbance was measured at 450 nm for each sample after 30 min in the dark [13]. Trolox was used as a standard in the range of 22.5 mM to 375 mM. The results are expressed as mM TE/g DM.
- *H_2O_2 assay*. For scavenging H_2O_2 radicals (H_2O_2 assay) 60 % methanolic tobacco extracts are analyzed using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). 0.1 ml plant extracts, 0.6 ml H_2O_2 and 3.3 ml PB placed in a test tube. After 10 min in the dark, the absorbance at 230 nm is measured for each sample. Trolox is used as a standard in the range of 0.09 mM to 3.00 mM. The results are expressed as mM TE/g DM.

Determination of total phenolic contents (TPC) using the Folin-Ciocalteu method (FC)

The determination of the amount of TPC was based on the FC method with some modification [13, 14]: 0.1 ml 60 % methanolic tobacco extract, 6 ml water and 0.5 ml 0.2 M FC reagent are placed into a test tube. After 4 min 3.4 ml 7.5 % Na_2CO_3 is added. All the samples and the blank are stored in the dark for 2 h and then are measured at 765 nm against a blank sample. The concentration of the phenolic compounds in the extracts is calculated using gallic acid as a standard. The results are expressed as mg GAE/g DM.

Statistical analysis

All experiments were performed at least three times. The results were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

Medicinal plants are a source of a wide variety of natural products, such as phenolic acids and flavonoids which possess antioxidant properties [3]. Total phenolic content in tobacco extracts, determined by Folin-Ciocalteu method, is presented in Table 1.

Table 1 shows that tobaccos from the Basmi variety group, organic and conventional production, are characterized by the highest TFC (average 43.01±5.33 mg GAE/g DM), followed by the tobaccos from the Virginia variety group – average 34.25±2.64 mg GAE/g DM. Tobaccos from the Kabakulak variety group have twice lower content of phenolic acids compared to Basmi and Virginia tobaccos – about 17.80±0.12 mg GAE/g DM. There is no significant difference in TPC between organic and conventional production.

Antioxidant activity

Two *in vitro* model systems were used to evaluate scavenging activity in tobacco extracts – CUPRAC method and H_2O_2 method, which have different mechanisms.

CUPRAC assay

CUPRAC transfer ET-based method permits overall quantification of all kinds of antioxidants. ET-based spectrophotometric assays measure the capacity of an antioxidant by the reduction of a chromogenic oxidant (probe) which changes color when reduced. The degree of color change (either an increase or decrease of absorbance at a given wavelength) is correlated with the concentration of antioxidants in the sample [10].

Antioxidant activity of tobacco extracts, evaluated by the CUPRAC method is presented in Fig. 1. It is obvious that the highest activity is manifested by the tobacco extracts obtained from organic production (Krumovgrad 58 and Nevrokop 1146, respectively, 609.14±42.60 mM TE/g and 648.20±44.20 mM TE/g) and tobacco Nevrokop 1146 conventional production – 751.45±52.6 mM TE/g, followed by the extracts from Virginia tobaccos, conventional production 695.64±48.65 mM TE/g (Virginia 0842) and 662.16±46.58 mM TE/g (Virginia 514) and the extract obtained from Krumovgrad 58, conventional production - 517.06±36.19 mM TE/g. Weaker activity is displayed by extracts obtained from the tobaccos of the variety group Kabakulak - Han Tervel 430.55±30.13 mM TE/g and Hanski 277 - 327.31±22.89 mM TE/g.

The antioxidant activity determined by the CUPRAC method of tobacco extracts is higher than that of dandelion extracts (99.9±7.0 mM TE/g) and lower than that of thyme extracts (868.6±60.8 mM TE/g) obtained under the same conditions in our earlier studies [15].

Due to the aggressive action of H_2O_2, it is extremely important to find substances that have the ability to bind to H_2O_2 and neutralize its action. The peroxide radical scavenging method (H_2O_2 method) is related to the ability of antioxidants such as flavonoids, simple phenolic acids, hydroxycinnamic acids and other to trap peroxide radicals with a simple, inexpensive universal colorimetric procedure [11].

Table 1. Total phenolic content in Bulgarian varieties of tobaccos grown under organic and conventional production, mg GAE/g DM

Variety group	Type of production	Variety	TPC
Basmi	Organic	Krumovgtad 58, II cl.	36.11±2.11
		Nevrokop, II cl.	46.14±2.36
	Conventional	Krumovgtad 58, II cl.	41.66±2.08
		Nevrokop, II cl.	48.13±2.42
Kabakulak	Conventional	Han Tervel 39, II cl.	17.89±1.45
		Hanski 277, II cl.	17.72±1.45
Virginia	Conventional	Virginia 514, II cl.	36.12±2.11
		Virginia 0842, II cl.	32.38±2.08

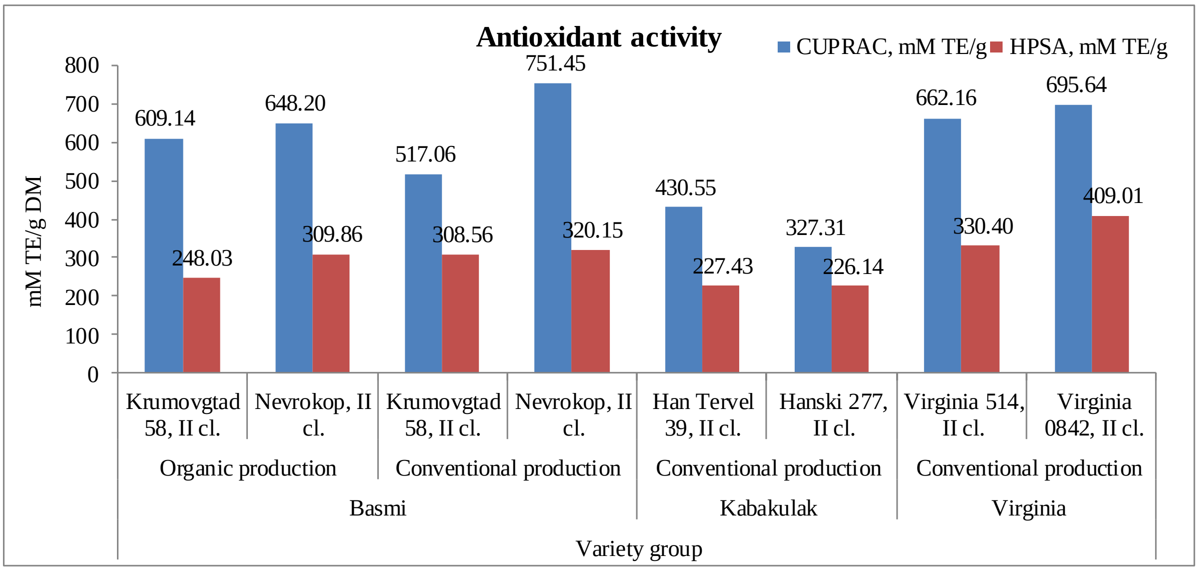

Fig. 1. Antioxidant capacities of tobacco extracts evaluated by CUPRAC method and H_2O_2 assay

The extracts from Virginia variety tobaccos are characterized by highest ability to neutralize peroxide radical group - 409.01±26.15 mM TE/g DM (Virginia 0842) and 330.4±21.12 mM TE/g DM (Virginia 514). Tobacco extracts obtained from Kabakulak varietal group are characterized by almost twice as low activity – 226.14±14.46 mM TE/g DM (variety Hanski 277) and 227.43±15.24 mM TE/g DM (Han Tervel 39) compared to the Virginia tobaccos.

The tobacco extracts, obtained from conventionally and organically produced Basmi variety group, occupy an intermediate position. Antioxidant activity, determined by H_2O_2 assay varied between 248.03 ±15.89 mM TE/g DM (Krumovgrad 58, organic production) and 320.15±20.47 mM TE/g DM (Nevrokop, conventional production). No significant difference in antioxidant activity was observed between the extracts obtained from conventional and biological tobacco production - Fig. 1.

The antioxidant activity determined by the H_2O_2 method of tobacco extracts is higher than antioxidant activity of dandelion extracts (42.2±2.7 mM TE/g) [15].

Correlation between TPC and antioxidant activity

To observe the dependence between TPC and AOA, the correlation dependence of the two free radical scavenging activity methods for tobacco extracts was investigated. Figures 2 and 3 present the correlation between TFC in tobacco extracts and antioxidant activity evaluated by CUPRAC assay and H_2O_2 assay.

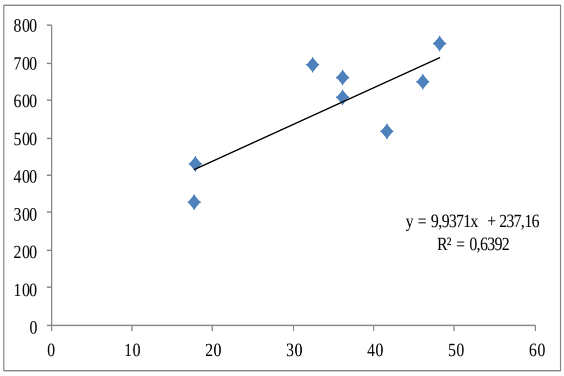

Fig. 2. Correlation between TPC and antioxidant activity, evaluation by CUPRAC assay

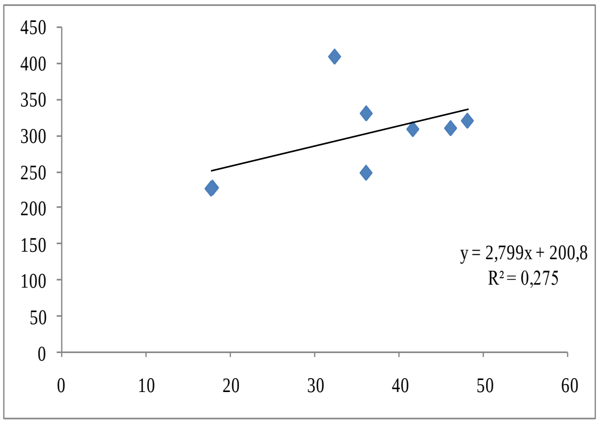

Fig. 3, Correlation between TPC and antioxidant activity, evaluation by H_2O_2 assay

As it is seen from Figs. 2 and 3, a better correlation is observed between TPC and CUPRAC method (R^2=0.639) than between TPC and H_2O_2 method (R^2=0.275).

CONCLUSION

Conventionally and organically grown Bulgarian tobaccos were used. Since it has been accepted nowadays that both hydrogen atom transfer (HAT)- and electron transfer (ET)-based assays are needed to give a reliable estimate of the antioxidant activity of plant extracts, in this work antioxidant activity of 60 % tobacco extracts was assayed by CUPRAC method (ET-based assay) and H_2O_2 method (HAT-based assay).

The results of the present study showed that:

- Tobacco extracts from Basmi and Virginia varietal groups, both conventional and organic production, have a higher antioxidant activity compared to the extracts from tobaccos from the Kabakulak varietal group in both investigated methods.
- The antioxidant activity of tobacco extracts determined by electron transfer methods (CUPRAC-method) is higher than the activity determined by hydrogen atom transfer methods (H_2O_2). Tobacco extracts have a higher ability to reduce Cu^{+2} to Cu^{+} compared to their ability to scavenge peroxide radicals.
- There is a better linear correlation between TPC and CUPRAC method, compared to the correlation between TPC and H_2O_2 method.

REFERENCES

1. A. Charlton, *J. R. Soc. Med*,. **97**(6), 292 (2004).
2. O. Alara, N. Abdurahman, C. Ukaegbu, *Curr. Res. Food Sci.*, **4** (March), 200 (2021).
3. M. Andersen, K. Markham, Boca Raton, FL: CRC Press, Taylor & Francis Group, 2006.
4. G. Tirzitis, G. Bartosz, *Acta Biochimica Polonica*, **57 (1),** 139 (2010).
5. K. Murthy, S. Кumar, PHARMATUTOR-ART, 1784 (2013). https://www.pharmatutor.org/articles/review-novel-vitro-antioxidant-method-new-insight-cuprac-assay.
6. A. Pleh, L. Mahmutović, A. Hromić-Jahjefendić, *Bioengineering Studies*, **2** (1), 1 (2021).
7. D. Huang, B. Ou, R. Prior, *J. Agric. Food Chem*., **53** (6), 1841 (2005).
8. H. Moharram, M. Youssef, *Alex. J. Fd. Sci. & Technol*., **11** (1), 31 (2014).
9. M. Antolovich, P. Prenzler, E. Patsalides, S. McDonald, K. Robards, *The Analyst* **127** (1), 183 (2002).
10. M. Ozyourek, K. Guclu, E. Tutem, K. Basxkan, E. Ercag, S. Celik, S. Baki, L. Yıldız, S.Karaman, R. Apak, *Analytical Methods*, **3,** 2439 (2011).
11. I. Munteanu, C. Apetre, *International Journal of Molecular Sciences*, **22** (7), 3380, (2021).
12. R. Apak, K. Güçlü, M. Ozyürek, S. EsinKarademir, E. Erçağ, *International Journal of Food Sciences and Nutrition*, **57** (5-6), 292 (2006).
13. D. Bojilov, S. Manolov, B. Bozadzhiev, J. Stremski1, I. Ivanov, *Journal of International Scientific Publications*, **6**, 314 (2018).
14. V. Singleton, J. Rossi, *Am. J. Enol. Vinic.*, **16** (48), 144 (1965).
15. D. Kirkova, M. Docheva, L. Stoyanova, Y. Stremski, S. Statkova-Abeghe, V. Petrova, Y. Kochev, *Bulgarian Chemical Communication*s, **56** (Special Issue D1), 172 (2024).

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Pathologic features in the oral cavity in adolescence with disturbances in the nutritional status

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The aim of the study is to investigate the carious process and gingival inflammation in adolescents with disturbances in nutritional status. The study was conducted in 2023-2024 and involved 162 children (86 boys and 76 girls) between 11-17 years old with a median age of 14. The nutritional status of the studied children was analyzed using an anthropometric indicator BMI-Z Percentile. The adolescents were divided into three groups: normal body weight, risk of obesity, and overweight. DMFT, SiC index, and PUFA index were used to evaluate the carious process. Gingival inflammation was evaluated using the Papillary Bleeding Index (PBI). Of all children studied, 68 (42%, 95% CI:34.3-50) were with normal body weight, 75 (46.3%, 95% CI:38.4-54.3) were at risk of obesity and 19 (11.7%, 95% CI:7.2-17.7) were overweight. In the three groups, there was no significant difference in DMFT, but median SiC index was increased in the second group - 9 (8-10.5) and in the third - 10 (8-12) ($p>0.05$). PUFA index was increased in the third group– 31.6%, in the second – 32% ($p<0.05$). PBI grew in the second group - 66%, while in the first and third -50%. ($p<0.05$). *Post hoc* analysis showed a statistically significant difference between the first and second groups' PBI. A moderate positive correlation was observed between PUFA and PBI in the first (ρ(rho) = 0.47, $p<0.001$) and the second group (ρ(rho) = 0.61, $p< 0.001$). Nutrition has a significant role in developing and complicating the carious process and gingival inflammation in adolescence.

Keywords: adolescent, carious process, gingival inflammation, nutritional status

INTRODUCTION

Obesity among adolescents is a worldwide public health problem. According to the World Obesity Federation, it is estimated that there will be 206 million obese children/adolescents in 2025 and 254 million in 2030 [1]. Failure to solve it leads to a predisposition to obesity in adulthood [2]. According to the American Academy of Pediatrics, Committee on Nutrition, overweight and obesity are now the most common medical conditions of childhood [3].

There is a similarity between caries and its complications, gingival inflammation, and obesity. All of them are of global importance, multifactorial, and related to lifestyle, which leads to interconnection between them. Important for their development are the way of eating, oral hygiene habits, active lifestyle, psychosocial behavior, socio-economic status, and parental support [4-6].

In our fast-paced everyday life, with fast food consumption, on-the-go eating and not enough time to prepare home-cooked meals, the results are there. Fast foods lack essential micro and macro elements such as vitamins, minerals, fiber, and amino acids, their nutritional value is extremely low. In contrast, they are rich in white flour, refined sugar, salt, polysaturated fats, trans fats, colorings and additives [7]. Diet and eating habits affect adolescents' growth, general and oral health [8, 9]. Adequate nutrition during childhood and adolescence is essential for children's growth and development [10]. Frequent consumption of carbohydrates in foods and drinks increases the risk of excess body weight, the development and progression of the carious process, and gingival inflammation [11]. In addition, according to the global disease burden from 2017, the most common condition is untreated and complicated caries of permanent teeth [12].

EXPERIMENTAL

The study was conducted in the period 2023-2024 at the Medical University "Prof. Dr. Paraskev Stoyanov"- Varna, University Medical and Dental Center at the Faculty of Dental Medicine and clinical halls at the Department of Pediatric Dentistry of the Faculty of Dental Medicine - Varna. A clinical examination was conducted to record the carious status by DMFT and SiC index, complication of the carious process by PUFA index, gingival

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inflammation by PBI.

In the study, we used one of the general health indicators, the overweight and obesity, BMI-Z score. It is used to diagnose the risk of obesity and overweight in children and adolescents. Children fall into the column "at risk of obesity" when the BMI Z-score is between the 85th and 95th percentile, "overweight" when they are above the 95th percentile and "severely obese" above the 99th percentile [13]. This index shows only total adipose tissue, and additional index systems are used to estimate its distribution [14].

The most universal index for reporting the prevalence of dental caries - DMFT - was used. D shows teeth with a carious lesion, and M shows Missing teeth in the dentition due to a complication of a carious process. F-teeth that have been restored after caries treatment. According to the WHO, the diagnosis of caries registered by DMFT-index is based only on visual diagnostics [15]. The DMFT index is a quick and convenient method, a widely used method for assessing the risk of caries [16]. In addition to the DMFT index, we used the Significant Caries Index (SiC Index). It draws attention to patients with the highest caries rates in each study population. It represents the average DMFT index of one-third of the studied adolescents in the population with the highest score [17].

However, these two indices, DMFT and SiC index, do not consider the severity and complications of the carious process. We therefore used a PUFA index created by Monse *et al*. [18], which was used to estimate the prevalence and severity of oral conditions associated with untreated caries. Reflects the presence of visible pulp (P/p), traumatic ulceration of the oral mucosa due to injury from residual sharp pieces of a tooth, root fragments (U/u), fistula in a suppurating fistulous course associated with a tooth or involvement of the pulp (F/ f) or abscess (A/a) when soft tissue swelling with purulent content is associated with pulp necrosis. The score of the examined patient is calculated in a cumulative way (P+U+F+A) and can vary from 0 to 32 for permanent dentition.

The Papillary Bleeding Index (PBI) is used to measure the level of gingival inflammation. It is beneficial for evaluating gingival inflammation in the area of the interdental papillae. The examination is carried out with the help of an atraumatic periodontal probe, which is inserted into the base of the papilla from the medial side and moved coronally to the tip of the papilla. The same movement is repeated distal to the tooth. After 20-30 seconds, the intensity of bleeding is assessed when the quadrant is fully examined. All available papillae in quadrants are examined. I and III quadrant are examined orally, and II and IV quadrant - vestibularly. The index is a sensitive indicator of the severity of gingival inflammation [19].

The intensity of the bleeding that occurred was measured by scoring on a scale of 0-4 [20]. The evaluation criteria are:

0: no bleeding; 1: a single discrete point of bleeding occurs; 2: bleeding points or a single bleeding line appear; 3: the interdental area is filled with blood after probing; 4: profuse bleeding on probing and blood flow to the marginal sulcus.

The amount of bleeding is calculated using a formula to find the PBI value. PBI is calculated by summing the results and dividing their sum by the number of papillae examined. Mean PBI was categorized according to the ranges [21]: 0—no inflammation, 0- 1.3—mild inflammation, 1.4 – 2.7—moderate inflammation, and 2.8 – 4 severe inflammation.

The collected data were coded, and a statistical analysis was carried out using Microsoft Excel 2010 and Jamovi Version 2.4. Analysis of the data was carried out by frequency distributions and descriptive statistics (chi-square test, Fisher's exact test, Mann–Whitney U test, Welch's test. For the strength and direction of the Correlation Relationship, the Spearman test was used. Level of significance - $p<0.05$.

RESULTS

Of all adolescents, 86 were boys and 76 were girls aged 11-17 with a median age of 14.

Of the 162 children examined, 68 (42%, 95% CI:34.3-50) were of normal body weight, 75 (46.3%, 95% CI:38.4-54.3) were at risk of obesity and 19 (11.7%, 95% CI:7.2-17.7) were overweight (Fig. 1).

In children with normal body weight, the median of the DMFT index is 4 (2-6.3), at risk of obesity - 5 (3-8), and with overweight - 3 (2-8) ($p>0.05$) (Table 1). An increase in the median SiC index was observed in children at risk of obesity 9 (8-10.5) and in those with overweight 10 (8-12), while in children of normal body weight its median was 8 (6.5-11).

Post hoc analysis showed a statistically significant difference between the PBI of normal weight children and those at risk of obesity.

The most affected by complications of the carious process, measured by the PUFA index, are adolescents with overweight – 31.6 % and at risk of obesity – 32 %.

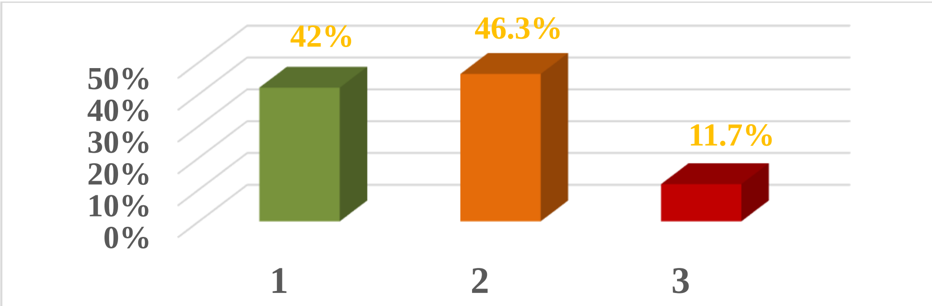
PBI increased significantly in the at-risk group–66%, while in normal and overweight adolescents it was 50% ($p<0.05$).

Table 1. Prevalence of DMFT, SiC, PUFA and PBI in the three studied groups

Index	Group*	N	Mean (SD)	Me (Q1-Q3)	p**
DMFT	1	68	4.5 (3.5)	4 (2-6.3)	0.24
	2	75	5.5 (3.6)	5 (3;8)	
	3	19	5.1 (4.3)	3 (2;8)	
SIC	1	23	8.5 (2.7)	8 (6.5;11)	0.23
	2	24	9.7 (2.5)	9 (8;10.5)	
	3	6	10.5 (3)	10 (8;12)	
PBI	1	68	0.6 (0.4)	0.5 (0.2;0.8)	<0.001
	2	75	1.0 (0.9)	0.66 (0.3;1.5)	
	3	19	0.5 (0.3)	0.5 (0.3;0.7)	
PUFA	1	68	0.2 (0.5)	0 (0;0)	0.018
	2	75	0.4 (0.9)	0 (0;1)	
	3	19	0.4 (0.8)	0 (0;1)	

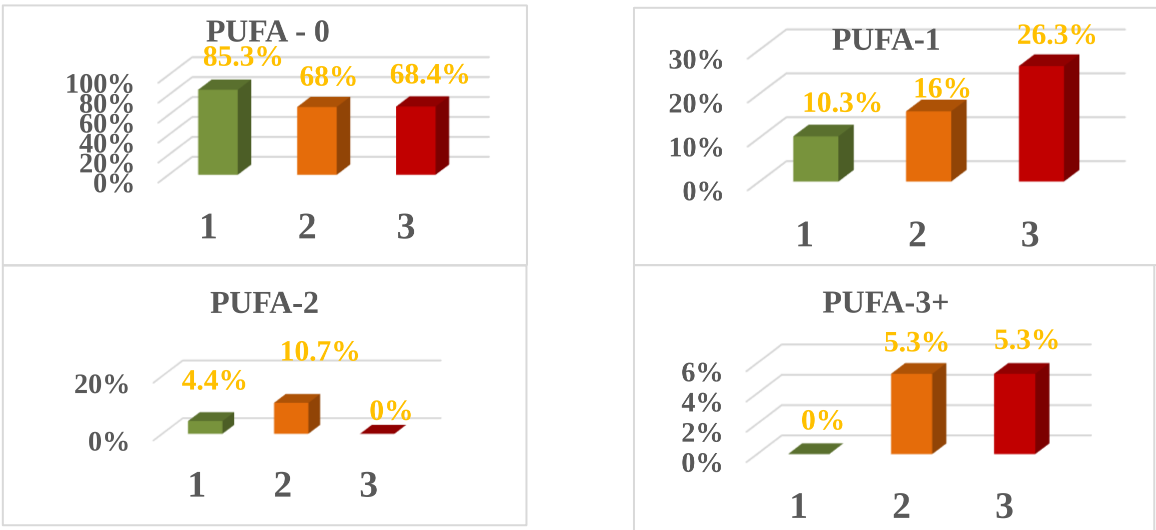
*1-group-adolescents with normal body weight; 2-group-adolescents at risk of obesity; 3-group-overweight adolescents; DMFT=Decay-missing-filled index; SiC=Significant Caries Index; PUFA=An index of clinical consequences of untreated dental caries; PBI=Papillary Bleeding Index; Q1-25th percentile; Q3-75th percentile;

**Welch's test



*BMI-Z Percentile=BMI-for-age; 1-group-adolescents with normal body weight; 2-group-adolescents at risk of obesity; 3-group-overweight adolescents

Fig. 1. Distribution of examined children according to BMI-Z Percentile*



**PUFA-0- without caries complications; PUFA-1- with one complication of caries; PUFA-2-with two complications of caries; PUFA-3+- with three or more caries complications

Fig. 2. Distribution of caries complications in the studied adolescent groups**

No complications of the carious process (PUFA-0) were observed in 85.3% of children with normal body weight, in 68% of children at risk of obesity and in 68.4% of children with overweight (Fig. 2). 26.3% of overweight adolescents had one tooth complicated by caries (PUFA-1), 16% of children at risk of obesity and 10.3% of normal-weight adolescents had PUFA-1. 10.7% of adolescents from the second studied group, 4.4% from the first group and 0% from the third group have two teeth complicated by caries. 5.3% of children at risk of obesity and overweight and 0% of children with normal body weight have three or more teeth complicated by caries (p=0.05).

A moderate positive correlation was also observed between PUFA and PBI in adolescents with normal weight (ρ(rho) = 0.47, p < 0 .001) and at risk of obesity (ρ (rho) = 0.61, p < 0 .001).

DISCUSSION

The prevalence of the carious process is more significant in children with disturbances in the nutritional status compared to children with normal body weight [22]. Willerhausen also reached this conclusion in 2004. In his study, 36% of children of normal weight were caries-free, while 28% at risk of obesity and 30% of overweight children had healthy teeth [23]. In their studies, Willerhausen and colleagues considered excess body weight responsible for the increased level of the carious process, but emphasized that weight is not the only etiological factor [24].

According to another study conducted by Marro *et al*, the median DMFT and prevalence of dental caries in overweight adolescents was significantly higher than in those of normal weight (p < 0.001) [25].

In a 2021 study of Panagiotou *et al*., the increased level of caries, plaque accumulation, and inflammation of the gingiva in adolescents with overweight and obesity is proven [26].

Regarding periodontal status, the study by Marro *et al*., showed significantly higher gingivitis scores (p < 0.001) in overweight adolescents [25]. A negative effect on the health of the gingival tissue in adolescents due to being overweight was also observed according to the study by Franchini *et al.* [27]. The results of our study confirm the theses of other studies finding a positive relationship between parameters of periodontal inflammation (bleeding on probing) and excess body weight [28]. Modeer *et al.* found in their studies that children with disturbances in the nutritional status had increased gingival inflammation [29, 30].

Poor nutritional status among adolescents can be a major reason for their inability to maintain oral health [31].

Therefore, screening for general health, oral health and assessment of nutritional status should be included as an essential part of health services. Our study showed higher values of DMFT, PUFA-2 and PUFA-3, PBI in children at risk of obesity compared to overweight children. Parents do not pay enough attention when their children fall into the "at risk of obesity" column to undertake corrections in their child's BMI and dental treatment accordingly. Studies by Kamran, Shahbong and Dixit found high PUFA index values among adolescents with disturbances in the nutritional status [32-34]. A survey by Chauhan *et al.* concluded that as the BMI index increases, the prevalence of the carious process and its severity among adolescents increases [35].

CONCLUSION

Nutrition plays a significant role in the development and complications of the carious process and gingival inflammation in adolescence. Continuous monitoring of this population is mandatory to improve the general and oral health of adolescents in the future.

REFERENCES

1. T. Lobstein, H. Brinsden. Atlas of Childhood Obesity; World Obesity Federation: London, UK, 2019.
2. WHO Report of the Commission on Ending Childhood Obesity. 2016. Available online: https://www.paho.org/en/documents/report-commission-ending-childhood-obesity (accessed on 15 October 2021).
3. American Academy of Pediatrics, Committee on Nutrition. Prevention of pediatric overweight and obesity, *Pediatrics*, **112**, 424 (2003).
4. L. Stangvaltaite-Mouhat, A.-S. Furberg, S.N. Drachev, T.A. Trovik. *BMC Oral Health*, **21**, 53 (2021).
5. L. Martens, S. De Smet, M.Y.P.M Yusof, S. Rajasekharan, *Eur. Arch. Paediatr. Dent*., **18**, 69 (2017).
6. K. Baxevanos, G. Menexes, A. Lazaridou, T. Coolidge, V. Topitsoglou, S. Kalfas. *Community Dent. Oral Epidemiol.*, **49**, 314 (2021).
7. J.C. Das, *Medical & Clinical Reviews*, **1**(1), 1 (2015).
8. B.V. Gunaseelan, V. Gunasekaran, *Journal of Scientific Dentistry,* **10**(1), 7 (2020).
9. P.D. Marsh, *BMC Oral Health. BioMed Central*, **6**(1), 1 (2006).
10. K. Mineva, M. Georgieva, Influence of water- and fat-soluble vitamins on oral health in adolescence, *Scripta Scientifica Medicinae Dentalis*, 2024. ISSN

2367-7244, http://dx.doi.org/10.14748/ssmd.v10i1.9172
11. L.M, Romito, *Dent. Clin. North Am.,* **47**, 187 (2003).
12. S.L. James, D. Abate, K.H. Abate, S.M. Abay, C. Abbafati, N.Abbasi, *Lancet*, **392** (10159), 1789 (2018).
13. M. Ferrillo, D. Calafiore, L. Lippi, A. Petri, A. Mastroianni, *J. Pers. Med.*, **13**, 1073 (2023). https://doi.org/10.3390/ jpm13071073
14. D.S. Freedman, B. Sherry. *Pediatrics*, **124** (Suppl. 1), S23 (2009).
15. WHO Oral health surveys: Basic Methods. 5th edn. World Health Organization: Geneva, Switzerland, 2013.
16. A.L.S. Castro, M.I.P. Vianna, C.M.C. Mendes, *BMC Oral Health,* **18**, Art. No 122 (2018).
17. D. Bratthal, *Int. Dent. J.,* **50,** 378 (2000).
18. B. Monse, R. Heinrich-Weltzien, H. Benzian, C.Holmgren, W. van Palenstein Helderman, *Community Dent Oral Epidemiol.,* **38**, 77 (2010).
19. H.F. Wolf, T.M. Hassel, Color Atlas of Dental Hygiene, 1st edn., Thieme, 2006.
20. S. Reddy. Essentials of Clinical Periodontology and Periodontics, New Delhi, Jaypee Brothers, 2011.
21. T.C. Andriani, Status Keradangan Gingiva pada Perempuan Paskamenopause (Pemeriksaan Klinis di Wilayah Bekasi), 2008.
22. G. Vallogini, V. Nobili, R. Rongo, S. De Rosa, F. Magliarditi, V. D'Antò, A. Galeotti. *European Journal of Pediatric Dentistry*, **18,** 4 (2017). DOI: 10.23804/ejpd.2017.18.04.02
23. B. Willershausen, *Eur. J. Med. Res.,* **9**, 400 (2004).
24. B. Willerhausen, *Clin. Oral Invest.,* **11,** 195 (2007).
25. F. Marro, S. De Smedt, S. Rajasekharan, L. Martens, P. Bottenberg, W. Jacquet. European Academy of Paediatric Dentistry, 2020. https://doi.org/10.1007/s40368-020-00534-w
26. E. Panagiotou, A. Agouropoulos, G. Vadiakas, P. Pervanidou, G. Chouliaras, C. Kanaka-Gantenbein, *Eur. Arch. Paediatr. Dent*., **22**, 861 (2021). https://doi.org/10.1007/s40368-021-00643-0
27. R. Franchini, A. Petri, M. Migliario, L. Rimondini, *J. Clin. Periodontol.,* **38,** 1021 (2011). doi: 10.1111/j.1600-051X.2011.01770.x.
28. J. Schmidt, M. Vogel, T. Poulain, W. Kiess, C. Hirsch, *Int. J. Environ. Res. Public Health,* **19**, 2905 (2022). https://doi.org/10.3390/ijerph19052905
29. T. Modéer, CC. Blomberg, B. Wondimu, A. Julihn, C. Marcus, *Obesity* **18**, 2367 (2010). doi:10.1038/oby.2010.63
30. T. Modéer, C. Blomberg, B. Wondimu, T. Yucel-Lindberg, C. Marcus, Association between obesity and periodontal disease in adolescents. IADR MIAMI 2009; abstract no. 829.
31. B. Santha, H. Sudheer, V. Saxena, M. Jain, V. Tiwari, *Journal of the College of Physicians and Surgeons Pakistan,* **26** (2), 125 (2016).
32. R. Kamran, W. Farooq, M.R. Faisal, F. Jahangir, *BMC Oral Health*, **17**, 1 (2017).
33. R. Shanbhog, B.S. Godhi, B. Nandlal, S.S. Kumar, V. Raju, S. Rashmi, *J. Int. Oral Health,* **5**(5), 1 (2013).
34. P.L. Dixit, A. Shakya, M. Shrestha, A. Shrestha. BMC Oral Health, **13**, 1 (2013)
35. P. Chauhan, I. Sharma, V. Galhotra, H. Duhan, M. Kaur, *International Healthcare Research Journal,* **2**(11), 283 (2019) DOI: 10.26440/ihrj.v2i11.204

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Physical and techno-functional properties of commercially available plant-based proteins

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In this study, the techno-functional and physical properties of various commercially available protein isolates and hydrolysates were compared to evaluate their potential application as alternative sources of proteins in liquid and solid food formulations. The foaming ability, foam stability, emulsifying ability, creaming stability and color of faba bean protein (FPI), pea protein (PPI), rice protein (RPI), hemp protein (HPI), and soy protein isolates (SPI) were measured. FPI and RPI possess a low coloring potential, because they have the lowest L*, a* and b* of color parameters. Of the investigated commercial protein isolates with the highest foaming ability, SPI (200% in 4% protein solutions) was significantly distinguished, followed by PPI (from 105 to 110% in 4% protein solutions), followed FPI (50%), RPI (35%) and HPI (15%). More than 60-70% of the formed foam was retained in a period of 60 min in 4% solutions. Emulsifying activity index (EAI) was the highest for SPI powder (33 m^2/g) followed by PPI, FRI, and RPI powders (from 22 to 28 m^2/g) and HPI (8 m^2/g). Soy (SPI) and pea (PPI) proteins were found to have the best foam stabilizing and emulsion stabilizing properties, as well as very good creaming and cream stabilizing ability. This information may help food formulators create a new generation of plant-based food and beverage products with improved nutritional properties.

Keywords: faba bean protein, pea protein, soy protein, rice protein, hemp protein, functional properties

INTRODUCTION

In the recent years, there has been an increased interest in the use of plant proteins as technological and functional ingredients in various food products [1]. The reasons for this shift are numerous and include growing consumer awareness of the environmental impact of food production, ethical and health concerns related to animal-based foods, and a general growing interest in eating more plants [1, 2]. Consumers are more willing to accept a new food if it resembles an already familiar food product. For this reason it is difficult to replace nutritional formulas containing animal proteins with satisfactory adequate plant-based alternatives [3]. Therefore, more plant proteins should be investigated for their functional and nutritional properties to facilitate the design of these replacement food products. Traditionally, soy protein has been the most popular plant protein for the production of food analogues, such as meat and milk alternatives, because it is cheap, available in large quantities and has very similar techno-functional properties associated with real meat and dairy products, such as high water holding capacity, ability to form semi-solid textures and ability to stabilize emulsions [4]. Additionally, soy proteins have been used in many other applications, such as baked goods, snacks and functional beverages, as well as plant-based cheeses and eggs [4]. Although soy is an established plant protein that provides a range of beneficial functions, it has some limitations [2]. There is a growing demand for protein and oil rich crops; this may change again in the future. For these reasons, it is important to establish the functional characteristics of other protein-rich plant sources in food matrices.

The functional properties of various proteins have been investigated as potential plant alternatives, including proteins from pea, chickpea, faba bean, and coconut [5]. Researchers have reported that proteins have potential in multiple food applications, including plant-based meat, pasta, and baked goods, due to their beneficial functional properties in these applications, but extensive comparisons of functional properties are lacking [5]. Most of the studies have focused on investigating the functional properties of proteins in laboratory settings, instead of commercially available protein ingredients [6]. For formulators, it is more important to establish the functional properties of commercial protein ingredients to be successfully applied in various innovative nutritional formulas [7]. A recent review highlighted some of the physicochemical and techno-functional properties of various plant proteins, including their solubility profile [1, 2].

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Foaming is one of the important techno-functional properties, which is commonly used in the food industry to produce items such as cream or ice cream [8]. During foaming, proteins are adsorbed at the air/water interface, reducing the surface tension between the air bubbles, thereby stabilizing the foam [9]. For these reasons, this study aimed to compare the physical and functional properties of commercial vegetable protein powders. The differences in foaming ability, foam stability emulsifying ability, creaming stability and color of the commercial ingredients were evaluated. This information can help food manufacturers to create a new generation of plant-based foods and beverages.

EXPERIMENTAL

Materials

The commercial proteins were purchased from the markets and have the following characteristics as origin, amino acid composition, nutritional composition, declared by the manufacturers, presented in Table 1.

METHODS

Physical properties

- *Color* Six g of powdered protein were weighed into a petri dish (60 mm × 15 mm) and the color coordinates were measured using Colorimeter 10 QC 200712. The instrument was calibrated using standard black and white tiles before sample standard black and white tiles before sample analysis. The L*, a*, and b* values of the samples were then determined. To determine the chroma (C) hue (h°), and browning index (BI), equations according to Granato and Masson were used [10].

- *Foaming abilities and foam stability* The foaming properties of the investigated plant-based proteins were studied by a stirring/shaking method. The series of concentrations of protein solutions were prepared (2%, 4%, and 8% w/w). All foam tests were performed in duplicate. Reproducibility of the results was typically expressed as mean ± 10%. The foaming ability (FA, %) was determined as an aliquot of 15 cm^3 sample solution whipped in a graduated 50 cm^3 cylinder by hand for 60 s. The foaming ability was determined by the volume increase (%) immediately after shaking and was calculated according to Cano-Medina *et al.* [11]. The foam stability (FS, %) is characterized by the volume of entrapped air still remaining in the foam after a certain period of time, t > 0. The foam stability was defined as the volume of the foam that remained after 60 min at room temperature (20°C) and was expressed as a percentage of the initial foam volume.

Table 1. Commercially available proteins studied, origin, batch number and manufacturer

Sample protein	Bach number	Producer	Abbreviation
Pea protein isolate, 85% of protein	7PPV023 28.02.2025	OstroVit Technology of nutrition	PPI1
Pea protein isolate PISANE™ C9 pea protein, 84% of protein	2022228876 04.06.2024	KUK-Austria GmbH	PPI2
SUPRO 670 IP Isolated soy protein, 92% of protein	RQ-228152, G010066352	KUK-Austria GmbH	SPI
VITESSENCE Prista P 360 Faba Bean Protein, 60% of protein	ABY2001, 25.01.2024	Ingredion UK Ltd.	FPI
Rice protein hydrolysate, 79% of protein	L:3GBLJ3, 08.08.2025	Gym Beam	OPI
HEMP protein, 49% of protein	L:3GBLJ2, 09.08 2025	Gym Beam	HPI

Table 2. The L*, a*, b* color values of plant protein powders measured using an instrumental colorimeter

	PPI1	PPI2	SPI	FPI	RPI	HPI
L*	87.2±0.1^c	84.3±0.3^c	89.9±0.2^b	93.2±0.2^a	91.7±0.1^b	62.7±0.2^d
a*	6.3±0.1^b	8.2±0.1^a	4.3±0.1^c	2.9±0.1^d	4.3±0.1^c	4.3±0.1^c
b*	21.9±0.2^b	23.1±0.2^b	18.8±0.5^c	19.3±0.3^c	17.6±0.1^d	29.8±0.2^a
C	22.8±0.2^b	24.5±0.2^b	19.3±0.5^c	19.5±0.3^c	18.1±0.1^d	30.1±0.2^a
h°	73.9±0.2^c	70.5±0.3^d	77.2±0.5^b	81.4±0.1^a	76.3±0.2^b	81.8±0.2^a
E	90.1±0.2^c	87.8±0.3^c	91.9±0.1^c	95.17±0.2^a	93.5±0.1^b	69.6±0.1^d
BI	5.2±0.1^b	7.0±0.1^a	3.5±0.1^c	2.3±0.1^d	3.4±0.1^c	5.1±0.1^b

$^{a, b, c, d}$ Means in each row followed by different letters are the Duncan groupings from highest to the lowest showing significant difference ($p<0.05$)

The test was performed as described by Ivanov *et al.* [12]. The foam stability was given by the parameter percentage volumetric foam stability.

- *Emulsifying properties* Emulsifying properties of the sample powders were studied using method as described by Naik *et al.* [13]. Refined sunflower oil (10 cm^3) and 4 % protein solution was homogenized using homogenizer (IKA model T18 ULTRA-TURRAX, Germany) for 1 min at room temperature. Emulsions (100 mm^3) were pipetted out at 0 and 10 min and diluted with 10 cm^3 water. The absorbance of the diluted emulsion was measured at 500 nm using water as blank. Emulsifying activity index (EAI) was expressed as square meter per gram of sample by Naik *et al.* [13], emulsion stability index (ESI) was determined by Naik *et al.* [13].
- *Creaming stability* The creaming index was measured according to a method described previously by Ma *et al.* [2] with slight modifications. Freshly prepared 50% oil-in-water emulsions (20 cm^3) were done by homogenizer (as previously described). The samples of these emulsions (20 cm^3) were then poured into 50 cm^3 sample vials (h = 9.5 cm; d = 2.5 cm) immediately after preparation and stored at 4°C until analysis. The creaming stability of the emulsions was determined over a 5 days period by using a calibre gage to measure the height of the clear serum layer (HS) formed at the bottom of the emulsions after the droplets moved upwards, as well as the total height of the emulsions (HT). The creaming index was then calculated as follows: CI (%) = (HS/HT) × 100.

Statistical analysis

Statistical analysis was performed using MS Excel 2010. The data were presented as mean values ± standard deviation (SD) from three replicates. Statistical analysis was done using ANOVA, with Tukey's range statistically significant at $p< 0.05$. The different letters within each column show significant differences according to Tukey’s test at $p <0.05$.

RESULTS AND DISCUSSION

Color is one of the initial cues that a consumer uses to evaluate the quality of a food product and so it is important to assess the potential impact of different plant proteins on food appearance. Ideally, plant protein powders should be free of residual pigments and other coloring compounds, which allow them to be used in a variety of different food products.

Therefore, instrumental colorimeter values (L*, a*, b*) were measured to quantify differences in the optical properties of the powders (Table 2). Here, high lightness (L*) and low a* and b* values are preferred, which means the powder has a white appearance with only low coloring components.The results are summerized in Table 2. The lightness value (L*) of the FPI was significantly higher than for the other protein isolates and this protein powder also had overall low coloring components. The redness (a*) of all the investigated protein isolates was significantly different from that of the soy protein isolate. Specifically, PPIs were slightly red (higher a*) whereas FPI was slightly less red (lower a*), and similar OPI and HPI. There was also a significant difference in the yellowness (b*) of the protein isolates. The HPI and PPI had the strongest yellow color (b*), followed by FPI, SPI, and then OPI.

These significant differences in color may have important implications when incorporating the proteins into different food products, such as yogurts, beverages, meat analogues, and others. For example, the adjustments are often needed to formulate meat analogues to recreate meat-like colors. Moreover, thesignificant differences in the color of guava juice have been reported after the addition of soy protein [10]. Thus, these results showed that especially FPI and OPI might be suited as a protein-rich ingredient with only low coloring properties.

An important functional property in the formulation of food products of vegetable proteins is to form and stabilize foam. Plant proteins can successfully adsorb at the air-water interface and create a protective film around the air bubbles. The ability of plant proteins to foam can be characterized by measuring the foaming capacity and the stability of the resulting foam over time [9].

This study aimed to investigate the foaming properties (Figure 1) and retention of the stability of the foam formed over a period of 60 minutes (Figure 2). Commercially available protein concentrates were tested at three different concentrations - 2, 4 and 8% w/w sample, as similar concentrations were commonly used in food products. SPI at all tested concentrations shows the highest foaming activity – from 190 to 200% (Figure 1). Furthermore, more than 60% of the foam generated was hold on within 1 h (Figure 2). Pea proteins formed over 100% foam at a concentration of 4% (Figure 1). This foam held on above 50% for 1 h (Figure 2).

The variation in foam structures among different

The variation in foam structures among different samples may have resulted from different conformations of relevant proteins such as large globulin-like proteins or by an altered ratio of foam-stabilizing and -destabilizing factors due to different extraction procedures.

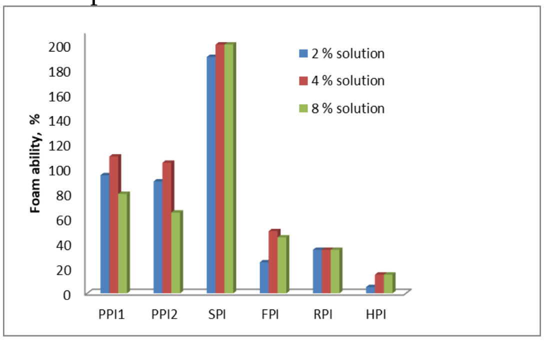

Figure 1. Foam ability (FA, %) of commercial available plant proteins

Furthermore, the variations in the foaming properties could be caused by non-proteinaceous material within the different concentrates, especially HPI and RPI containing 24% and 13% of dietary fibres.Plant protein isolates can act as emulsifiers in various food systems [5]. The type and amino acid composition of proteins determine the degree of adsorption to the surfaces of lipid droplets in the formation of emulsions. Emulsifying agents reduce the interfacial tension between the aqueous and lipid phases and form a protective coating that can prevent droplets from aggregating with each other. In many cases it is important that emulsifiers can form small uniform droplets during homogenization. For this reason, we measured the effect of protein type on its emulsifying ability in model 50% oil-water emulsions and 4% protein sample concentration. This type of information is important for commercial products because it determines how much emulsifier should be added to the product to prevent gravitational phase separation [14].

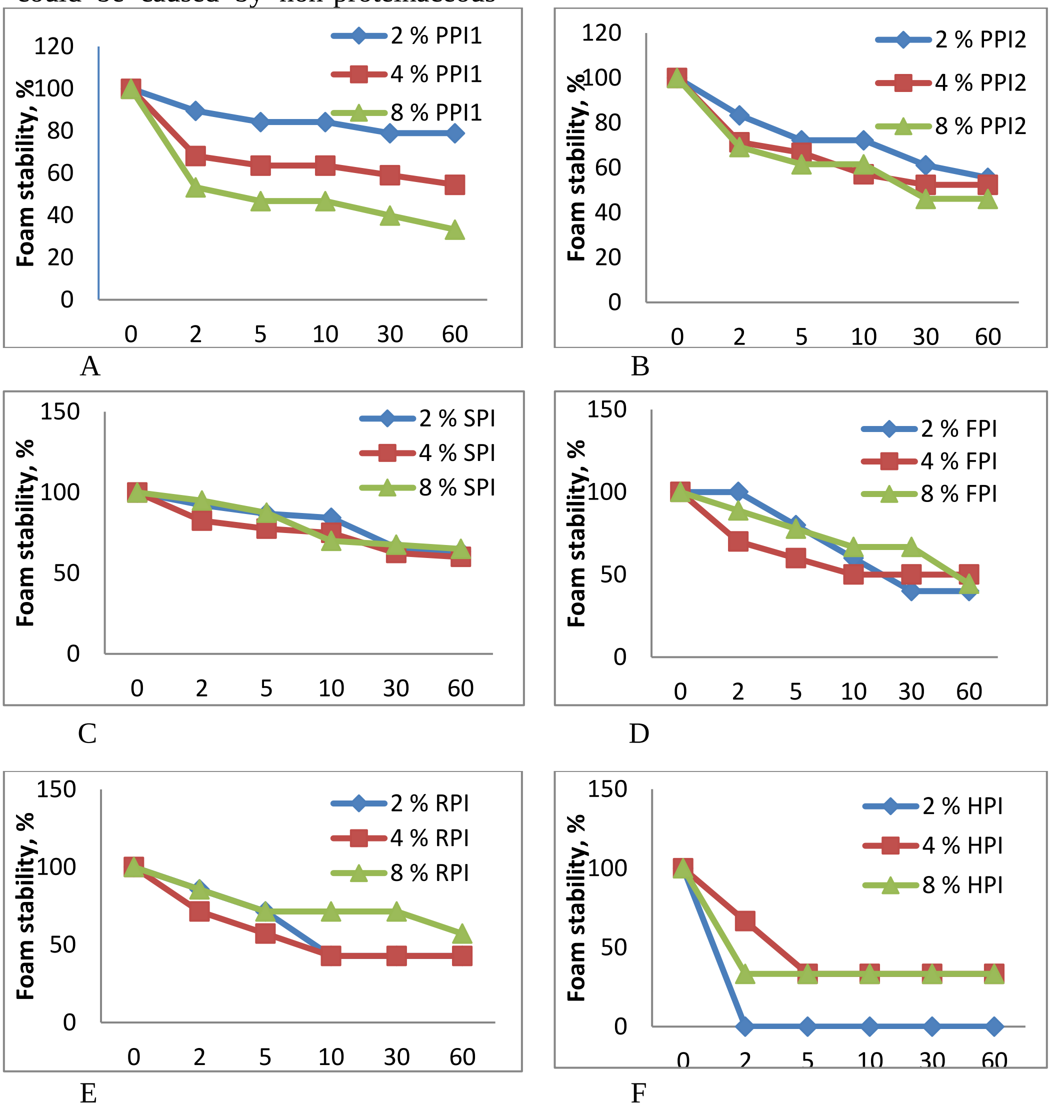

Figure 2. Foam stability (FS, %) of commercial available plant proteins; A – PPI1; B – PPI2; C – SPI; D – FPI; E – RPI; F – HPI.

Emulsifying activity index (EAI) was highest for SPI powder (33 m^2/g) followed by PPI, FRI, and RPI powder (from 22 to 28 m^2/g) and HPI (8 m^2/g). EAI indicated the area of interface (water/oil) stabilized per unit weight of powder. Similar results have been reported by different authors for pea protein, soy protein and faba protein [15-17].

Although, the EAIs were similar in value, the emulsion stability index (ESI) of the obtained emulsions was different. The highest stability of the emulsions was obtained for SPI (326 min), followed by PPI (83 min), and the protein powders HPI, RPI, and FPI (38, 35, 21 min, respectively) were of low stability.

Table 3. Emulsifying activity index (EAI) and emulsion stability index (ESI) of investigated 50% oil-water emulsions and 4% protein sample concentration

Sample	EAI, m^2/g	ESI, min
PPI1	23.58±1.25[c]	83.00±0.55[b]
PPI2	28.46±1.05[b]	83.17±0.61[b]
SPI	33.16±0.95[a]	326.81±3.22[a]
FPI	24.55±1.20[c]	38.04±2.12[c]
RPI	22.66±1.10[c]	35.01±1.32[c]
HPI	8.06±1.10[d]	21.87±1.24[d]

[a, b, c, d] Means in each column followed by different letters are the Duncan groupings from highest to the lowest showing significant difference ($p<0.05$).

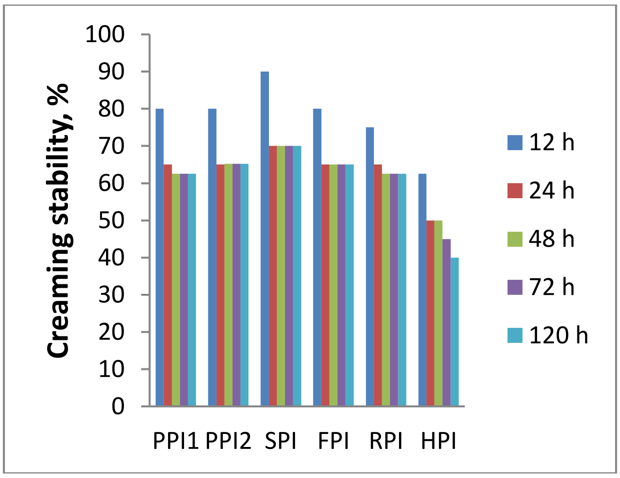

Figure 3. Creaming stability at investigated plant-based commercial available proteins for 120 h period

The rate of creaming in the prepered emulsions increases with increasing droplet size and decreasing aqueous phase viscosity. Therefore, phase separation due to this mechanism is particularly rapid in oil-in-water emulsions where the size of the oil droplet is large and the viscosity of the water phase is low [2]. Therefore, it is important in the development of the food product to ensure that plant-based proteins can maintain good stability of the formed cream to prevent phase separation. For this reason, we measured the cream stability of 50% oil-in-water emulsions containing different commercially available protein types and concentrations (4%) during storage at ambient conditions for 5 days (Fig. 3). The best creaming ability was observed with SPI (90% for fried 12 h), followed by PPI, FPI and RPI (over 65%). The formed emulsion is preserved in 65-70% during the studied period. This defines the investigated commercial proteins as good ingredients in creamy foods. The creaming of HPI emulsions is similar to the rest of the investigated emulsions in the first 12 h, but the stability of the emulsions in the investigated period significantly decreases, the observed phenomenon is not surprising since they have relatively low EIAs (Table. 2). This effect may be due to the fact, that the droplets in these emulsions are weakly flocculated and the resulting emulsions break up.

CONCLUSION

Plant-based proteins are gaining more and more popularity in various protein-enriched food products intended for dietary nutrition. The investigated pea, faba, rice, hemp and soy protein powders have significantly different functional characteristics, with good emulsifying and foaming properties possessed by soy and pea protein. All investigated proteins can form 50 % oil/water emulsions, but hemp protein stands out with the worst characteristics. The colors of the plant protein isolates were significantly different from those of the soy protein isolate, with faba bean protein powder having the lowest coloring potential. In summary, we have shown that the investigated plant proteins have relatively similar functional characteristics to soy proteins, but most of them are not as versatile as soy and therefore the specific type of protein should be selected based on the end application. This information will be very important for the design of the next generation of alternative plant-based food products. In the future, it will be important to also investigate the functionality of actual food products, as well as perform a sensory analysis of their quality characteristics.

REFERENCES

1. K. K. Ma, G. Lutz, A. A. Nolden, D. J. McClements, A J. Kinchla, *Future Foods*, **6**, 100155 (2022)
2. K. K. Ma, M. Greis, J. Lu, A. A. Nolden, D. J. McClements, A. J. Kinchl, *Foods*, **11(4)**, 594 (2022)
3. F. Michel, C. Hartmann, M. Siegrist, *Food Qual. Prefer.*, **87**, 104063 (2021)

4. P. Singh, R. Kumar, S. N. Sabapathy, A. S. Bawa, *Compr. Rev. Food Sci. Food Saf.*, **7(1)**, 14 (2008)
5. A. Singhal, A.C. Karaca, R. Tyler, M. Nickerson, Pulse proteins: from processing to structure-function relationships. in: A. K. Goyal (ed.) Grain Legumes, InTech. India, 2016.
6. T.G. Burger, Y. Zhang, *Trends Food Sci. Technol.*, **86**, 25 (2019)
7. M.C. Añón, D.A. Sorgentini, J.R. Wagner, *J. Agric. Food Chem.*, **49(10)**, 4852 (2001)
8. L. Amagliani, J. Silva, M. Saffon, J. Dombrowski, *Trends Food Sci. Technol.*, **118**, 261 (2021)
9. G. Narsimhan, N. Xiang, *Annu. Rev. Food Sci. Technol.*, **9**, 45 (2018)
10. D. Granato, M. L. Masson, *Food Sci. Technol.*, **30(4)**, 1090 (2010)
11. A. H. Cano-Medina, L. Jiménez-Islas, R. Dendooven, P. Herrera, G. González-Alatorre, E. M. Escamilla-Silva, *Food Res. Int.*, **44(3)**, 684 (2011).
12. I. Ivanov, N. Petkova, P. Denev, *Ind. Technol.*, **4**, 21 (2017)
13. A. Naik, S. N. Raghavendra, K. S. Raghavarao, *Appl. Biochem. Biotechnol.*, **167(5)**, 1290 (2012)
14. V. Yanakieva, A. Parzhanova, D. Dimitrov, I. Vasileva, P. Raeva, S. Ivanova, *Agric. For.*, **69(4)**, 71 (2023)
15. A. C. Karaca, N. Low, M. Nickerson, *Food Res. Int.*, **44(9)**, 2742 (2011)
16. K. Shevkani, N. Singh, A. Kaur, J. Rana, *Food Hydrocoll.*, **43**, 679 (2015)
17. L. P. Gouvêa, R. Caldeira, T. L. Azevedo, M. C. Galdeano, I. Felberg, J. R. Lima, C. G. Mellinger, *Food Hydrocoll.*, **137**, 108351 (2023)

Innovative applications of *Spirulina platensis* L. and phycocyanin in food products: effects on the quality of sponge cakes

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The physicochemical properties of muffins incorporating different concentrations of *Spirulina platensis* L. (1 %, 3 %, and 6 %) were evaluated and compared to control muffins. The addition of *Spirulina platensis* increased the specific gravity and volume of the muffins, with the highest volume observed in muffins containing 1 % *Spirulina*. The pH of the muffins remained neutral across all samples, while the water absorption capacity decreased as the *Spirulina* concentration increased. Ash content showed a marked increase, particularly with higher *Spirulina* concentrations, indicating a boost in the muffins' mineral content. Sensory analysis revealed enhanced color, aroma, and flavor with 6 % *Spirulina*, though muffins with 1 % and 3 % *Spirulina* exhibited higher scores for shape and pore uniformity. Similarly, the incorporation of phycocyanin led to a decrease in relative mass, water absorption, and volume, while improving the color, aroma, and overall sensory attributes compared to the control. Color analysis using the CIELab system showed no significant difference in lightness between the experimental and control samples, though the red component (a*) was significantly reduced in *Spirulina* and phycocyanin muffins. The findings suggest that both *Spirulina platensis* L. and phycocyanin significantly enhance the nutritional and sensory properties of muffins, providing a functional and aesthetically appealing food product.

Keywords: physicochemical properties, cakes, *Spirulina platensis L.*, phycocyanin, foods.

INTRODUCTION

Microalgae are natural sources of important bioactive compounds such as vitamins, essential amino acids, polyunsaturated fatty acids, minerals, carotenoids, enzymes, and fibers. They represent a valuable source of nearly all essential vitamins (A, B1, B2, B6, B12, C, E, niacin-B3, biotin, folic acid, and pantothenic acid-B5) [1]. Food products often incorporate them due to their rich chemical composition and nutritional value. Recognized as "superfoods," they have the potential to become valuable raw materials, improving both the nutritional and functional qualities of food [2, 3]. They are high in protein content with a balanced amino acid profile [4]. Certain types of microalgae have higher protein content compared to traditional animal or plant sources. For example, the protein content in *Spirulina platensis* L. is 65 % - higher than in skimmed dry milk (36 %), soy flour (37 %), chicken (24 %), fish (24 %), beef (22 %), and peanuts (26 %) [5]. Proteins build every cell, tissue, and organ in the human body and are involved in forming structural components such as the musculoskeletal system, enzymes, hormones, hemoglobin, actin, myosin, blood serum, and blood cells [6]. Proteins from microalgae are valuable for supporting human health, especially for vegans or vegetarians, as well as individuals on dietary regimens where maintaining a balance of essential amino acids is challenging.

Some researchers [7] say that *Spirulina platensis* L. makes superoxide dismutase and catalase work better, stops lipid peroxidation and DNA damage, and turns on antioxidant enzymes in cells. It is rich in phycobiliproteins, known for their hepato-protective, anti-inflammatory, immunomodulatory,

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anticancer, and antioxidant properties [1].

The goal of this study is to develop recipe ideas and technical methods for making muffins with *Spirulina platensis* L. and phycocyanin and to investigate their physical, chemical, sensory, and color properties.

MATERIALS AND METHODS

Materials

To produce sponge cake batter, the following materials were used, sourced from major retail chains in Bulgaria: wheat flour “Extra” (type 500), produced by "Sofia MEL" JSC, Sofia; chicken eggs; sugar (crystal, refined); and deionized water.

The used *Spirulina platensis* L. was grown in a bioreactor in Varvara, Bulgaria. Nikolova *et al.* [8] did a full study of its chemical makeup and properties. Phycocyanin was also used, extracted using a green method described in Nikolova *et al.* [9].

The following recipe composition formulations were made: (1) a control batter for muffins; (2) mixtures that replaced some of the wheat flour with 1 %, 3 %, and 6 % *Spirulina platensis* L. flour and phycocyanin, in that order. Table 1 provides the precise recipe compositions of the control and newly developed formulations.

We will use the following designations in the upcoming discussions: Variant 1-1 % *Spirulina platensis* L. (V1), Variant 2-3 % *Spirulina platensis* L. (V2), Variant 3-6 % *Spirulina platensis* L. (V3), Variant 4-1 % phycocyanin (V4), Variant 5-3 % phycocyanin (V5), Variant 6-6 % phycocyanin (V6).

Preparation technology of muffins

Table 1 displays the muffin recipe composition. Egg whites and two-thirds of the sugar were mixed using a mixer for 5 min at a medium speed of 3000 rad/s^{-1}. The remaining sugar was mixed with the egg yolks. The flour and *Spirulina platensis* L. were mixed for 5 min. The powdered *spirulina* was added at 1 %, 3 %, and 6 % levels as a flour substitute in the muffin recipe. Phycocyanin (1 %, 3 %, and 6 %) was included as a substitute for egg yolk. The ingredients were homogenized for 5 min (t = 20 °C). The muffins were baked for 20 min at 180 °C in an oven with upper and lower heating. After cooling to room temperature, they were stored in plastic bags.

Methods for evaluating muffins

pH measurement. A digital pH meter (HANNA-SN0111053Nq, electrode: HL1230B (Czech Republic) with a range of pH 0.00 to 14.00 and an accuracy of 0.01 pH was used.

Researchers analyzed *total moisture content* using standard methods [10-12].

Weight measurement. Samples of muffins with *Spirulina platensis* L. and phycocyanin were cooled for 2 h, then weighed on an electronic scale.

Water absorption capacity. Measured by determining the swelling of biscuits per BDS 15221-81 [13].

Specific gravity for cake batter. Calculated by dividing the cake batter weight in a standard cup by the weight of an equal volume of distilled water [14].

Volume: Measured with a volumeter using small, uniform grains (in cm^3) per AACC method [14].

Ash content. The total ash content of the muffins (%) was determined by incineration in a muffle furnace (Germany) at 700°C until a constant weight was achieved [11]. The results represent the arithmetic mean of three parallel samples.

To assess the structural characteristics of the muffins, photographs of cross-sections were taken, and color parameters were measured.

The color of the muffin crust was evaluated using a high-quality technical colorimeter, PCE-CSM 5, PCE Instruments UK Ltd (Southampton, UK). The measurements were recorded in the CIELab system [15]. The parameters a and b, as well as the lightness (L*), were determined during the color measurement process.

Table 1. Recipe composition of muffins (control) and with *Spirulina platensis* L. (1, 3 and 6 %) and phycocyanin (1.. 3 and 6 %)

	Control	*Spirulina platensis* L.			Phycocyanin		
Ingredients (%)		1 (%)	3 (%)	6 (%)	1 (%)	3 (%)	6 (%)
Egg yolk	13.35	13.35	13.35	13.35	13.21	12.95	12.55
Egg white	29.88	29.88	29.88	29.88	29.88	29.88	29.88
Sugar	25.90	25.90	25.90	25.90	25.90	25.90	25.90
Wheat flour	30.88	30.57	29.95	29.02	30.88	30.88	30.88
Spirulina platensis L.	0.00	0.31	0.93	1.85	0.00	0.00	0.00
Phycocyanin	0.00	0.00	0.00	0.00	0.13	0.40	0.80

Sensory evaluation: quantitative descriptive test for sensory profiling was applied to assess the sensory attributes. Sensory parameters (shape, color, aroma, size, sweetness, residual taste, aftertaste, and crumb softness) of the muffins were evaluated six hours after baking, in accordance with BDS EN ISO [16, 17].

Statistical analysis: All parameters examined were assessed following three repetitions. Data from all studies were analyzed to get the mean and standard deviation (SD). The statistical analyses were conducted using IBM SPSS software at a significance level of $p < 0.01$.

The sensory analysis data of sponge cake blots were analyzed using Kendall's concordance method to evaluate the agreement in the ranks of trained tasters about the tested samples [18].

RESULTS AND DISCUSSION

The physicochemical parameters of muffins without (Control) and with *Spirulina platensis* L. (1, 3 and 6 %) were determined. The relative mass of muffins with 1 %, 3 %, and 6 % *Spirulina platensis* L. showed higher values than the control (0.72 ± 0.03). Increasing the amount of *Spirulina platensis* L. from 3 % to 6 % did not significantly affect the relative mass. The control muffin had a volume of 207.00 ± 6.71 cm³, close to that of muffins with 6 % *Spirulina platensis L.* (208.33 ± 2.89 cm³). Muffins with 1% *Spirulina platensis* L. exhibited the most significant volume (216.67 ± 5.77 cm³), followed by those with 3 % *Spirulina platensis* L. (215.00 ± 5.00 cm³). It can be concluded that incorporating *Spirulina platensis* L. in concentrations of 1 % to 3 % increased both the relative mass of the batter and the volume (cm³) of the muffins (Table 2).

Table 2. Physicochemical parameters of muffins without and with *Spirulina platensis* L. (1, 3 and 6 **%)**

Physical parameters	Amount added of *Spirulina platensis*			
	Control	1 % *Spirulina platensis* L.	3 % *Spirulina platensis* L.	6 % *Spirulina platensis* L .
Specific gravity for cake batter	0.72 ± 0.03^{b}	0.82 ± 0.02^{a}	0.86 ± 0.04^{a}	0.86 ± 0.04^{a}
Volume (cm^3)	207.00 ± 6.71^{b}	216.67 ± 5.77^{a}	215.00 ± 5.00^{a}	208.33 ± 2.89^{b}
Water absorption capacity (%)	332.50 ± 7.72^{a}	262.00 ± 6.24^{b}	261.67 ± 4.047^{b}	267.67 ± 6.51^{b}
pH	8.00 ± 0.06^{a}	7.10 ± 0.02^{b}	7.17 ± 0.10^{b}	7.10 ± 0.03^{b}
Ash content	0.57 ± 0.04^{d}	0.73 ± 0.02^{c}	0.94 ± 0.06^{b}	1.57 ± 0.04^{a}
Moisture (%)	28.49 ± 0.36^{a}	27.82 ± 0.37^{b}	$27.70 \pm 0.54^{b.c}$	27.16 ± 0.17^{c}

Means in a row with a common superscript letter (a–c) differ ($p < 0.05$) as analyzed by Duncan test.

Table 3. Physicochemical parameters of muffins without and with phycocyanin (1, 3, 6 %)

Physical parameters	Amount added of phycocyanin			
	Control	1 % phycocyanin	3 %phycocyanin	6 %phycocyanin
Specific gravity for cake batter	0.72 ± 0.03^{a}	$0.68 \pm 0.03^{a.b}$	0.63 ± 0.03^{b}	0.56 ± 0.03^{c}
Volume (cm^3)	207.00 ± 6.71^{a}	193.33 ± 2.89^{b}	178.33 ± 7.64^{c}	171.67 ± 7.64^{c}
Water absorption capacity (%)	332.50 ± 7.72^{a}	309.00 ± 7.21^{b}	291.00 ± 5.00^{c}	273.33 ± 2.89^{d}
pH	8.00 ± 0.06^{a}	$7.71 \pm 0.20^{a.b}$	7.32 ± 0.26^{b}	7.30 ± 0.28^{b}
Ash content	0.57 ± 0.04^{a}	0.56 ± 0.04^{a}	0.60 ± 0.05^{a}	0.62 ± 0.02^{a}
Moisture (%)	28.49 ± 0.36^{b}	28.67 ± 0.52^{b}	$27.70 \pm 0.54^{b.c}$	29.23 ± 0.10^{a}

Means in a row with a common superscript letter (a–d) differ ($p < 0.05$) as analyzed by Duncan test.

The pH of the new products tended toward alkaline (slightly above 7.00), which is considered balanced according to literature data [19, 20].

The newly developed muffins' water absorption capacity (%) decreased by 21-20 % compared to the control (332.50 ± 7.72) % as the *Spirulina platensis* L. concentration increased from 1 % to 6 %. This indicates that water absorption capacity decreases proportionally with the increasing concentration of *Spirulina platensis* L.

Adding 1% *Spirulina platensis* L. increased the ash content by 1.3 times, while 3 % *Spirulina platensis* L. resulted in a 1.6-fold increase, and 6% *Spirulina platensis* L. led to a 2.8-fold increase compared to the control (0.57 ± 0.04). Thus, adding *Spirulina platensis* L. significantly enhanced the mineral content of the muffins, contributing to their nutritional and biological value. The addition of *Spirulina platensis* L. (1 %, 3 %, and 6 %) did not significantly affect the moisture content (%). The addition of phycocyanin reduced the relative mass of the batter in the newly developed muffins by 23 %. A similar trend, although less pronounced (17 %), was observed for the volume (cm^3).

Phycocyanin addition visibly influenced the products' pH. Increasing the phycocyanin concentration from 3 % to 6 % had no significant effect on this parameter. It is important to note that the newly developed products retained a neutral pH, similar to the control, which is considered balanced according to literature data. When 1 %, 3 %, and 6 % phycocyanin were added, the ability to absorb water dropped by about half at each concentration. Muffins with 6 % phycocyanin showed a 14 % increase in ash content compared to the control (Table 3).

A color analysis of the muffin surface and a sensory evaluation of their consumer qualities were conducted. Figure 1 illustrates the morphological features of the muffins' surface and interior compared to the control.

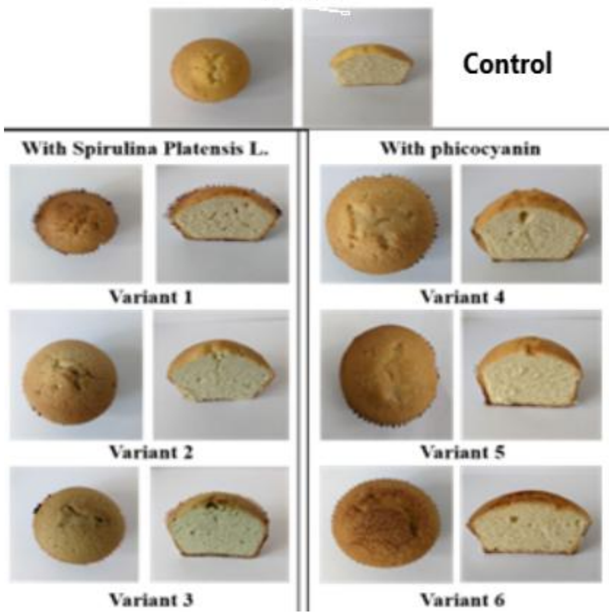

Fig. 1. Photographs of top surfaces and cross-sections of a muffin without *Spirulina platensis* L., with *Spirulina platensis* L, and with phycocyanin.

A quantitative descriptive sensory profiling test (Meilgaard, 1991) was used to determine the sensory attributes (shape, color, aroma, pore size and uniformity, sweet taste, residual taste (aftertaste)) of the newly developed muffins.

To fully describe the product characteristics, specific indicators and quantitative scales were developed to measure their intensity. The intensity results were statistically analyzed to determine significant differences among the evaluated muffins by a selected panel of ten trained sensory assessors, ensuring the accuracy of the evaluation [21].

The crumb softness of the muffins was also determined six hours after baking, Muffin samples were prepared one hour before evaluation. The crumb layers from each type were cut into 1.5 × 1.5 × 1.5 cm samples, which were stored in coded containers covered with aluminum foil. The sensory assessors evaluated the coded samples in random order, presented simultaneously.

As shown in Figure 2, for the shape parameter, muffins with 1 % and 3 % *Spirulina platensis* L. achieved the highest values, equal to those of the control. The intensity of color and aroma perception was highest in muffins with 6 % *Spirulina platensis L.* Uniform pore size was observed in samples with 1 % and 3 % *Spirulina platensis* L.*,* while those with 6 % were similar to the control.

A stronger residual taste than that of the control was noted in all muffins with *Spirulina platensis L.*, with intensity increasing with higher concentrations, though less noticeable at lower levels. The tasters did not detect a difference in the softness of the developed muffins, but the values were lower than those of the control.

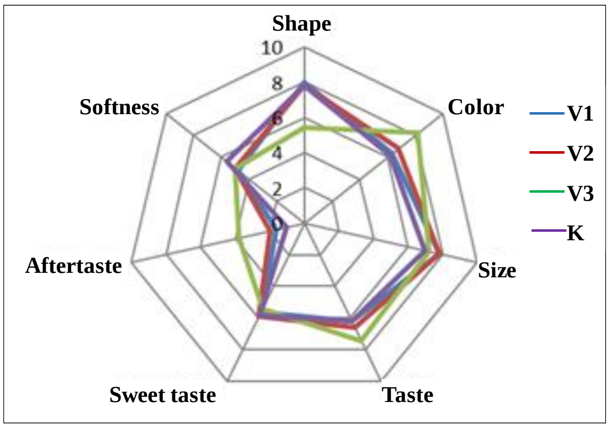

Fig. 2. Sensory profile of muffins without (Control) and with *Spirulina platensis* L. (1, 3 and 6 %): V1 – 1 % *Spirulina platensis* L. V2 – 3 % *Spirulina platensis* L. V3 – 6 % *Spirulina platensis* L.

The 1 %, 3 %, and 6 % phycocyanin muffins showed highly pronounced sensory attributes of shape, color, aroma, sweet taste, and softness (Figure 3). The intensity of perception of color, smell, residual taste, and softness was higher than that of the control.

For all concentrations of phycocyanin (1 % to 6 %), the values for color and aroma were distinctly different from those of the control. A concentration of 1 % phycocyanin did not affect the size of the muffins, whereas muffins with 3 % and 6 % of phycocyanin had similar and higher size values. Adding 3 % and 6 % of phycocyanin resulted in a more pronounced increase in muffin size.

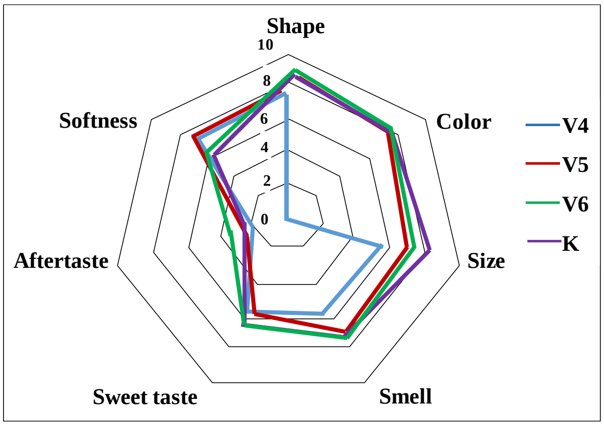

Fig. 3. Sensory profile of muffins without (Control) and with phycocyanin (1, 3 and 6 %): V4 - 1 % phycocyanin V5 - 3 % phycocyanin V6 - 6 % phycocyanin.

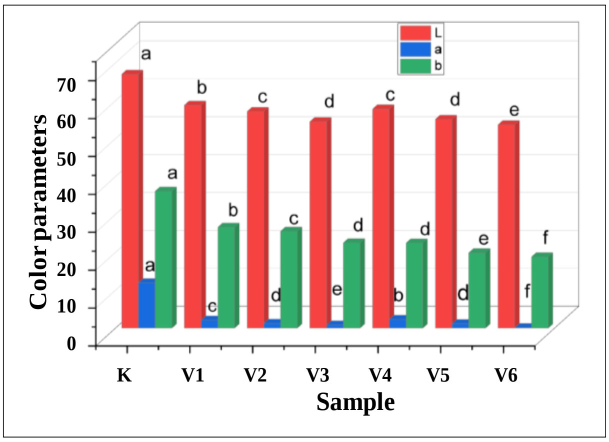

Fig. 4. Color parameters of muffin crusts with phycocyanin (1, 3, and 6 %), *Spirulina platensis* L. (1, 3, and 6 %), and control.

The results for the color parameters in the CIELab colorimetric system for the muffin crusts with phycocyanin and *Spirulina platensis* L. are presented in Figure 4.

It was found that the L* parameter which measures how light the muffin crust is, was the same for samples containing 1 %, 3 %, and 6 % of *Spirulina platensis* L. and 1 %, 3 %, and 6 % of phycocyanin as it was for the control sample. This shows that adding *Spirulina platensis* L. and phycocyanin at the chosen concentrations doesn't have a big effect on how light the crust is of the new products.

The a* values, reflecting the contribution of the red component to the crust's color, showed a significant decrease compared to the control sample. For samples with 1 % concentrations of *Spirulina platensis* L. and phycocyanin, the a* values were nearly five times lower than those of the control. We observed a similar trend for the other two concentrations, particularly in samples containing phycocyanin, where the effect was more pronounced.

The b* values, which represent the yellow component in the color of the crust, showed no significant differences among the muffin samples with *Spirulina platensis* L., added at the selected concentrations, did not alter the color of the crust.

CONCLUSION

✓ *Increased nutritional value*: The incorporation of *Spirulina platensis* L. into muffins significantly enhanced their mineral content, particularly with higher concentrations of *Spirulina* (3 % and 6 %), which resulted in increased ash content. This suggests an improvement in the nutritional value of the muffins, providing additional health benefits.

✓ *Physicochemical properties*: The addition of *Spirulina platensis* L. led to a higher relative mass and increased volume, especially at the 1 % and 3 % concentrations. However, increasing the *Spirulina* content beyond 3 % did not significantly affect these parameters. The pH of the muffins remained balanced, slightly alkaline, regardless of *Spirulina* concentration. Water absorption capacity decreased with increasing *Spirulina* content, indicating its impact on dough consistency.

✓ *Sensory qualities*: Sensory evaluation revealed that muffins with 1 % and 3 % *Spirulina platensis* L. were preferred for their shape, color, and uniformity. Muffins with 6% *Spirulina* exhibited a stronger aroma and flavor, although the aftertaste increased with higher concentrations. The sensory attributes of muffins with *Spirulina* showed an overall enhancement in aroma, color, and taste, with lower concentrations being preferable for texture and shape.

✓ *Effect of phycocyanin*: The addition of phycocyanin reduced the relative mass and volume of muffins, particularly at 3 % and 6 %. Despite this, phycocyanin significantly improved color and aroma, contributing positively to the sensory appeal of the muffins. Phycocyanin also caused a decrease in water absorption and a slight increase in ash content, though its effect was less pronounced than that of *Spirulina*.

✓ *Color analysis*: Both *Spirulina platensis* L. and phycocyanin influenced the color of the muffin crust, with reductions in the red component (a*) being particularly notable in phycocyanin-containing muffins. However, these additions did not significantly affect the lightness (L*) of the crust, indicating minimal impact on the overall appearance.

✓ *Functional ingredient potential:* Both *Spirulina platensis* L. and phycocyanin proved to be valuable functional ingredients in muffin production, enhancing the product's nutritional profile and sensory properties. Their incorporation into bakery products can contribute to the development of healthier, more appealing food options, with positive implications for product innovation in the bakery industry.

REFERENCES

1. E.W. Becker, Microalgae in human and animal nutrition, in: A. Richmond (ed.), Handbook of Microalgal Culture: Applied Phycology and Biotechnology, Second edn., 2013, p. 312.
2. A. Marzec, P. Kramarczuk, H. Kowalska, J. Kowalska, *Applied Sciences*, **13** (13), 632 (2023).
3. A. Barka, C. Blecker, *Biotechnol. Agron. Soc. Environ.,* **20**, 427 (2016).
4. Y. Wang, S.M. Tibbetts, P.J. McGinn, *Foods,* **10,** (12), 3002 (2021).
5. K. Moorhead, B. Capelli, G. R. Cysewski, Spirulina Nature's Superfood, Cyanotech Corporation, 3rd edn., 2012.
6. **B.** Popov, Hygiene, nutrition and occupational diseases, 2018. ISBN 978-954-9977-62-2.
7. P. Grover, A. Bhatnagar, N. Kumari, A.N. Bhatt, D.K. Nishad, J. Purkayastha, *Saudi Journal of Biological Sciences,* **28**, 3, 853 (2021).
8. Kr. Nikolova, G. Gentscheva, D. Gyurova, V. Pavlova, I. Dincheva, M. Velikova, A. Gerasimova, L. Makedonski, G. Gergov, *Life*, **14(2)**, 174 (2024).
9. BDS 3412:1979. Bread and bread products. Regulation for taking samples and testing methods (1979).
10. BDS 5313-1985. Confectionery products. Physical and chemical tests. Methods for determination of dry material, moisture, ash and ash alkalinity (2015). American Association of Cereal Chemists Approved Methods. Stati Uniti: The Committee, (2000) Method 44-15.02 Moisture—Air-Oven Methods.
11. BDS 15221-1981. Confectionery. Method for determination the swelling of biscuits (1981).
12. American Association of Cereal Chemists Approved Methods (10-05. 01 and 10-95): Guidelines for Measurement of Volume by Rapeseed Displacement; American Association of Cereal Chemists: St. Paul, MN, USA, (1998)
13. International Commission on Illumination, Vienna, Austria, **2** (1978).
14. BDS EN ISO 8586:2023. Sensory analysis - Selection and training of sensory assessors (2023).
15. BDS EN ISO 13299:2016. Sensory analysis - Methodology - General guidance for establishing a sensory profile (2016).
16. F.S. Daniel, Wood, John Wiley & Sons, Hoboken, NJ, USA, **20,** 1980.
17. AOAC. Official methods of analysis, 25th end. (2010).
18. American Association of Cereal Chemists Approved Methods. Stati Uniti: The Committee, (2000) Method 02-31.01.
19. M.R. Baeva, I.N. Panchev, V.V. Terzieva, *Food/Nahrung,* **44** (4), 242 (2000).

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Microwave-assisted isolation of inulin from shatavari roots - chemical characterization and functional properties

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The aim of the current study was to isolate and characterize inulin from roots of shatavari (*Asparagus racemosus* Willd.) using microwave-assisted extraction. Physico-chemical properties of the isolated polysaccharide as yield, purity, degree of polymerization, molecular weight, polydispersity index and color characteristics were evaluated. The structural elucidation was performed by FTIR spectroscopy. The functional properties as swelling, water and oil holding capacities, tapped and bulk densities, flowability, cohesiveness, hygroscopicity and wettability were evaluated. The isolated inulin was characterized by low degree of polymerization 7-10 and molecular mass of 1.6 kDa. The structure of inulin-type fructan was confirmed by FT-IR spectroscopy, where 2-ketose and β (2→1) bonds were found. Inulin showed better oil-holding capacity than water-holding one, good swelling properties (6 g/cm^3), high cohesiveness and fair flowability. The conducted research was the first detailed study for the elucidation of structure and functional properties of inulin from shatavari (*Asparagus racemosus* Willd.) roots. The isolated inulin is characterized as low-molecular fructan with good swelling and oil-holding capacities and it can be used in food or pharmaceutical formulations as taste enhancer and potential prebiotic.

Keywords: shatavari *(Asparagus racemosus* Willd.), inulin, FTIR spectroscopy, functional properties

INTRODUCTION

Shatavari *(Asparagus racemosus*), known also as satawar, or shatamuli is a medicinal plant originated from India and the Himalayas that belongs to the Asparagus genus, *Liliaceae* family [1, 2]. The roots of *A. racemosus* are finger-shaped, bitter in taste and they are sweet, oleaginous, indigestible, cooling, and an appetizer [2]. This plant is considered as one of the most popular herbs in Ayurvedic system, the category of 'Ayurvedic Rasayana' which essentially comprises herbs possessing adaptogenic and immuostimulating properties [1]. *A. racemosus* possesses aphrodisiac, immune-modulatory, adaptogenic, anaphylactic, antiulcer, molluscicidal and radioprotective properties [1-4]. The extracts of shatavari roots contain many bioactive compounds, especially carbohydrates, phenolic compounds, steroidal glycosides. Tannins were detected in ethanolic and aqueous extracts, whereas steroids, terpenes, and saponins were found in ethanolic extracts. The major compounds that bring about its biological activities are steroidal saponins, especially Shatavarins I–IX [3]. Shatavarins (I–IV) are antimicrobial compounds that inhibit mold growth and possess insecticidal activity [2]. However, the information about carbohydrate composition is unsufficient. Some recent studies have demonstrated that fructooligoacchrides (FOSs) from the roots of *A. racemosus* showed *in vitro* immunomodulatory activity, higher stimulatory activity of 51.8 ± 1.2% and low cytotoxicity [1]. The aqueous extracts of the roots of *Asparagus racemosus* rich in 2→1 type FOS were evaluated for their efficacy against streptozotocin- and alloxan-induced diabetes leading to sexual dysfunction in rats [5]. Some authors reported that the content of inulin is 12 % with degree of polymerization 7-8 [1, 6, 7]. However, there is no detailed study so far of the characteristics of inulin from shatavari roots and evaluation of its functional properties. Revealing the polysacharide characteristics of shatavari roots will bring about its future application. Therefore, the aim of the current study was to isolate inulin from roots of shatavari (*Asparagus racemosus* Willd.) using microwave-assisted extraction and to characterize its physicochemical and functional properties.

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EXPERIMENTAL

All used solvents and chemicals were of analytical grade.

Plant material

The finely ground powder of shatavari (*Asparagus racemosus*) roots was purchased from on-line shop Zoya (Bulgaria) with LOT 2006030006, with declared origin from India. The roots were used as they were received without any pretreatment.

Carbohydrate analysis in aqueous extracts

The aqueous extracts of shatavari roots (2.5 g) were obtained using an ultrasonic bath (Isolab, Germany) as previously decribed [8]. The total fructan content in the extracts was determined by resorcinol-thiourea spectrophotometric method at 480 nm [9]. The individual sugars and inulin were analyzed by HPLC-RID method [9].

Microwave-assisted isolation of fructan from shatavari roots

The shatavari root sample (50 g) was extracted with 500 ml of boiling distilled water in duplicate using a microwave device Crown (2452 kW), with microwave power of 700 W for 5 min [10]. The hot water extract was filtered through a nylon cloth. The extract was cooled to room temperature and then was precipitated with acetone (1:4 v/v). The obtained sample was separated by centrifugation and then purified by recrystallization from boiling water and precipitated with acetone (1:5 v/v). The obtained polysaccharide was filtered and dried at 40 °C.

Characterization of fructan from shatavari roots

The melting point was measured with a Kofler apparatus. The reducing groups were determined by the PAHBAH method at 410 nm [10], while total fructose content - by resorcinol-thiourea reagent at 480 nm. The purity of inulin was analyzed by HPLC on an Elite Chrome - Hitachi instrument with a Shodex® Sugar SP0810 column (300 mm × 8.0 mm i.d.) at 85°C, flow rate of 1.0 ml/min and injection volume of 20 µl [10]. High-performance size-exclusion chromatography (HPLC-SEC) was used for the determination of number average molecular weight (Mn), and weight average molecular weight (Mw). The analysis of isolated inulin was performed on HPLC ELITE LaChrome (VWR Hitachi, Japan) chromatograph with a column Shodex OH-pack 806 M (ID 8 mm and length 300 mm), (Shodex Co., Tokyo, Japan) at 30°C and a RI detector (VWR Hitachi Chromaster, 5450, Japan) with 0.1M $NaNO_3$ [9]. Polydispersity index (X) of inulin was calculated as the ratio of the two molecular weights (Mw/Mn).

FTIR spectroscopy

The FTIR spectrum of isolated shatavari fructan (2 mg) was collected in KBr tablet on a Nicolet FTIR Avatar Nicolet (Thermo Scientific, USA) spectrometer in the wavelength range of 4000–400 cm^{-1} after 132 scans at a resolution of 2 cm^{-1}.

The color measurement of shatavari inulin was done using a portable colorimeter Model WR-10QC D 65 lighting, following the CIELAB (L*, a*, b*) system [11]. The swelling properties, water- and oil-holding capacities of shatawari inulin were evaluated according to Robertson *et al.* [12]. Other characteristics as true, bulk, and tapped densities, angle of repose, flowability and wettability were determined according to the methods described in [13].

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation.

RESULTS AND DISCUSSION

Before isolation of polysaccharide, a screening of the aqueous extract for carbohydrate composition is needed. The results from the analysis of the aqueous extract from shatavari roots are summarized in Table 1. The carbohydrate profile is presented on Figure 1. The total fructan content in the analyzed sample was 24 g/100 g dw, as 21.22 g/100 g dw was inulin. In the aqueous extract of shatavari roots were found also 1-kestose (2.91 g/100 g), sucrose, glucose and fructose. Therefore, inulin is the dominating polysaccharide in shatavari roots (88 % from the total detected fructans), followed by prebiotic 1-kestose. The inulin level in *Asparagus racemosus* found [14] in some studies varied between 10 − 15 g/100 g fw [14, 15]. *A. falcatus, A. racemosus* and *A. officinalis* have shown inulin content in the roots of 17.74, 11.83 and 15.3% on a fresh weight basis, respectively [16]. According to some authors [16] asparagus roots contain around 28% fructans on a dry weight basis, which was near to our results (24% dw).

Table 1. Fructan and sugars content in shatavari roots, g/100 g dw

Characteristics	Values, g/100 g dw
Total fructans	24.07±2.68
Inulin	21.22±2.30
Nystose	-
1-Kestose	2.91±0.05
Sucrose	2.73±0.25
Glucose	0.70±0.21
Fructose	1.90±0.19

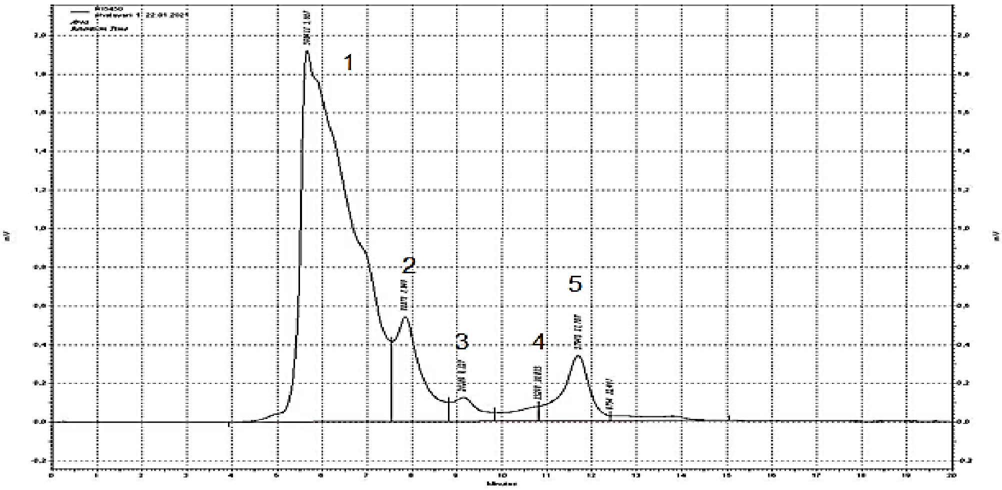

Fig. 1. HPLC-RID chromatogram of an aqueous extract of shatavari roots, where 1. inulin, 2. 1-kestose, 3-sucrose, 4-glucose and 5-fructose.

Isalation and characterization of inulin-type fructan from Asparagus racemosus roots

The yield was low (2.6 % dw), with 19 % fructose content. The purity of the obtained inulin was 58%. The degree of polymerization was evaluated to be 7-10, and the obtained inulin from *Asparagus racemosus* can be evaluated as low-molecular fructan, part of fructooligosacchrides with molecular mass 1655 Da. The polydispersity index is 1.03, which is near to the chicory inulin Frutafit HD with DP 8-12 used as a reference (Table 2). In some early study about phyto-chemical evaluation 26.7% yield of 2 → 1 linked fructo-oligosaccharides (FOS) with degree of polymerization (DP) of nearly 7–8 in *Asparagus racemosus* roots was reported [1]. Sun *et al*. [17] reported a new inulin-type fructan from *Asparagus cochinchinensis* with molecular weight of 2690 Da isolated using distilled water and it could be used as a dietary supplement to improve health. The average degrees of polymerization were 8.8 in raw inulin (*Asparagus racemosus*) according to another study [18]. Therefore, our data about inulin with low molecular mass and low degree of polymerization coincided with most of the reports in literature for *Asparagus racemosus* [1, 18]. HPLC-RID chromatogram of the polysaccharide from shatawari isolated by microwave–assisted extraction is presented in Fig. 2. The purity of the obtained inulin was 58% and from the chromatogram can be seen also presence of sucrose and fructose, which are degradation products of inulin-type fructan during the sample preparation. The low molecular weight of shatavari inulin led to the presence of a small amout of free fructose as degradation product during heating of the sample.

The HPLC-SEC chromatogram (Fig. 3) shows the molecular weight distribution pattern of the isolated polysaccharides from shatavari roots. Four peaks were observed, the first two being due to polysaccharides. The first peak with a retention time of 11.49 min was due to a polysaccharide fraction with Mw 43038 Da and Mn 38765 Da, while the second peak with the retention time of 13.2 min is inulin with Mw 1655 Da and Mn 1605 Da with degree of polymerization 10.

Some authors have described up to 14 different isomers of DP 4-8 from the roots of *A. officinalis,* belonging to both inulin and neo series [19-21]. Inulin with DP 7 and three neo series (F-β(2→6)-G-β(2→1)-F5, F2-β(2→6)-G-β(2→1)-F4 and F4-β(2→6)-G-β(2→1)-F2) were previously reported in the roots of *A. officinalis and A. racemosus*, as the greatest variety of oligomers of DP 3–10 reported in *A. officinalis* [19]. Therefore, the isolated short-chained inulin-type fructan in our study was in accordance of the data reported in the literature for short-chained fructans in Asparagus genus.

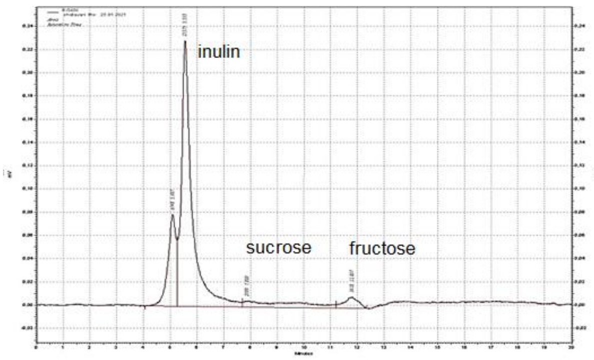

Fig. 2. The purity of inulin from shatavari isolated by microwave–assisted extraction

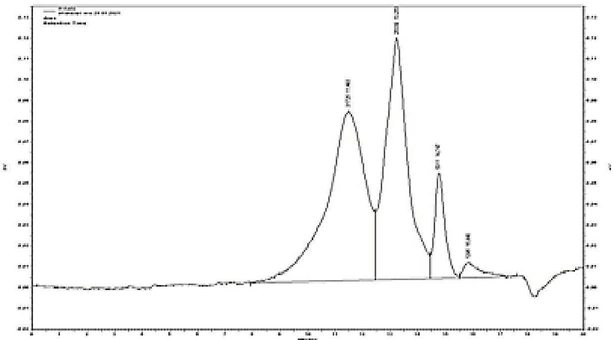

Fig. 3. HPLC-SEC of polysaccharide from shatavari isolated by microwave–assisted extraction

Table 2. Physicochemical characteristics of inulin isolated from shatavari roots by microwave-assisted extraction

Characteristics	Shatavari inulin MW	Inulin Frutafit HD DP 8-12
Yield , %	2.6±0.4	-
Purity, %	58.0±0.6	82.5±0.3
Fructose content, %	19.1±0.6	75.0±0.4
Reducing groups, %	3.0±0.3	5.4±0.5
DP by spectrophotometric method	7	15
DP by HPLC-SEC	10	12
Mw, Da	1655	2185
Mn, Da	1605	2105
Polydispersity index	1.03	1.04
Color characteristics		
L	63.72±1.64	100.00±0.00
a	5.04±0.99	0.08±0.04
b	5.17±1.29	6.79±0.10
C	7.22±1.61	6.79±0.10
h	45.34±1.84	92.29±5.16
Δ E	32.39±0.87	4.81±0.55

The color of shatavari inulin was darker than that of commercial low molecular inulin from chicory (Table 2). Shatawari inulin was pale-white and darker than that obtained from *Asparagus falcatus* [6]. The possible explanation for this dark color could be the presence of polyphenolic compounds, as previously described by some authors [6, 16]. The color of shatavari inulin was clearly different from that of chicory inulin because the asparagus extract was dark brown. The L* (lightness) value decreased and the b* value (yellowness) increased. The similar observation was made for other unpurified fructans from natural sources such as Jerusalem artichoke, *Asparagus falcatus* and *Taraxacum javanicum* plants [14, 16]. The reported by Hamdi *et al.* [16] data for color characteristic for asparagus inulin demonstrated even higher results for darker inulin (L=50.5203; a=3.9032; b=17.3581) in comparison to our study.

Functional properties of shatavari inulin

The degree of polymerization is one of the most important fructan characteristics for further application in food products. Oligofructose with DP ≤ 10 has some degree of sweetness (30–65% compared to sucrose). It is very soluble in water, not texturizing, but is easily and quickly fermented by colonic microbiota with high prebiotic activity [16]. Therefore, the evaluation of functional properties of the obtained short-chained inulin from shartavari will reveal its potential for futher application in food products.

The functional properties of inulin isolated from shatavari roots are summarized in Table 3.

Table 3. Functional properties of inulin isolated from shatavari roots

Functional properties	Shatavari iunulin MW	Inulin Raftiline HD
Swelling, g/cm^3	6.70	0.74
Water-holding capacity, g water/g	2.90	0.40
Oil-holding capacity, g oil/g	3.10	0.40
Bulk density, g/mL	1.01	24
Tapped density, g/mL	0.61	18.5
True density, g/mL	1.17	1.11
Carr’s index	40	23
Hausner ratio	1.67	1.30
Angle of repose,°	5.14	25.64
Flowability	Very poor	Fair
Cohesiveness	Very poor	Fair
Hygroscopicity, %	7.9	3.7
Wettability, s	225	9

Shatavari inulin demonstrated good swelling properties (6.7 g/cm^3), better oil-holding and water-holding capacity in comparison with commercial chicory inulin Frutafit DP 8-12. In our case, inulin from *Asparagus racemosus* demonstrated 3.1 g/g sample oil-holding capacity, which was three times higher in comparision to chicory inulin used as reference with DP 8-12.

However, inulin from other asparagus sources demonstrated higher oil-holding capacity, more than 3 times higher than our results. For example, Hamdi *et al*. [16] reported oil-holding capacity for asparasus inulin about 94 g/100 g, and explained this fact with low purity of inulin (near 58% in fructans), while inulin from *Asparagus falcatus* demonstrated oil-holding capacity of- 101.62 g/100 g inulin [6]. Functional characteristics regarding swelling properties, oil-holding capacity of this polysaccharide (3.1 g oil/g sample), and water-holding capacity (2.90 g water/g sample) were similar to those of echinacea inulin obtained by microwave-assisted extraction [11, 22]. The values for water- and oil-holding capacities of shatawari inulin were close to previously reported data for long-chained chicory (1.59 g water/g sample and 3.4 g oil/g sample, respectively) [23]. However, our results for oil-holding capacity were higher in comparision to commercial chicory inulin and globe artichoke inulin (1.37 and 1.38 g oil/g sample, respectively) and Jerusalem artichoke 1.02 g oil/g sample [24].

Shatavari inulin with oil-holding properties can be used as a functional ingredient to improve taste. In addition, this is the first detailed study that evaluates functional properties of shatavari inulin. Based on Nandi's classification [25] shatavari inulin demostated high cohesiveness and fair flowability, based on Carr`s index (40) and Hausner ratio (1.67) (Table 3). Shatavari inulin is more hygroscopic (7.9 %) than chicory inulin (Table 3), that could be explained with low degree of polymerization DP 7-10 (Table 2). Moreover, the higher sharavari wettability could be explained with presence of other fractions of high-molecular polysaccarides (Fig. 3). The flow properties—the angle of repose, Carr's index, and Hausner ratio are very important for the packaging and transportation of powdered pharmaceutical and food additives. Based on these characteristics, the isolated shatavari inulin can be evaluated as hygroscopic with very poor cohesiveness and flowability and low wettability.

FTIR specroscopy

The structure of shatawari inulin isolated by microwave-assisted water extraction was confirmed by FT-IR spectroscopy (Fig. 4).

In general, the FTIR spectrum of shatavari inulin mainly shows all typical bands for inulin-type fructans [16, 26, 27]. A broad band at 3470 cm^{-1} was due to O–H stretching vibrations assossiated with inter- and intramolecular hydrogen bonds in the inulin structure. Smilar bands between 3600 and 3200 cm^{-1} due to the stretching of OH-groups from carbohydrate were detected earlier in asparagus inulin [6, 16]. The bands at 2967 cm^{-1} were due to C–H asymmetric stretching vibrations. The bands at 2894 cm^{-1} were characteristic for symmetric stretching vibrations of C–H from CH_2. The bands at 1458 cm^{-1} were due to symmetric stretching vibrations C–H in pyranosyl ring and $\beta_{O\text{–}H}$ (OH). The band at 1654 cm^{-1} was assigned to the absorption of water.

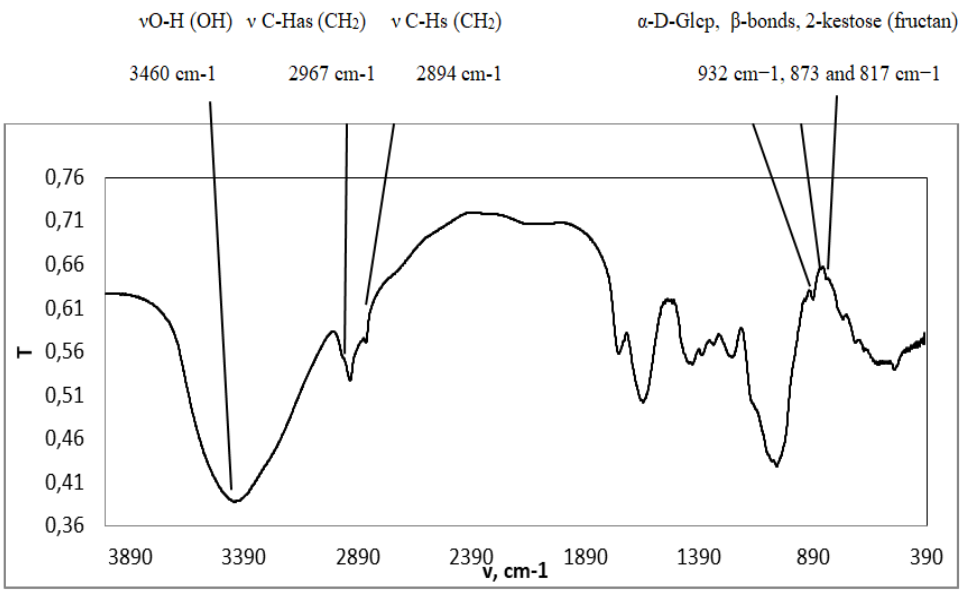

Fig. 4. FT-IR spectrum of shatavari inulin isolated by microwave-assisted extraction with DP 7-10

The bands at 1166 cm^{-1} were assigned to C–O–C ring stretching vibrations from glycoside linkage. The band at 1024 cm^{-1} was assigned to C–O stretching vibrations. The band at 932 cm^{-1} showed the presence of α-D-glucopyranosyl residue in the carbohydrate chain. A band for 2-ketofuranose and β-anomer bendings in C1–H was detected at 873 cm^{-1} and the occurence of a typical band at 817 cm^{-1} confirmed the presence of 2-ketose in pyranosyl or furanosyl ring. Moreover, the band at 783 cm^{-1} was a confirmation for the presence of ketopyranoses, as well as aldopyranoses with symmetrical ring breathing vibrations [29]. A band between 950 and 920 cm^{-1} was diagnostic for fructans, previously detected in other fructan-bearing species such as *A. potatorum* and *A. angustifolia* [27]. In our spectrum a small band at 940 cm^{-1} (Fig. 4) was detected that was due to 2→6 as in levan structure described previously [26]. Moreover, the bands at 932.62 and 822.22 cm^{-1} indicated the presence of fructose (Fru) with a *β*-configuration glycosidic bond in inulin-type fructan from *A. cochinchinensis* [17]. The observed bands were in accordance with previous reports [16, 17, 27]. The bands at fingerprint region in the FT-IR spectrum were reported earlier for inulin-type fructan. The bands at 932, 873, and 817 cm^{-1} were typical for inulin from different plant sources such as burdock, echinacea, dahlia, chicory [10, 11, 15, 22, 23, 28, 29]. In addition, asparagus plant is rich in fructooligosaccharides and contains neokestose type (2→1, 2→6) fructopyranosyl linked units of fructans [1, 16, 20, 21]. In the FTIR spectrum bands at 2938 cm^{-1} typical for lipids, and at 1080 cm^{-1} for carbohydrates were found [26]. However, the band at 1741 cm^{-1} was typical for C=O stretching vibration that could be due to the protein or phenolic compounds bound to the carbohydrate. A similar observation was reported earlier for the FTIR spectrum of asparagus inulin-type fructan in the zone 1700–1500 cm^{-1}, where bands were assigned to proteins (amide I and amide II vibrations) and to aromatic compounds (C-H and C=C-C aromatic bond stretching) [16]. In conclusion, inulin, isolated from shatavari in the present study, contained mainly bands typical for inulin-type fructan with *β* 2→1 bonds.

CONCLUSION

The conducted research elucidated the structure and functional properties of inulin from shatavari (*Asparagus racemosus* Willd.) roots. The obtained shatavari inulin was low-molecular with DP 7-10, good swelling properties and oil-holding capacity, high hygroscopicity, very poor cohesiveness and flowability and low wettability. Inulin molecular-weight properties revealed its potential to be used as taste modifier in food products with potential prebiotic effect, due to its low-molecular weight and degree of polymerization.

REFERENCES

1. M. Thakur, P. Connellan, M. Deseo, C. Morris, W. Praznik, R. Loeppert, V.K. Dixit, Willd, I*nt. J. Biol. Macromol.*, **50**, 77 (2012).
2. A. Ranjan, P. Prabhakar, in: Food Bioactives, S. Ch. Deka (ed.), Apple Academic Press, 2019, p. 191.
3. P.Y. Hayes, A. H. Jahidin, R. Lehmann, K. Penman, W. Kitching, J.J. De Voss, *Phytochem.*, **69,** 796 (2008).
4. R. Govindarajan, M. Vijayakumar, P. Pushpangadan, *J. Ethnopharmacol.*, **99**, 165 (2005).
5. N. Singh, M. Garg, P. Prajapati, P. Singh, R. Chopra, A. Kumari, A. Mittal, *Heliyon*, **9**, e14932 (2023).
6. D. Mudannayake, K. M. S. Wimalasiri, K. Silva, S.Ajlouni, *J. Food Sci*., **80**, C950 (2015).
7. M. Thakur, R. Loeppert, W. Praznik, W, V. Dixit, *J. Compl. Integr. Med.*, **5**, 5 (2008).
8. N. Petkova, I. Ivanov, D. Mihaylova, A. Lante, *Acta Sci. Pol. Hortorum Cultus*., **3**, 125 (2020).
9. N. Petkova, I. Hambarlyiska, Y. Tumbarski, R. Vrancheva, M. Raeva, I. Ivanov, *Biointerface Res. Appl. Chem.*, **12**, 2826 (2022).
10. N. Petkova, G. Sherova, P. Denev, *Int. Food Res. J.*, **25,** 1876 (2018).
11. N. Petkova, A. Petrova, I. Ivanov, I. Hambarlyiska, Y. Tumbarski, I. Dincheva, M. Ognyanov, P. Denev, *ChemEng.*,**7**, 94 (2023).
12. F. Robertson, D. de Monredon, P. Dysseler, F. Guillon, R. Amado, J.-F. Thibault, *LWT*, **33**, 72 (2000).
13. R. Jirayucharoensak, K. Khuenpet, W. Jittanit, S. Sirisansaneeyakul, *Dry. Technol.*, **37**, 1215 (2019).
14. D.C. Mudannayake, K.M.S. Wimalasiri, K.F.S.T. Silva, S. Ajlouni, *Jour. Nat. Sci. Found. of Sri Lanka*, **43**(1), 2015.
15. N. Kaur, A. K. Gupta, *Jour.Biosci.*, **27**(7), 703 (2002).
16. A. Hamdi, I. Viera-Alcaide, R. Guillén-Bejarano, R. Rodríguez-Arcos, M. Muñoz, *Foods*, **12**(1), 81 (2022).
17. Q. Sun, L. Zhu, Y. Li, Y. Cui, S. Jiang, N. Tao, C. Dong, *Carb. Polymer*s, **247**, 116761 (2020).
18. R.S. Singh, R. Dhaliwal, M. Puri, *J. Ind. Microbiol. Biotechnol.*, **34**, 649 (2007).
19. I. Viera-Alcaide, A. Hamdi, R. Guillén-Bejarano, R. Rodríguez-Arcos, J.A. Espejo-Calvo, A. Jiménez-Araujo, *Foods*, **11**, 652 (2022).
20. N. Shiomi, *New Phytol.*, **123**, 263 (1993).
21. N. Shiomi, J. Yamada, M. Izawa, *Agric. Biol. Chem.*, **40**, 567 (1976).
22. N. Petkova, P. Denev, in: Proceedings of the 14th International Conference on Polysaccharides-Glycoscience, Prague, Czech Republic, 7–9 November, 2018.

23. M. A. Bouaziz, R. Rassaoui, S. Besbes, *J. Chem.* **2014**, 758697 (2014).
24. S. Rashid, A. Rakha, M.S. Butt, M. Asghar, *Progr. Nutr*. **20,** 191 (2018).
25. K. Nandi, D.J. Sen, F. Patra, B. Nandy, K. Bera, B. Mahanti, *World J. Pharm. Pharm. Sci*., **9**, 1565 (2020).
26. M. Grube, M. Bekers, D. Upite, E. Kaminska, *Spectroscopy*, **16,** 289 (2002).
27. L. F. Salomé-Abarca, R. E. Márquez-López, M.G. López, *Sci. Reports*, **14**, 4776 (2024).
28. L. Xiaoming, Zh.Ningyang, Zh. Renjie, M. Cui, X. Xu, Y. Qiao, *Front.Nutr.*, **8,** 798450 (2021).
29. R. S. Tipson, NBS Monograph, Washington, DC, 1968, p. 1.

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Hemispheric asymmetry in learning and memory after unilateral infusion of cannabinoid ligands into the CA1 hippocampal area of bulbectomized rats

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The effects of the ligands of cannabinoid receptor HU 210 (CB-receptor agonist) and SR 141716A (CB1 receptor antagonist) infused unilaterally (left or right) into the CA1 hippocampal area on learning and memory (two-way active avoidance task) in rats with a depression model (olfactory bulbectomy, OBX) were examined. We found that HU 210 (5 µg/ 0.1 µl) microinjected unilaterally into the CA1 area of rats impaired learning and memory processes, the effect being more pronounced on the right side. SR 141716A (3 µg/0.1 µl) infused into the left or right CA1 area had no significant effect on (sham-operated) rat performance in the active avoidance test. The most important findings are that the administration of HU 210 in the right CA1 area reversed the OBX-induced learning and memory deficits, while microinjection into the left CA1 area ameliorated the acquisition and retention deficits in OBX rats. SR 141716A further impaired the rat's performance and its suppressive effect was present only on the right side. The results demonstrate that CB receptors are involved in learning and memory in OBX rats. Our study provides for the first time, data about differential and asymmetrical effects of cannabinoid ligands on learning and memory in OBX rats, suggesting a lateralization of CB receptors in the brain hemispheres. The asymmetrical distribution of CB receptors in the left and right CA1 hippocampal area could contribute to the asymmetry in the cognitive effects of the CB receptor ligands.

Keywords: CB ligands, hemispheric asymmetry, learning and memory, depression

INTRODUCTION

Hemispheric asymmetry, characterized by differences between left and right hemispheres, exists on structural, functional, and molecular levels [1, 2]. Evidence supports the presence of receptor or structural asymmetry in pathways mediating behavioral responses, contributing to lateralized behavioral patterns. Neuropharmacological studies have explored asymmetry in behavioral responses (such as exploratory behavior, motor activity, anxiety, pain perception, and learning and memory) across various brain structures, and have established asymmetry in neurotransmitter systems such as dopamine, gamma-aminobutyric acid, serotonin neurotransmitter system, as well as asymmetry involving neuropeptides - cholecystokinin, somatostatin, vasoactive intestinal peptide, and angiotensin [3-5].

Studies have found significant alterations in brain regions in depressive disorder patients, such as the hippocampus, amygdala, and prefrontal and frontal cortex; similar changes have been observed in OBX rats [6-8]. The hippocampus, a limbic system structure, plays a crucial role in depression. Patients with major depression often exhibit reduced hippocampal volume; structural and functional changes in the hippocampus have been linked to cognitive impairment. In addition to its role in memory formation, recent studies have associated the hippocampus with emotional and cognitive functions.

Bilateral olfactory bulbectomy (OBX) is a valid animal model for depression and it is also used for modeling Alzheimer's disease. Following OBX, rats display behavioral changes including hyperlocomotion, memory disturbances, reduced sexual activity, aggressive behavior, and hyperemotionality [8]. The correlation between structural, functional, and biochemical alterations in the brains of both depressed patients and OBX rats supports the use of OBX as a model to study the mechanisms underlying the pathophysiology of depression.

Accumulating evidence has underscored the significance of the endocannabinoid system in modulating behavioral, neurochemical, neuroendocrine, and neuroimmune responses to various stimuli [9]. Cannabinoids have been shown to alleviate hippocampal neuronal loss following cerebral ischemia and acute brain injury, providing neuroprotection in neurodegenerative conditions

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including Alzheimer's, Parkinson's disease, multiple sclerosis, Huntington's chorea, Tourette's syndrome, epilepsy, stroke, and various dementias [10]. Several mechanisms have been proposed to elucidate the neuroprotective effects of cannabinoids, including neuromodulation and calcium homeostasis modulation, protein kinase activation, and antioxidant activity, among others [10, 11]. Findings suggest the involvement of endogenous cannabinoids in regulating cognitive processes. Moreover, the endocannabinoid system has been implicated in the pathogenesis of depressive disorders which often manifest with cognitive impairment, including memory deficits [12, 13]. The two major cannabinoid receptors (CB1 and CB2) belong to the family of G-protein coupled receptors [10]. CB1 receptors are densely distributed in brain areas related to motor control, cognition, emotional responses, and motivated behavior [10, 12].

The relationship between hemispheric brain asymmetry and depression has been actively investigated, and abnormal asymmetry has also been reported to be associated with poor cognitive function. Previously, we demonstrated impaired learning and memory in OBX rats after intracerebroventricular (i.c.v) application of CB receptor ligands (HU 210 and SR 141716A) using both active and passive avoidance tests [14, 15]. HU 210 improved the memory deficits of OBX rats, while SR 141716A impaired their performance. Based on the data mentioned above, this study aimed to investigate the hemispheric asymmetry in learning and memory in response to unilateral infusion of the CB receptor agonist (HU 210) and the CB1 receptor antagonist (SR 141716A) into the CA1 hippocampal area in rats with a depression model, using a two-way active avoidance test.

EXPERIMENTAL

Animals

The experiments were carried out on 42 male Wistar rats weighing 200–220 g at the time of surgery. Behavioral experiments were performed between 10:00 am and 1:00 pm. The experiments were conducted following the guidelines of the local Ethics Committee, which fully comply with EC Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering and reduce the number of animals used in the study.

Surgical procedures. Experimental model of depression (OBX). Stereotaxic implantation and drug microinjection into the CA1 hippocampal area

Bilateral olfactory bulbectomy (OBX) was carried out according to the method described by Kelly *et al.* [16]. Seven days after bilateral olfactory bulbectomy cannulae were surgically implanted bilaterally into the CA1 area as described previously [5]. After cannulae implantation, the rats were allowed 7 days to recover. HU-210 (Tocris) and SR 141716A (Sanofi) were dissolved *ex tempore* in saline and 0.1 µl of HU-210 (5 µg) or 0.1 µl of SR 141716A (3 µg) (pH 7.4) were microinjected over 1 min into the left or right CA1 hippocampal area. The injection cannula was left in place for an additional 30 sec. Five min after the infusion the rats were tested in the shuttle box. Sham-operated rats were subjected to a procedure identical to that described for the OBX rats.

Learning and memory test

- *Two-way active avoidance (shuttle-box).* The behavioral tests were performed 14 days after OBX-surgery, i.e. when a depressive-like state was developed. The animals were tested in a two-way active avoidance test (shuttle box) described previously [5]. HU-210, SR 141716A, or saline were infused 5 min before the tests.
- *Verification*. Before the scarification, the rats were microinjected into the CA1 hippocampal area with 1µl 2% Fastgreen dye. Bulbectomy and the injection sites were verified *post-mortem*. Animals with misplaced or asymmetrical cannulas were excluded from the statistical analysis.
- *Statistical analysis.* Separate two-factor analysis of variance (ANOVA) was used to analyze the data obtained for number of avoidances for learning (1st and 2nd training day) and memory test (24 h after the 2nd training day) between subject factors: substances (three levels: HU-210, SR 141716A, and saline) and side of injection (two levels: left and right). ANOVA data were further analyzed by *SNK* test for *post hoc* group comparisons where appropriate.

RESULTS

Effects of HU 210 and SR 141716A microinjected unilaterally into CA1 hippocampal area of sham-operated rats

After the unilateral infusion of HU 210 and SR 141716A in the CA1 area, the two-factor ANOVA showed a significant effect for the factor "drug" for the 1st training day ($F_{1,35}$ = 9.2129; P ≤ 0.0001) and the 2nd training day ($F_{2,35}$ = 28.3762; P ≤ 0.0001) and a nonsignificant effect for the factor "side" for the two training days ($F_{2,35}$ = 2.9629; P =NS) and ($F_{1,35}$ = 0.9278; P =NS). For the memory test, there was a significant effect for the factors "drug" ($F_{2,35}$ = 4.7107; P ≤ 0.05) and "side" ($F_{1,35}$ = 62.11957; P ≤ 0.0001) and for the interaction "drug" X " side" ($F_{2,\ 35}$ = 4.9053, P ≤ 0.02).

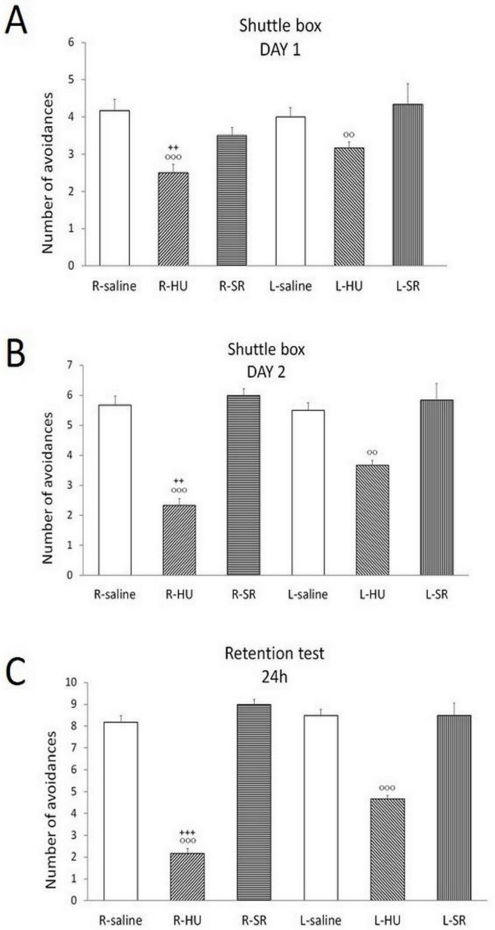

Fig. 1 (A, B, C). Effects of HU-210 (5 µg/0.1 µl) or SR 141716A (3 µg/0.1 µl) microinjected into the left (L) or right (R) CA1 hippocampal area of sham-operated rats on the number of avoidances (N Av). A) 1st training day; B) 2nd training day; C) retention test. ^{oo}P ≤ 0:01; ^{ooo}P ≤ 0:001 – comparisons of the N Av, following drug injections *vs*. resp. saline injections into the CA1 area. $^{++}$P ≤ 0.01; $^{+++}$P ≤ 0:001 – comparisons of the N Av, following injections into the right CA1 *vs*. left CA1 area. n = 6. Means (± S.E.M.) are presented.

HU 210 injected unilaterally into the CA1 area impaired the learning and memory processes of sham-operated rats. The *post hoc* SNK test showed that HU 210 microinjected into the right (R) or left (L) CA1 area reduced the number of avoidances compared to the corresponding saline-treated controls: on the 1st training day (R - P ≤ 0.001; L - P ≤ 0.01), 2nd training day (R - P ≤ 0.001; L - P ≤ 0.004) and the retention test (R - P ≤ 0.001; L -P ≤ 0.001) (Fig. 1. A, B, C).

Comparing the unilateral HU 210 infusions (left *vs.* right), the right-side infusions resulted in significantly fewer avoidances than left-side ones on the 1st and 2nd days (P ≤ 0.01; P ≤ 0.003) and at the memory test (P ≤ 0.001) (Fig. 1, A, B, C). *Post-hoc* test showed that SR141716A infused into the right or left CA1 area did not change the number of avoidances on the 1st and 2nd (P =NS) training day and at retention test (P =NS) compared to the respective controls treated with saline: on the 1st (P =NS) and 2nd (P =NS) training day and at retention test (P =NS) (Fig. 1. A, B, C).

Comparing the effects of SR141716A in the right *vs*. left CA1 hippocampal areas, no significant differences were found – on 1st (P = NS), 2nd (P = NS) days, and retention test (P = NS) (Fig. 1. A, B, C).

Effects of HU 210 and SR 141716A microinjected unilaterally into CA1 hippocampal area of olfactory bulbectomized (OBX) rats.

Two-factor ANOVA after unilateral microinjections of HU210 or SR141716A into the CA1 hippocampal area of OBX-rats showed, for the 1st training day, a significant effect for the factors "drug" ($F_{2,35}$ = 77.8666; P ≤ 0.0001), "side" ($F_{1,35}$ = 19.2666; P ≤ 0.0001) and significant interactions between " side" X "drug" ($F_{2,35}$ = 19.4666; P ≤ 0.0001); for the 2nd training day - a significant effect for the factors "drug" ($F_{2,35}$ = 15.9210; P ≤ 0.0001), "side" ($F_{1,35}$ = 58.9144; P ≤ 0.0001) and significant interactions "side" X "drug" " ($F_{2,35}$ = 20.5592; P ≤ 0.0001) and at the retention test – "drug" ($F_{2,35}$ = 146.6562; P ≤ 0.0001), "side" ($F_{1,35}$ = 40.500; P ≤ 0.0001) and "side" X "drug" ($F_{2,35}$ = 86.71875; P ≤ 0.0001).

HU 210 infused into the right CA1 area increased the number of avoidances in OBX-rats compared to both right-side saline-microinjected OBX rats and to sham-operated rats on 1st (resp. P ≤ 0.001; P ≤ 0.02) and 2nd (resp. P ≤ 0.0001; P ≤ 0.04) training day and at the memory test (resp. P ≤ 0.001; P ≤ 0.04). HU-210 microinjected into the left CA1 area increased the number of avoidances in OBX-rats compared to the left-side saline-microinjected OBX-rats on 1st (P ≤ 0.002) and 2nd (P ≤ 0.04) training day and at the retention test (P ≤ 0.002). Compared with sham-operated rats the number was lower on 1st (P ≤ 0.002) and 2nd (P ≤ 0.001) training day and at the retention test ($P \leq 0.001$). HU 210 infused into the right-CA1 significantly increased the number of avoidances on the 1st ($P \leq 0.001$), 2nd ($P \leq 0.001$) training day and the retention test ($P \leq 0.0001$) as compared to the left-CA1 injections (Fig. 2. A, B, C).

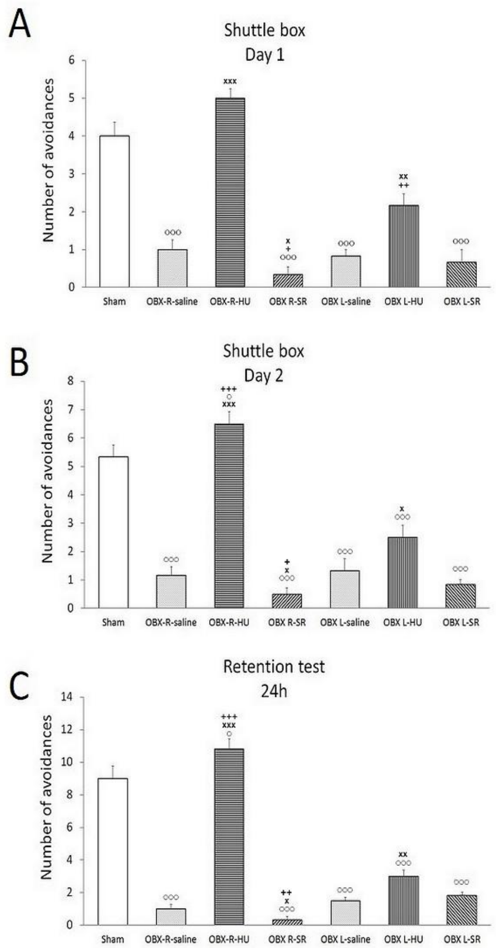

Fig. 2. (A, B, C). Effects of HU-210 (5 µg/0.1 µl) or SR 141716A (3 µg/0.1 µl) microinjected into the left (L) or right (R) CA1 hippocampal area of OBX rats on the number of avoidances (N Av). A) 1st training day; B) 2nd training day; C) retention test. $^{\circ}P \leq 0.05$; $^{\circ\circ}P \leq 0.01$;$^{\circ\circ\circ}P \leq 0.001$ – comparisons of the N AV, following drug injections *vs.* sham-operated rats; $^{x}P \leq 0{:}05$; $^{xx}P \leq 0{:}05$; $^{xxx}P \leq 0{:}001$ - comparisons of the N AV, following drug injections to OBX rats *vs.* OBX-saline injections into the respective CA1 area $^{+++}P \leq 0{:}001$ – comparisons of the N AV, following injections into the right CA1 area *vs.* left CA1 area in OBX rats. n = 6. Means (± S.E.M.) are presented.

SR141716A microinjected into the right CA1 area of OBX-rats decreased the number of avoidances compared to the corresponding OBX R-saline controls on 1st ($P \leq 0.03$), 2nd ($P \leq 0.05$) training day and at the memory test ($P \leq 0.03$); in the left CA1 area, it did not significantly change the number of avoidances compared to the OBX L-saline controls on 1st (P =NS), 2nd (P =NS) training day and at the retention test (P =NS). SR141716A-treated OBX rats showed fewer avoidances in all tests than the sham-operated rats ($P \leq 0.001$) (Fig.2. A, B, C). Comparing the effects of SR141716A into the right *vs.* left CA1 area of OBX-rats, it was found that SR141716A infused into the right side significantly decreased the number of avoidances on the 1st training day ($P \leq 0.05$) and at the retention test ($P \leq 0.05$) as compared to the left side (Fig.2. A, B, C)

DISCUSSION

Olfactory bulbectomy is an established animal model of depression. It triggers behavioral, immune, endocrine, neurochemical, etc. alterations in rodents, which resemble the ones observed in patients with depressive disorders. Behavioral disturbances, including memory deficit, are present two weeks after the surgical intervention [16]. The behavioral deficits occurring after OBX are associated with neurodegenerative changes and disrupted communication between different brain regions. The reduced spine density in the hippocampal CA1, CA3, and dentate gyrus most likely contributes to the impaired cognitive processes [17].

In a previous work we reported that CB receptor agonist HU 210, administered *i.c.v.*, deteriorates the learning and memory of rats and ameliorates the memory deficits of OBX rats. In contrast, SR 141716A had opposite effects on the rat avoidance performance [18]. This study extends our understanding of the learning and memory effects of cannabinoid ligands (HU 210 and SR 141716A) applied to the brain in rats with a model of depression (OBX). We have chosen the hippocampus because it is known to be a brain structure involved in learning and memory processes. Additionally, it is characterized by a high concentration of cannabinoid receptors. CB_1 receptors are expressed at a high density in the CA1, CA3 regions and dentate gyrus [19]. CB_2 receptors have low physiological expression, but it is enhanced in certain pathological conditions (e.g., neurodegenerative diseases, brain injuries, etc.).

In the present study, HU 210 microinjected unilaterally, into the left or right CA1 hippocampal area deteriorated learning and memory of sham-operated rats, tested in an active avoidance paradigm and the effect was more pronounced on the right side. The unilateral infusions of SR 141716A did not significantly affect the rat's performance. Our data contradict the findings of Wise et al. [20], who reported memory impairment following intrahippocampal administration of the CB1 receptor antagonist SR 141716A.

Previously we have demonstrated that OBX impairs rat performance in both active and passive avoidance tasks [14]. We have also observed asymmetric learning and memory impairing effects after angiotensin 1 (AT1) receptor stimulation in the left amygdala of OBX rats, while inhibition of AT1 receptors in the left amygdala improved these processes and prevented memory deficits in OBX model of depression [21]. In the present study, the acute administration of HU 210 in the right CA1 area reversed the OBX-induced learning and memory deficits in the two-way active avoidance task, while microinjection into the left CA1 area only attenuated the acquisition and retention deficits. SR 141716A further impaired the rat's performance and its suppressive effect was present only on the right side. We hypothesize that the learning and memory-improving effects of HU 210 could be due to a modulatory influence on the altered activity of some neurotransmitter systems (GABA, 5-HT, Ach, etc.) which have been reported following olfactory bulbectomy. Following OBX, a significant reduction of long-term potentiation (LTP) in the hippocampal CA1 region is also observed [22]. It is well known that CB_1R agonists impair cognition and prevent LTP of synaptic transmission. A recent work demonstrated that CB_1R activation may affect CA1 LTP in an opposing way, depending on the strength of LTP induction and magnitude. It was shown that both a CB_1R inverse agonist, AM251, and a CB_1R antagonist Rimonabant lead to a facilitation of weakly induced LTP but to an inhibition of strongly induced LTP [23]. The opposing effects of cannabinoid ligands in sham-operated and OVX rats may also be related to the dual influence of endocannabinoids on LTP.

This study is the first to provide information on a positive effect of HU 210 on the acquisition and retention of active avoidance learning when injected into the CA1 hippocampal area in OBX rats. An important finding is that the cannabinoid ligands exerted an asymmetric effect on learning and memory. The differential responses to the unilateral (left or right) drug administration in OBX rats suggest an asymmetric distribution of CB1 receptors in the left and right CA1 areas. There is evidence for functional asymmetries in LTP, showing that exposure to a novel environment early in life enhances LTP selectively in the right CA1 area of adult rats, whereas the left hippocampus shows no differences [24]. Considering the above data, we can assume, albeit speculatively, that the CB receptor stimulation is most likely involved in the modulation of excitatory and inhibitory synaptic transmission, and hence in hippocampal LTP.

Our data on the effects of cannabinoid ligands infused unilaterally into the CA1 area of OBX rats are original and suggest that CB1 receptors are probably involved in the development of the depressive state.

CONCLUSION

The present study is the first to provide information on the modulatory effect of cannabinoid receptor ligands microinjected into the hippocampal CA1 area on the learning and memory of olfactory bulbectomized rats. It is demonstrated that stimulation of hippocampal CB receptors in a rat depression model (olfactory bulbectomy) improves rat performance in the active avoidance task. We observed also asymmetrical effects of cannabinoid ligands on learning and memory, suggesting a differential distribution of CB receptors in the brain hemispheres. The asymmetrical distribution of CB receptors in the left and right CA1 hippocampal area could contribute to the asymmetry in the cognitive effects of the CB receptor ligands.

REFERENCES

1. A. Mundorf, J. Peterburs, S. Ocklenburg, *Front. Syst. Neurosci.*, **15,** (2021).
2. X.Z. Kong, M.C. Postema, T. Guadalupe , C. de Kovel , P. Boedhoe, M. Hoogman, S.R. Mathias, D. van Rooij ,D. Schijven , D.C. Glahn, S.E. Medland, N. Jahanshad, S.I. Thomopoulos, J.A. Turner, J. Buitelaar , T.G. van Erp, B. Franke, S.E. Fisher, O.A. van den Heuvel, L. Schmaal, P.M. Thompson, C. Francks, *Hum. Brain Mapp.*, **43,** 167 (2022).
3. R. Tashev, *C.R. Acad. Bulg. Sci*, **63**, 3, 427 (2010).
4. I. Belcheva, R. Tashev, S. Belcheva, *Laterality*, **12,** 475 (2007).
5. M. Ivanova, S. Belcheva, I. Belcheva, N. Negrev, R. Tashev, *Psychopharmacology*, **221,** 561 (2012).
6. J.D. Bremner, M. Vythilingam, E. Vermetten, A. Nazeer, J. Adil, J. Sarfraz Khan, L.H. Staib, D.S. Charney, *Biological Psychiatry*, **51,** 273 (2002).
7. W.C. Drevets, J.L. Price, M.L. Furey, *Brain Struct. Funct.* **213,** 93 (2008).
8. K.H. Franks, M.I. Chuah, A.E. King, J.C. Vickers, *Front.Aging.Neurosci.*, **7,** 234 (2015).
9. D.P. Finn, M.P. Viveros, E.M. Marco, *J Psychopharmacol.*, **26**, 3 (2012).
10. K. Mackie, in: Handbook of Experimental Pharmacology, R.G. Pertwee (ed.), vol. 168, Springer, Berlin, Heidelberg, 2005, p.299.
11. A.J. Drysdale, B. Platt, *Curr. Med. Chem.*,**10**, 2719 (2003).
12. F. Rodríguez de Fonseca, I. Del Arco, F.J. Bermudez-Silva, A. Bilbao, A. Cippitelli, M. Navarro, *Alcohol and Alcoholism*, **40,** 2 (2005).
13. D. Marazziti, G. Consoli, M. Picchetti, M. Carlini, L. Faravelli, *Eur. J. Pharmacol.*, **626**, 83 (2010 .

14. R. Tashev, M. Ivanova, T. Toromanov, M. Marinov, S. Belcheva, I. Belcheva, *C. R. Acad. Bulg. Sci.* **63**, 617 (2010).
15. M. Marinov, M. Ivanova, S. Belcheva, I. Belcheva, R. Tashev, *C. R. Acad. Bulg. Sci.* **66,** 1331 (2013).
16. J.P. Kelly, A. Wrynn, B.E. Leonard, *Pharmacol. Ther.* **74,** 299 (1997).
17. S.D. Norrholm, C.C. Ouimet, *Synapse,* **42**, 151 (2001).
18. M. Velikova, D. Doncheva, R. Tashev, *Acta Neurobiol. Exp.*, **80**, 286 (2020).
19. K. Tsou, S. Brown, M.C. Sañudo-Peña, K. Mackie, J.M. Walker, *Neuroscience,* **83**, 393 (1998).
20. L. E. Wise, A. J. Thorpe, A. H. Lichtman, *Neuropsychopharm.*, **34**, 2072 (2009).
21. R. Tashev, H. Nocheva, M. Velikova, *C. R. Acad .Bulg. Sci.*, **77,** 48 (2024).
22. V. Vorobyov, N. Medvinskaya, A. Deev A. F. Sengpiel, N. Bobkova, N. S. Lunin, *Int. J. Neurosci.*, **130**, 1225 (2020).
23. A. Silva-Cruz, M. Carlström, J. A. Ribeiro, A. M. Sebastião, *Front. Pharmacol.,* **8,** 921 (2017).
24. A. C. Tang in: The Asymmetrical Brain. K. Hugdahl, R.J. Davidson (eds.) Cambridge, Mass: MIT Press, 2003, p. xiii, 796.

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Investigation of antibacterial activity of combinations of *Thymus vulgaris* essential oil and some conventional antibiotics

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The essential oils of *T. vulgaris* have antiseptic, antiviral, and antimicrobial properties. Many studies have reported that the synergistic combination of essential oils (EOs), and conventional antimicrobial agents is an effective solution for developing preparations with increased antimicrobial properties and low toxicity to the organism. The present study aims to evaluate the antimicrobial activity of thyme essential oil and standard antibiotics combination against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The essential oil was analyzed using gas chromatography coupled with mass spectrometry (GC-MS). The analysis of the essential oil of *Thymus vulgaris* revealed the main components to be thymol (45.74%), *p*-cymene (21.05%), ɣ-terpinene (12.37%). For *S. aureus*, we combined thyme essential oil (TEO) with penicillin (P), cefoxitin (FOX), erythromycin (ERY), gentamicin (GEN), and tetracycline (TET), and we combined *Escherichia coli* and *Klebsiella pneumoniae,* respectively, with ampicillin, ceftriaxone, meropenem, ciprofloxacin and gentamicin, as suggested by EUCAST, 2024.

TEO showed *in vitro* antibacterial activity against all tested bacterial strains and increased the antimicrobial activity of the tested antibiotics. For all tested combinations of TEO with antibiotics against *S. aureus* (penicillin, cefoxitin, erythromycin, gentamicin and tetracycline), an increase in the zone of inhibition was observed in a large part of the strains - respectively P-TEO in 3/6 strains, FOX-TEO in 5/ 6 strains, ERY-TEO - 3/6 strains, GEN-TEO - 5/6 and TET-TEO – 4/6 strains of *S. aureus.* TEO also increased the antimicrobial action of ampicillin, ceftriaxone, meropenem, ciprofloxacin and gentamycin against most of the *Escherichia coli* and *Klebsiella pneumoniae* strains. The joint application of TEO and classic antibiotics can be one of the ways to overcome the development of bacterial resistance and side effects of the antibiotic preparations application.

Keywords: antimicrobial effects, antibiotics, thymus essential oil, combination

INTRODUCTION

In 2019, the World Health Organization (WHO) defined antimicrobial resistance (AMR) as one of the 10 global health threats facing humanity [1]. Antimicrobial resistance is observed after introducing almost every microbial agent in the clinical practice. For instance, in the mid-1940s, only a few years after the introduction of penicillin, penicillin-resistant *Staphylococcus aureus* spread in the hospital environment and within a decade became a serious public problem [2, 3]. The incidence of antibiotic resistance to ciprofloxacin, commonly used to treat UTIs, was 43.1% for *Escherichia coli* and 36.4% for *Klebsiella pneumoniae* in the countries reporting data to the Global Antimicrobial Resistance Surveillance System in 2019 [4]. In some countries, nearly half of the patients infected with *Klebsiella pneumoniae* carry strains that are resistant to carbapenems, significantly limiting the options for effective treatment [5]. The AMR problem requires using new, safe, and effective substances [6]. In recent years, special attention has been paid to products of natural origin due to their low toxicity, biodegradability, and broad spectrum of action

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compared to synthetic essential oils (EOs) due to their strong and broad-spectrum action against microorganisms, their relative safety for humans, and undetermined (so far) microbial resistance to their components [6, 7], therefore they may help in limiting antibiotic resistance. The action mechanism of EOs has been reported in detail in the scientific literature including bacterial cell wall degradation, enzyme and membrane protein destruction, and cell contents leakage after disruption of the cytoplasmic membrane. Therefore, positive effects against bacteria may occur with joint implementation of antibiotics and EOs [8, 9].

Many studies have reported that the synergistic combination of EOs with conventional antimicrobials is an effective solution for developing preparations with increased antimicrobial properties and low toxicity to the microorganisms [7].

One of them is thyme essential oil (TEO), obtained from the plant *Thymus vulgaris* L. belonging to the genus *Thymus* (thyme) which consists of about 215 species of herbaceous perennials and shrubs. *Thymus vulgaris* L. is a low-growing herbaceous plant native to Southern Europe and for centuries widely used as herbal tea, spice, perfume, and insecticide [10]. The EOs of *T. vulgaris* have antiseptic, antiviral, and antimicrobial properties, and according to some studies [11], they have a better antimicrobial effect on Gram-positive strains. Other studies reported a higher antimicrobial activity of TEO against *Escherichia coli* compared to the tested Gram-positive strains [12, 13].

In our previous study [14], the antimicrobial activity of two (for external and internal use) commercial essential oils from *Thymus vulgaris* against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 was demonstrated.

The aim of the current study was to evaluate the use of TEO to improve the effectiveness of the standard antibiotics against *S. aureus*, *E. coli*, and *K. pneumoniae*.

MATERIALS AND METHODS

The study was conducted at the Medical College – Varna, Bulgaria. We used the statistical software IBM SPSS, version 25, to present and analyze the data.

Thyme essential oil

The thyme essential oil used in this study was purchased from the commercial market and is 100% pure with certified organic ingredients.

Gas chromatography-mass spectrometry

For the purposes of the analysis, the equipment consisting of gas chromatograph 7890A, flame ionization detector, and mass spectrometer 5975C (Agilent Technologies) was used. A Stabilwax column (Restek) with the following parameters was employed: length 30 m, diameter 0.25 mm, and film thickness 0.25 µm. The temperature program was as follows: initial temperature 65°C, ramped to 170°C at 1.5°C/min; total analysis time 70 min; injector and detector temperatures 250°C, FID temperature: 250°C; carrier gas hydrogen with a flow rate of 0.8 ml/min; carrier gas helium with a flow rate of 0.8 ml/min; mass spectrometer scan range m/z = 40-450; sample injection volume 1.0 µl in split mode 100:1. The compounds were identified by comparing the retention times and Kovats retention indices (RI) with those of standard substances and mass spectral data from the NIST'08 (National Institute of Standards and Technology, USA) and Adams Libraries.

Tested bacterial strains

The antimicrobial activity of combinations between antibiotics and TEO was studied against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, presented in Table 1. Nine of the tested strains were clinical isolates and three were reference strains of microorganisms. The bacterial strains were stored at -18°C in glycerol medium in a microorganism bank at the Varna Medical College.

Table 1. The strains of *S. aureus*, *E. coli*, and *K. pneumoniae* tested in a study of the antimicrobial activity of conventional antibiotics with TEO.

Bacterial species	Clinical material/Source
Staphylococcus aureus	throat swab
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus ATCC 29213	reference strain
Esherichia coli	urine
Esherichia coli	urine
Esherichia coli ATCC25922	reference strain
Klebsiella pneumoniae	sputum
Klebsiella pneumoniae	wound
Klebsiella pneumoniae ATCC13883	reference strain

For the purposes of the research in this work, they were cultured initially in brain heart infusion broth for 24 h and then on blood agar for another 24 h. The referent strains were provided from MicroSwap, Ridacom, Bulgaria.

Antimicrobial activity evaluation test

The antimicrobial activity of antibiotic combinations with TEO was investigated by the Kirby-Bauer disk diffusion test [15]. The action of each of the studied antimicrobial agents was tested separately. The antibiotics selection against each of the test strains, presented in Table 1, was made according to the EUCAST, 2024 instructions *(NCIPD, 2024)* [16].

Standardized bacterial culture was prepared (0.5 MF) and spread on the surface of Mueller-Hinton agar media (*HiMedia*, provided by Ridacom, Bulgaria). We previously prepared a stock solution of 2.5% (v/v) TEO in DMSO (1% (v/v)). After that, in each culture medium with the culture of the corresponding microbe, three disks (d=6 mm) in the following combinations and concentrations of active agents were added:

- Disc soaked in 100 µl of 2.5% (v/v) TEO;
- Factory-prepared antibiotic disk in concentration according to EUCAST, 2024 standards;
- A second identical antibiotic disk additionally soaked in 100 µl of 2.5% (v/v) TEO.

Controls were set for the solvent used to prepare suspensions for the active substances – DMSO. All samples were triplicated, after which, we incubated the nutrient media for 24 h at 37°C. The obtained inhibition zones were measured in mm and compared to each other; for all antibiotics, results were determined for their effect on the respective microbe: S – sensitive or R – resistant, following the EUCAST, 2024 instructions [16].

A list of antibiotics included in the study is presented in Table 2.

Table 2. List of antibiotics studied

Antibiotics (ATBs)	Abbreviation	Chemical Family
penicillin G	P	penicillins
cefoxitin	FOX	2nd generation cephalosporins
erythromycin	E	macrolides
gentamycin	GEN	aminoglycosides
tetracyclin	TET	tetracyclines
ampicillin	A	aminopenicillins
ceftriaxone	CRO	3rd generation cephalosporin
meropenem	MEM	carbapenems
ciprofloxacin	CIP	quinolones

Statistical analysis

The statistical software IBM SPSS Statistics 25 was used to present and analyze the data. A Paired samples t-test was applied to investigate whether there was a statistically significant difference between the mean antimicrobial effects of antibiotics and of the combination of TEO with antibiotics. We used the level of significance for the 2-tailed test $\alpha = 0{,}05$.

RESULTS AND DISCUSSION

The chemical components of TEO, which were identified, are reported in Table 3. 24 components were identified in the Thyme EO representing 99.7% of the total, the major components being thymol (45.74%), p-cymene (21.05%), ɣ-terpinene (12.37%).

Table 3. Chemical composition of *Thymus vulgaris* essential oil.

No. Compound	Retention time (min)	% of total ion current
	Thyme EO	
1. α-Thujene	9.11	1.34
2. α-Pinene	9.32	1.26
3. Camphene	9.84	1.11
4. β-Pinene	10.75	0.15
5. 1-Octen-3-ol	10.97	0.20
6. β-Myrcene	11.23	0.88
7. α-Terpinene	12.06	1.13
8. p-Cymene	*12.31*	*21.05*
9. Limonene	12.47	0.50
10. ɣ-Terpinene	*13.41*	*12.37*
11. Sabinene hydrate	13.79	0.57
12. β-Linalool	14.74	2.03
13. Camphor	16.12	1.01
14. Borneol	16.90	1.79
15. Terpinen-4-ol	17.16	0.87
16. Thymol methyl ether	18.58	0.14
17. Carvacrol, methyl ether	18.84	1.33
18. Bornyl acetate	20.20	0.66
19.Thymol	*20.37*	*45.74*
20. Carvacrol	20.75	2.01
21.β-Caryophyllene	23.77	2.79
22.ɣ-Cadinene	26.07	0.12
23. δ-Cadinene	26.19	0.18
24. Caryophyllene oxide	27.73	0.48

Table 4. Antimicrobial activity of combining TEO with conventional antibiotics against *S. aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* tested by disk-diffusion method (diameter of inhibition zones - mm).

Bacterial strain	TEO	P	P+ TEO	FOX	FOX+ TEO	ERY	ERY+ TEO	GEN	GEN+ TEO	TET	TET+ TEO
EUCAST, 2024		S≥18		S≥27		S≥21		S≥18		S≥22	
		R<18		R<27		R<21		R<18		R<22	
S. aureus 1	15	16	18	16	18	28	26	28	34	14	18
S. aureus 2	16	14	16	18	19	28	28	26	30	12	18
S. aureus 3	17	26	26	28	29	24	26	20	22	22	22
S. aureus 4	19	28	28	29	32	24	29	20	22	26	28
S. aureus 5	19	30	30	30	30	26	26	26	30	26	28
S. aureus ATCC 29213	20	19	20	28	30	35	37	44	44	35	35
Bacterial strain		A	A+ TEO	CRO	CRO+ TEO	MEM	MEM+ TEO	CIP	CIP+ TEO	GEN	GEN+ TEO
EUCAST, 2024		S≥14		S≥25		S≥22		S≥25		S≥17	
		R<14		R<22		R<16		R<22		R<17	
E. coli 1	21	12	17	20	26	32	35	16	16	12	24
E. coli 2	20	12	26	20	29	34	37	14	22	18	26
E. coli ATCC 25922	23	12	32	34	39	38	42	45	49	25	34
K. pneumoniae 1	18	0	15	24	24	35	37	19	23	22	22
K. pneumoniae 2	17	0	16	22	27	35	38	29	30	22	23
K. pneumoniae ATCC 13883	20	14	21	35	35	38	42	45	45	25	25

TEO – Thyme essential oil; P - penicillin G; FOX – cefoxitin; ERY – erythromycin; GEN – gentamycin; TET – tetracyclin; A – ampicillin; CRO – ceftriaxone; MEM – meropenem; CIP – ciprofloxacin.

Table 4 shows the effect of TEO on the antibacterial action of antibiotics for clinical use. The influence of TEO on the antibacterial power of the antibiotics used on the tested strains of microorganisms can be determined by comparing the diameters of the inhibition zones provoked from the antimicrobial agents alone and in combination with essential oils. In our study, the reference strain *Escherichia coli ATCC25922* was the most sensitive to TEO, showing the largest (23 mm) zone of inhibition. TEO demonstrated stronger inhibitory effects against *E. coli* and weaker against *S. aureus*.

In the study, we investigated the antimicrobial effects of combining TEO with antibiotics using agar media techniques. The greatest increase in the zone of growth inhibition in *S. aureus* was observed with the combined use of TET-TEO (up to 6 mm) *(p=0.058, t=-2.445)* and ERY-TEO (5 mm) *(p=0.287, t=-1.190, CI [-3.687-1.353])*. In the remaining three combinations of TEO with penicillin (P), cefoxitin (FOX) and gentamycin (GEN), an increase in the zone of inhibition was also observed in a large proportion of strains - respectively P-TEO in 3/6 strains *(p=0.093, t=-2.076)*, FOX-TEO in 5/6 strains *(p=0.017, t=-3.503)* and GEN-TEO - 5/6 strains *(p=0,017, t=-3.503)*.

The zone of growth inhibition of tested *E. coli* increased between 3 and 20 mm in almost all studied combinations. Two strains of *E. coli* were resistant to ceftriaxone, but when it was combined with TEO, the growth inhibition zones increased to 26-29 mm. Studies were conducted on the synergistic interactions of active components of thyme essential oil, but we did not find any other studies conducted that investigated the interaction of TEO with different antibiotic groups.

In *Klebsiella pneumoniae*, administration of a combination of ampicillin and TEO resulted in a decrease in the zone of inhibition of neat oil, possibly due to an antagonistic effect of ampicillin on TEO *(p=0.003, t=-5.508)*. In the combinations of TEO with ceftriaxone *(p=0.035, t=-2.879)* and gentamycin *(p=0.069, t=-2.315)*, enhancement of antimicrobial activities was reported in 1/3 strain each, ciprofloxacine with TEO - 2/3 *(p=0.077, t=-2.221)* and TEO combined with meropenem *(p=0.001, t=-10,304)* - 3/3 strains of *K. pneumoniae*.

DISCUSSION

Thyme essential oil is characterized by a high concentration of thymol (45.74%), p-cymene (21.05%), and γ-terpinene (12.37%) (Table 3). Through chemical composition analysis, we have confirmed that the tested essential oil belongs to the thymol chemotype. Similar findings have been reported by other authors, who also classified their thyme essential oil samples as belonging to the thymol chemotype. Galovičová *et al*. observed a thymol content comparable to that found in our sample, at 48%. However, the levels of γ-terpinene and p-cymene were approximately half of those observed in our analysis [17]. In a similar study, Al-Asmari *et al.* identified the essential oil of *T. vulgaris* as thymol-type, with furan (12.19%) and p-cymene (2.78%) as additional key components [18]. The Food and Drug Administration (FDA) recognizes thymol as safe for consumption, which further supports its potential use as a natural antimicrobial agent [19].

Thymol exhibits a broad range of pharmacological properties including antioxidant, anti-inflammatory, antimicrobial, analgesic, and antitumor activities. Its antimicrobial action is particularly notable, as it effectively inhibits the growth of various pathogens, including *Salmonella spp.* and *Staphylococcus aureus*, which are capable of forming biofilms that enhance their adhesion to surfaces. This ability poses a significant risk to food safety, underscoring thymol's potential as an effective agent against a variety of infectious pathogens, in line with its FDA-approved safety for consumption [20, 21].

There are numerous studies on the antimicrobial activity of TEO against Gram-positive and Gram-negative microorganisms [11-13]. The main compounds in TEO are thymol and carvacrol, which are active against *Staphylococci*, *Streptococci*, and *Salmonella sp*. TEO has higher activity against *E. coli* compared to Gram-positive microorganisms, according to some studies [12, 13, 22, 23]. In our study, the reference strain *Escherichia coli* ATCC25922 was also the most sensitive to TEO, showing the largest (23 mm) zone of inhibition and smaller against *S. aureus*.

Investigating potential synergic interactions of TEO with antibiotics against *E. coli*, the zone of growth inhibition increased between 3 and 20 mm in almost all tested combinations. Two strains of *E. coli* were resistant to ceftriaxone, but when it was combined with TEO, the growth inhibition zones increased to 26-29 mm. In the study of Moussaoui and Alaoui [24], a synergistic effect was observed with a combination of *Thymus willdenowii* Boiss and gentamycin against *E. coli*. Another study by Amassmoud *et al*. demonstrated a strong synergistic effect between EO of *T. broussonnetii* and *T. pallidus* and ciprofloxacin against *S. aureus, S. enterica,* and *E. coli* [7].

Combining TEO with antibiotics, the greatest increase in the zone of growth inhibition in *S. aureus* was observed with the combined use of TET-TEO and ERY-TEO. In a study conducted by Pancu *et al*., the positive influence of TEO in combination with tetracycline against *S. aureus* was also found [25], and Rosato *et al.* proved the synergistic effects between gentamycin and TEO against *S. aureus* [9]. In the remaining three combinations of TEO with P, FOX and GEN, an increase in the zone of inhibition was also observed in most of the samples.

For *Klebsiella pneumoniae*, administration of a combination of ampicillin and TEO resulted in a decrease in the antimicrobial activity. This effect is probably due to the innate resistance of *Klebsiella pneumoniae* to ampicillin. In the combinations of TEO with FOX, GEN and CIP, antimicrobial activity enhancement was reported in 6 from 9 samples. Studies were conducted that investigate synergistic interactions of individual active components of thyme essential oil and antibiotics against *K. pneumoniae*, but we did not find any other studies that investigated the interaction of TEO with different antibiotic groups.

CONCLUSION

The present study highlights the potential of TEO to enhance the antimicrobial effects of conventional antibiotics. The combination of TEO with antibiotics exhibited a synergistic effect, indicating that this approach may contribute to mitigating the emerging issue of bacterial resistance and reducing the adverse effects associated with antibiotic therapies. Further investigation is warranted to fully elucidate the therapeutic potential of TEO in combination therapies and its broader clinical applicability.

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REFERENCES

1. WHO, Ten threats to global health in 2019. Available on-line on: https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019
2. H. F. Chambers, F. R. Deleo, *Nat. Rev. Microbiol.*, **7**, 629 (2009).
3. B.S. Ilić, B.D. Kocić, V.M. Cirić, O.G. Ćvetković, D.L. Miladinović, *Scientific World Journa,*. **1**, 1 (2014).

4. Global antimicrobial resistance and use surveillance system (GLASS) report 2021.
5. J. Ma, X. Song, M. Li, Z. Yu, W. Cheng, Z. Yu, W. Zhang, Y. Zhang, A. Shen, H. Sun, L. Li. *Microbiol Res.*, ***266***, 127249 (2023).
6. C. Mollea, F. Bosco, D. Fissore, *Antibiotics*, **11**, 1809 (2022).
7. O. Amassmoud, I. Abbad, M. Iriti, L. Hassani, N. Mezrioui, A. Abbad. *Curr Microbiol*., **1,** 80(12), 398 (2023).
8. M. Aljaafari, M. Sultan Alhosani, A. Abushelaibi, K.S. Lai, S.H. Erin Lim, in: H. El-Shemy (ed.) Essential Oils - Oils of Nature, Chapter 2, 2019.
9. A. Rosato, S. Sblano, L. Salvagno, A. Carocci, M. L. Clodoveo, F. Corbo, G. Fracchiolla. *Antibiotics* (Basel). **24**,9(10), 637 (2020).
10. S. Anžlovar, D. Baričevič, J. A. Avguštin, J. D. Koce. *Technology and Food Biotechnology*, **52**, 263 (2014).
11. M. Marino, C. Bersani, G. Comi. *J. Food Prot*., **62**(9),1017 (1999).
12. D. Gheorghita, A. Robu, A. Antoniac, I. Antoniac, L.M. Ditu, A.-D. Raiciu, J. Tomescu, E. Grosu, A. Saceleanu. *Appl Sci*. **12**, 9482 (2022).
13. A. Puškárová, M. Bučková, L. Kraková, et al., *Sci. Rep.*, **7**, 8211 (2017)
14. N. Ermenlieva, E. Georgieva, S. Stamova, G. Tsankova, S. Mihaylova, A. Tsvetkova, *Supplement Journal of IMAB*, 87 (2021).
15. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. Available online in: https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf
16. EUCAST, 2024. Available online in: https://ncipd.org/index.php?lang=bg
17. L. Galovičová, P. Borotová, V. Valková, N.L.Vukovic, M. Vukic, J. Štefániková, H. Ďúranová, P. L. Kowalczewsk, N. Čmikov, M. Kačániová. *Plants* (Basel) (2021).
18. A. K. Al-Asmari, M. T. Athar, A. A. Al-Faraidy, M. S. Almuhaiza. *Asian Pac. J. Trop. Biomed*., **7**, 147 (2017).
19. M.S. Igoe, Y.H. Hui, in: R.S. Igoe, Y.H. Hui (eds.) *Environ. Microbiol*. **78,** 2586 (2012).
20. A. Al-Mariri, M. Safi. *Iran. J. Med. Sci*.. **39**(1), 36 (2014).
21. C.A. Semeniuc, C.R. Pop, A.M. Rotar. *J, Drug Anal*., **25**(2),403 (2017).
22. F. Moussaoui, F. Alaoui. *Asian Pac. J. Trop. Biomed*. **6**, 32 (2016).
23. D.F. Pancu, R. Buzatu, R. Milutinovici, S. Iurciuc, A. Dolghi, M. Poenaru. *Curr Health Sci J.*,**48**(1), 18 (2022).

Attitudes toward healthy lifestyle among students from two schools in Varna

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This research aims to investigate the attitudes towards a healthy lifestyle among students aged 12-19 from two schools in Varna. The healthy habits acquired in youth are key to maintaining good health in adulthood and old age. The research is anonymous, questionnaire-based, and conducted electronically using a Google Form from September to October 2022. It includes 125 students. The survey contains 28 questions related to various aspects of a healthy lifestyle - dietary habits, alcohol consumption, smoking, and physical activity. The survey results have been processed in Excel and percentages have been calculated. Regarding healthy eating, one of the criteria is the consumption of fruits and vegetables, which is reported by all respondents. Fish is part of the menu for a minority, with only 19.2% including it in their weekly diet. Fatty meats such as pork are preferred by 27.2% of students, while 56.8% prefer chicken meat. A significant percentage, 43.2%, consume alcohol, with 21.7% of them doing so daily. Smokers account for 21.6%. As for physical activity, the results favor a healthy lifestyle. Actively participating in physical education and sports classes are 81.4% of students. The study emphasizes the need for educational programs in schools related to the principles of nutritious eating and a healthy lifestyle, avoiding bad eating habits, and the benefits of physical activity for our health.

Keywords: healthy lifestyle, healthy eating, dietary questionnaire, adolescents, school-aged students, nutrition, activities

INTRODUCTION

Adolescence, which spans the ages of 12 to 19, is a critical period of growth and development. Establishing healthy eating and lifestyle habits during these years has a lasting impact on both physical and mental health. The World Health Organization (WHO) emphasizes the importance of a balanced diet and active lifestyle for adolescents to support their growth and cognitive development. Proper nutrition and healthy habits formed during adolescence can lay the foundation for a healthy adult life. This stage of life is crucial for promoting long-term health and well-being.

Many of the habits acquired during the teenage years persist into adulthood. For example, alcohol consumption and smoking habits developed during adolescence are often carried into later life. The likelihood of alcohol use in youth leading to addiction in adulthood is particularly high [1]. Overweight and obesity are risk factors for the development of various non-communicable diseases, including diabetes, cardiovascular diseases, and several types of cancer, as well as premature mortality [2-5].

In many countries and regions, childhood obesity is increasing [6,7]. If this trend continues, it is projected that by 2025, 20% or more of children and adolescents in over 30 countries around the world will be obese [8].

Healthy eating habits acquired during adolescence are essential for maintaining those habits throughout life [9]. Some chronic diseases begin and progress specifically during this age [10]. For these reasons, young people need to acquire healthy lifestyle habits during adolescence, which are crucial determinants of their long-term health. Motivated by this, we surveyed the approximate percentage of children and adolescents aged 12 to 19 who attempt to live healthily in two schools in Varna. The study was conducted as part of the project *"Healthy Lifestyle? But How and Since When? Not Tomorrow, but Today and Now!*", funded by the Municipality of Varna, Directorate of Youth Activities and Sports.

METHODS AND MATERIALS

Objectives of the Study

The study was conducted as a survey to examine the attitudes of school-aged students toward a healthy lifestyle. The participants were students from two schools in the city of Varna: the „Dr. Ivan Bogorov" - Vocational High School of Economics and seventh-grade students from the „Otets Paisiy" - Primary School.

The main objectives of the study are:

- *Calculating the percentage of participants* categorized as underweight, normal weight, overweight, or at risk of obesity;

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- *Calculating the percentage of participants* categorized as underweight, normal weight, overweight, or at risk of obesity;
- *Analysis of students' dietary and physical activity habits*: Determining the frequency and quality of meals, food preferences, and the level of physical activity among youth aged 12-19;
- *Assessment of the prevalence of harmful habits*: Investigating the frequency of alcohol consumption, smoking, and the use of carbonated drinks and unhealthy foods among young people;
- *Formulating recommendations for future research*: Providing guidance on expanding the scope of the study with larger samples and conducting a more in-depth analysis of the factors influencing students' healthy lifestyles.

Procedures and Participants

The survey was conducted by high school and medical students under the supervision of Senior Assistant Professor Y. Eneva, in collaboration with biology and computer science teachers from the "Dr. Ivan Bogorov" Vocational High School of Economics. It was administered using Google Forms and was conducted anonymously. The survey took place from September to October 2022 in an electronic format. Participation was voluntary, with 125 students taking part. No incentives were offered for participation. Consent for the anonymous use of the survey results was obtained from both participants and their parents or guardians.

The first two questions of the survey aimed to determine the age and gender of the participants. The results showed that 56% of the respondents were girls and 44% were boys. The age distribution was as follows: 72.8% were aged 16 to 19 years, while 27.2% were aged 12 to 15 years.

Determination of Body Mass Index (BMI) of the Participants

The last two questions collected information on the respondents' height and weight to calculate their Body Mass Index (BMI) [11]. BMI is a medico-biological indicator used to assess healthy weight relative to height and weight, and it helps diagnose underweight, normal weight, overweight, and obesity. Developed by Belgian sociologist and statistician Adolphe Quetelet in 1869, BMI is calculated using the formula:

$$BMI = \frac{W}{h^2},$$

where, *BMI* - *Body Mass Index*, *W* - weight (kg), h^2 - height (m)

Socio-economic status and ethnic origin were not subjects of our study, and questions related to these topics were not included in the survey.

Questions on Healthy Lifestyle Behaviors

Through 24 questions, we gathered information about the students' lifestyles. Twelve of these questions focused on their eating habits and how closely these align with healthy eating guidelines. The questions included:

- ✓ How many times a day do you eat?
- ✓ Do you eat between main meals?
- ✓ What do you like to eat between main meals?
- ✓ How do you eat (e.g., sit-down meals, eat on the go)?
- ✓ Do you have breakfast?
- ✓ How often do you consume sweets, fruits, vegetables, fish, and seafood?
- ✓ What do you prefer to eat during breaks?
- ✓ What types of meat do you enjoy?
- ✓ Do you follow the rule of not eating 2 hours before bed?

The next four questions focus on the use of alcohol and carbonated beverages. While it is well known that alcohol is highly detrimental to adolescents, studies indicate that many teenagers experiment with alcohol, sometimes frequently, and may view it as prestigious. Early alcohol consumption increases the risk of addiction in adulthood. Therefore, we included the following questions:

- ✓ Do you drink alcohol?
- ✓ How often do you drink alcohol?

Carbonated drinks are typically excluded from a healthy lifestyle, so we aimed to assess their prevalence in the daily lives of young people. To this end, we included the following questions:

- ✓ Do you drink carbonated beverages?
- ✓ How often do you drink carbonated beverages?

Another harmful habit that often develops during adolescence is smoking. To assess its prevalence among teenagers, we included two questions in the survey: "Do you smoke?" and "How often do you smoke?" For those who responded affirmatively to the first question, we also asked, "How many cigarettes do you smoke per day?".

An important indicator of a healthy lifestyle is physical activity. To assess this aspect, we included five questions in the survey:

- ✓ Do you actively participate in physical education and sports classes?
- ✓ How do you feel after physical education and sports classes?

- ✓ Do you play sports outside of physical education and sports classes?
- ✓ How often do you exercise?
- ✓ Do you enjoy walking in nature?

Physical education and sports classes are mandatory in the curriculum. The first question aims to assess students' attitudes towards these classes, while the second evaluates their impact – whether they feel tired or stimulated and if they help them relax. Many young people are active in sports, engaging in activities such as going to the gym, running, or playing tennis. The next two questions are designed to determine the percentage of active sports participants. Additionally, we included a question about walking in nature to understand students' attitudes towards this activity and its role in relaxation and fresh air. Walking outdoors is particularly important for a healthy lifestyle, as it helps the body to relax and recover.

We processed the survey results using Microsoft Excel, calculated the percentage for each response, and created pie charts to visualize the data. The results are presented in the figures.

Data Analysis

Using the data from the table, we determined the percentage of participants in various weight categories: 11.2% were overweight, 22.4% were underweight, 2.4% were obese, and 64% were of normal weight. These percentages are based on survey responses and provide an estimate of the participants' weight status. Further personal research is needed to refine these results.

According to the WHO, over 340 million children and adolescents aged 5-19 were overweight or obese in 2016, underscoring the urgent need to promote healthy eating habits and physical activity. Additionally, iron deficiency anemia affects approximately 25% of the global adolescent population, which can hinder cognitive and physical development.

A healthy lifestyle encompasses habits related to nutrition, physical activity, rest, and stress reduction. Proper nutrition is particularly crucial for adolescents, as their bodies are developing and growing, requiring adequate energy from food. Healthy eating for adolescents involves:

- ✓ consuming wholesome and varied foods;
- ✓ ensuring sufficient intake of vegetables and fruits;
- ✓ limiting the consumption of fats, sugars, and salts;
- ✓ drinking sufficient fluids.

A varied diet is achieved by including at least one item from each of the following main food groups in the daily menu:

- ✓ cereals and/or potatoes;
- ✓ vegetables;
- ✓ fruits;
- ✓ milk and dairy products;
- ✓ protein-rich foods such as meat, poultry, fish, eggs, and legumes;
- ✓ added vegetable oils and/or dairy fat/butter.

Boys typically require more calories and higher protein intake compared to girls. It is recommended that their diet predominantly includes animal-based proteins—such as milk, meat, fish, and eggs—since these provide essential amino acids necessary for growth.

In early childhood, individual energy and nutritional needs vary based on factors like growth, sex, health status, and others. Inadequate intake of essential nutrients (proteins, calcium) and energy can impair bone mineralization, growth, physical activity, and resistance to infections.

After age 10, gender differences in bone structure and muscle mass become more pronounced, affecting energy requirements. For boys aged 10-14, energy needs range from 1900 to 2350 kcal per day, while for girls, the range is 1780 to 2180 kcal. For adolescents aged 14-19, the energy requirements are between 2820 and 3250 kcal for boys and 2000 to 2270 kcal for girls. The increased rate of growth during this period heightens the body's need for both structural and catalytic nutrients.

Leading nutrition experts recommend eating five times daily, consisting of three main meals and two snacks. The suggested distribution of energy intake is as follows: breakfast should provide 20%, the morning snack 2-5%, lunch 30-33%, the afternoon snack 20%, and dinner 25%. Irregular eating patterns, especially skipping breakfast, can negatively impact children’s behavior, leading to poorer concentration, increased distractibility, and irritability. As shown in the diagram (Fig. 1), 34.4% of respondents reported eating fewer than three times a day, which does not align with healthy eating principles. Additionally, 50.4% of respondents reported that they eat slowly and seated, while the remainder eat quickly, often in front of the computer, while reading, or multitasking (Fig. 1). Eating more than five times a day is reported by 3.2% of respondents, while 4.8% eat five times a day, and 15.2% eat four times a day. Assuming that eating five or more times a day is considered healthy, only 8% of adolescents adhere to this principle. If we accept eating four times a day as a reasonable compromise, then 23.2% of adolescents follow this healthier eating pattern. This indicates a need for educational programs on healthy eating. It is possible that those who reported eating more than five times a day were also including snacks between main meals, despite the survey question asking, "Do you eat anything between main meals?". To this question, 78.4% of respondents indicated that they like to snack, but only 32.4% of them choose fruit, which is considered healthy (Fig. 1). The rest prefer less healthy options.

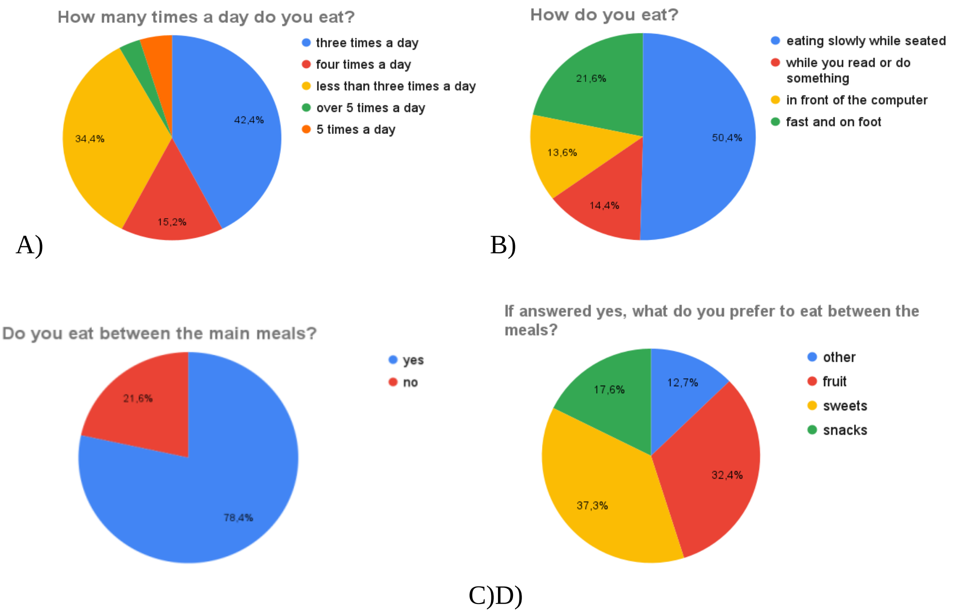

Figure 1. Illustration of respondents' answers to the questions: A) "How many times a day do you eat?"; B) "How do you eat (e.g., sit-down meals, eat on the go)?"; C) "Do you eat between main meals?"; and D) "What do you like to eat between main meals?"

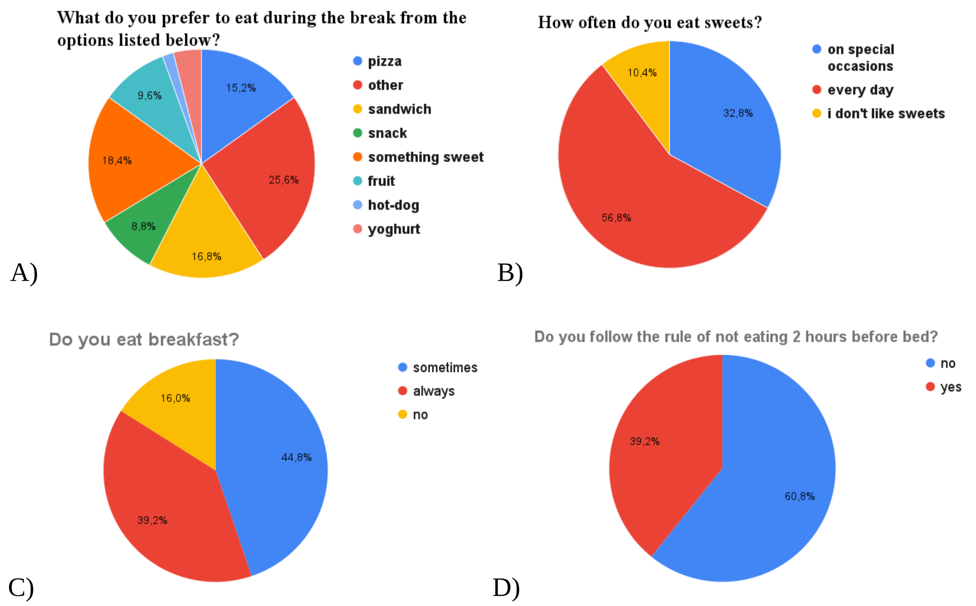

Figure 2. Illustration of respondents' answers to the questions: A) "What do you prefer to eat during breaks?"; B) "How often do you eat sweets?"; C) "Do you have breakfast?"; and D) "Do you follow the rule of not eating 2 hours before bed?".

To further explore eating habits, we asked, “What do you like to eat during breaks from the list below?” The results show that only 9.6% choose fruit, and 18.4% choose milk; the rest prefer items like pizza, sandwiches, breakfast foods, sweets, hot dogs, or other options (Fig. 2). Sweets are particularly popular, with 56.8% of students consuming them daily and 32.8% consuming them occasionally. The remaining 10.4% do not like sweets (Fig. 2). It is important to note that sweets and products with added sugars are not part of a healthy diet. Carbohydrates [12] should make up about 45-65% of an adolescent's daily caloric intake, serving as the primary energy source necessary for physical activity and brain function. Complex carbohydrates found in whole grains, fruits, and vegetables are preferable to simple carbohydrates because they provide sustained energy and are rich in dietary fiber. For instance, 100 grams of brown rice or oatmeal can offer substantial energy while supporting digestive health. Simple carbohydrates, such as those in sweet snacks and drinks, should be limited, as excessive consumption can lead to resistance changes, allergic reactions, dental caries, and obesity. The WHO recommends a daily intake of at least 400 grams of vegetables and fruits and at least 25 grams of fiber for individuals aged 10 and older.

Breakfast is one of the main meals of the day, yet only 39.2% of respondents report eating it regularly (Fig. 2). While eating breakfast is important, the nutritional quality of the breakfast is also crucial; however, the survey did not include questions about the specifics of breakfast consumption. Another healthy principle is to avoid going to bed within two hours of eating, but 60.8% of students do not follow this guideline, meaning only 39.2% adhere to it (Fig. 2). Eating right before bed can lead to calorie storage around the waist, as the body metabolizes food more slowly and stores unprocessed food as fat. Additionally, this practice can impair the absorption of essential micronutrients, such as vitamins and minerals (e.g., vitamins B and D, iron, selenium, zinc, and chromium), leading to deficiencies that further slow down metabolism [13]. On the other hand, recent research [14] suggests that the effects of late eating, which refers to having dinner 1 hour before bedtime in the context of the study, may not be universally negative, particularly in healthy individuals, while traditional advice often recommends avoiding food close to bedtime to prevent impaired sleep quality. However, individual responses can vary, and factors such as the type of food consumed and personal health conditions should also be considered.

What teenagers consume – such as fruits, vegetables, fish, and seafood – along with their preferred types of meat, are key indicators of healthy eating. According to the survey, 45.6% of respondents eat fruit daily, and 67.2% consume vegetables daily (Fig. 3).

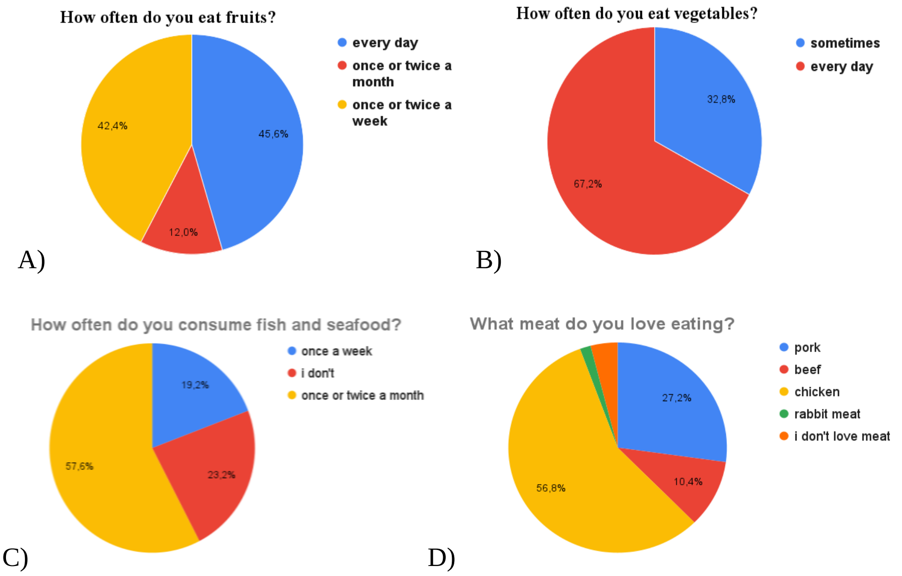

Figure 3. Illustration of respondents' answers to the questions: A) “How often do you eat fruits?”; B) “How often do you eat vegetables?”; C) “How often do you consume fish and seafood?”; and D) “What types of meat do you enjoy?”.

Only 19.2% of respondents consume fish once a week (Fig. 3). Fish is a well-known source of omega-3 fatty acids and proteins, which are essential for body growth. While fatty meats are not preferred, 27.2% of respondents favor pork, and 56.8% prefer chicken. The percentage of those who prefer rabbit or do not consume meat is very small—only 5.6% in total (Fig. 3). Protein is crucial for muscle development, tissue repair, and immune function. Adolescents need approximately 0.85 grams of protein per kilogram of body weight daily, which equates to about 50-60 grams per day for most. Protein sources should be varied and include lean meats (e.g., 100 grams of chicken breast), fish, eggs (one large egg contains around 6 grams of protein), beans, lentils, nuts (e.g., 30 grams of almonds), and dairy products. These foods not only provide the necessary protein but also essential amino acids and other nutrients that support overall growth and health [15].

Healthy fats are essential for brain development, hormone production, and overall health, contributing 25-35% of daily caloric intake. Unsaturated fats, found in foods such as avocados, nuts (e.g., 30g of walnuts), seeds, olive oil (one tablespoon contains about 14g of fat), and oily fish (e.g., 100g of salmon), are recommended. These fats help reduce inflammation and support cardiovascular health. In contrast, trans fats and saturated fats, commonly found in processed foods, should be limited, as they can increase the risk of heart disease and other health problems.

Adequate intake of iron, calcium, and vitamins is critical for adolescents. Iron is particularly important for preventing anemia and aiding oxygen transport. According to the WHO iron deficiency anemia affects approximately 25% of the global adolescent population, which can hinder theire cognitive and physical development. Girls, due to menstruation, require about 15 mg of iron per day, while boys need about 11 mg. Good sources of iron include 100 g of red meat, poultry, fish, lentils, beans, and fortified cereals. Calcium is essential for bone growth, with a recommended intake of 1,300 mg per day. This can be found in dairy products (e.g., one cup of milk provides about 300 mg), cheese, yogurt, fortified plant-based milk, and green leafy vegetables. Vitamin D, which is necessary for calcium absorption and bone health, should be consumed at around 15 mcg per day. Sources include fatty fish like salmon, fortified foods, and adequate sunlight exposure.

The age range of 12-19 is particularly vulnerable. Many lifelong habits are formed during this period, and unfortunately, vices such as alcohol abuse and smoking often begin at this age. WHO reports that in developed countries, 47% of boys and 36% of girls aged 15 consume alcohol more than once a week. Alcohol consumption is frequently linked to inadequate intake of certain vitamins and minerals, with folate deficiency often associated with chronic alcohol use. According to the survey results (Fig. 4), 43.2% of respondents reported drinking alcohol, with 6.7% consuming it daily, 21.7% weekly, 11.7% once or twice a month, and 60% only on occasion. Alarmingly, 7.7% of those who drink alcohol admitted to abusing it whenever they drank, and 29.9% had done so once or several times (Fig. 4).

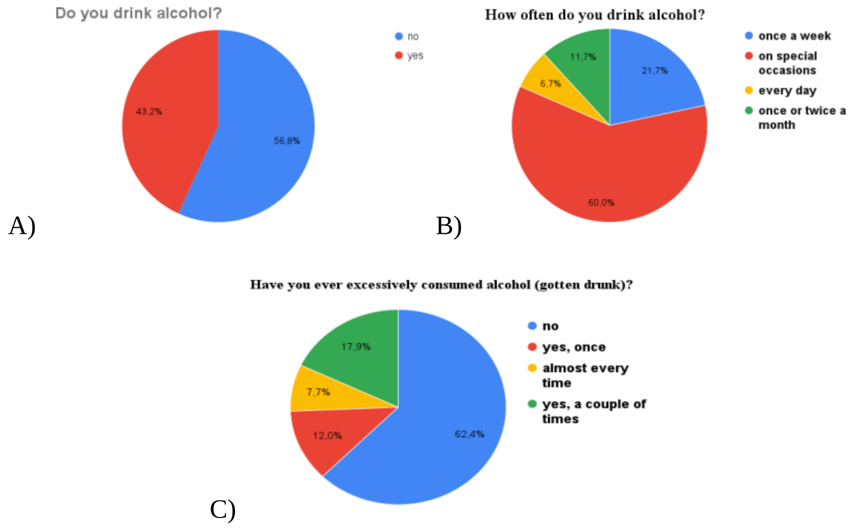

Figure 4. Illustration of respondents' answers to the questions: A) “Do you drink alcohol?”; B) “How often do you drink alcohol”; and C) “Have you ever excessively consumed alcohol?”.

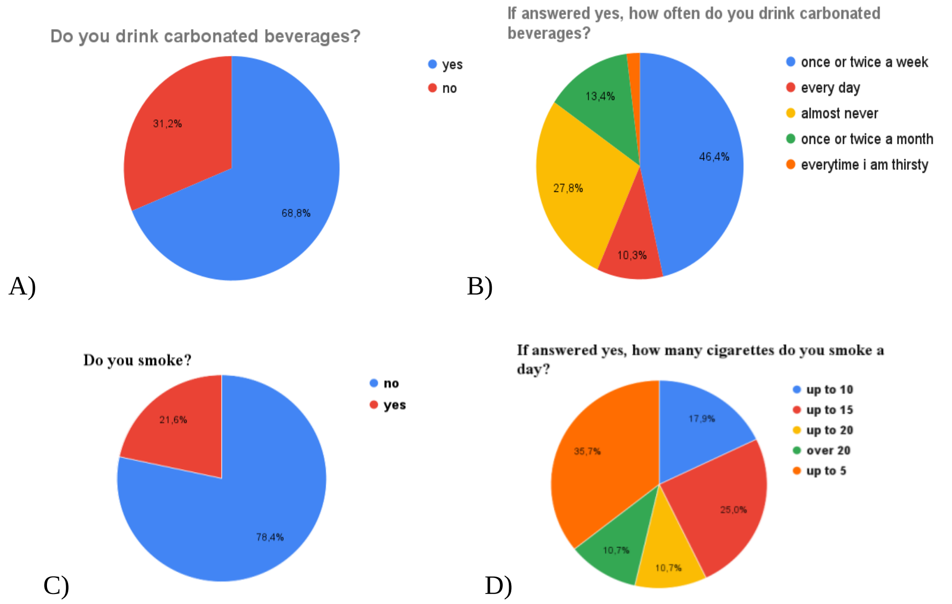

Figure 5. Illustration of respondents' answers to the questions: A) "Do you drink carbonated beverages?"; B) "How often do you drink carbonated beverages?"; C) "Do you smoke?"; and D) "How many cigarettes do you smoke per day?".

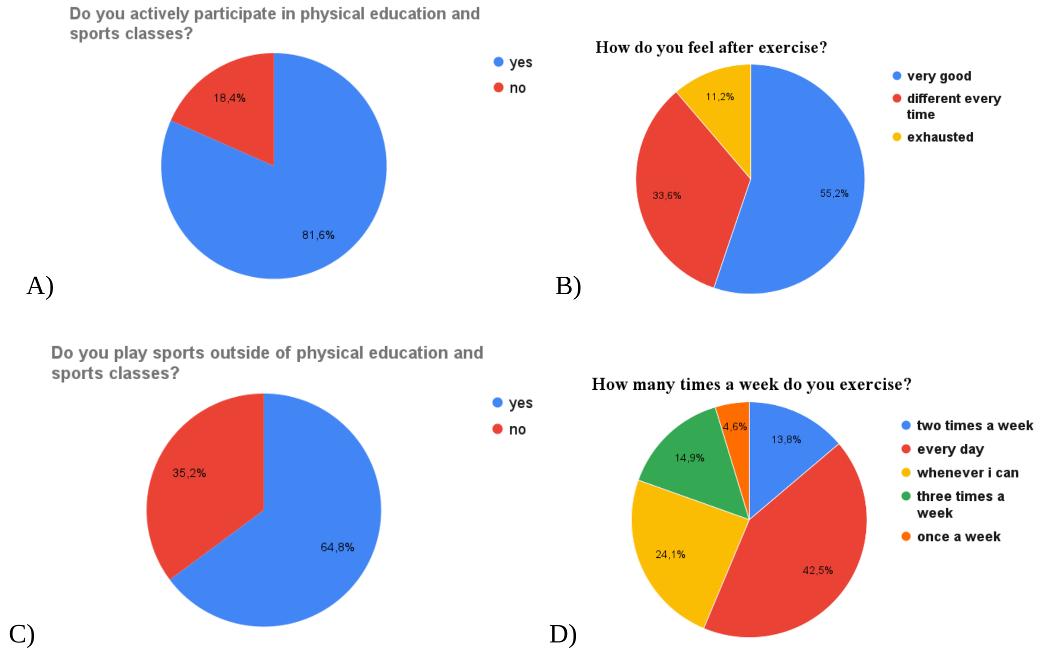

Figure 6. Illustration of respondents' answers to the questions: A) "Do you actively participate in physical education and sports classes?"; B) "How do you feel after physical education and sports classes?"; C) "Do you play sports outside of physical education and sports classes"; and D) "How often do you exercise?".

The consumption of carbonated drinks is also unhealthy, yet 68.8% of respondents indicated a preference for these beverages, with 10.3% consuming them daily and 27.8% consuming them only extremely rarely (Fig. 5). Carbonated beverages, particularly those high in sugar, can contribute to weight gain, tooth decay, and digestive issues such as bloating and acid reflux. Regular consumption can increase the risk of chronic diseases like type 2 diabetes, heart disease, and osteoporosis, while also raising the likelihood of kidney stones and dehydration [16].

Smoking during adolescence can lead to addiction, respiratory issues, and an increased risk of chronic diseases such as lung cancer, heart disease, and stroke. Around 90% of smokers begin before the age of 18, and early initiation is linked to more intense smoking habits and greater challenges in quitting later in life.

To the question "Do you smoke?" 21.6% of respondents answered Yes. Among the smokers, 35.7% limit themselves to up to 5 cigarettes a day, 17.9% smoke up to 10, 25% up to 15, 10.7% up to 20, and 10.7% smoke more than 20 cigarettes a day, which is extremely harmful to their health (Fig. 5). Despite the inclusion of smoking-related questions in educational programs, the survey shows that over 20% of respondents still smoke. Preventive strategies should focus on educating adolescents about the health risks, enforcing smoke-free policies, and reducing access to tobacco products. By raising awareness of the dangers of smoking and fostering a supportive environment, we can help reduce smoking initiation and promote healthier choices.

In terms of physical activity, the results are encouraging. Young people enjoy participating in sports. A significant 82% of respondents are actively involved in physical education and sports classes, and 54.9% report feeling very good afterward (Fig. 6). 64.8% of respondents identify as actively exercising outside of class, with 42.5% of them claiming to exercise daily (Fig. 6). The final question related to physical activity asks, "Do you enjoy walking in nature?" An impressive 93.6% responded positively. According to WHO, at least 60 minutes of moderate to vigorous physical activity daily is recommended. This can include sports, walking, cycling, running, swimming, and more. Regular exercise helps maintain a healthy weight, improves cardiovascular health, strengthens bones and muscles, and promotes mental well-being.

The analysis shows that the respondents' physical activity habits align more closely with the principles of a healthy lifestyle than their eating habits, which are concerning. Although some students exhibit an understanding of the principles of healthy living, their practical application in everyday life is limited. Factors such as insufficient knowledge, social influences, and the availability of unhealthy food options act as significant barriers to developing sustainable healthy habits. Additionally, physical activity, although widely practiced, needs to be paired with healthier dietary choices to ensure comprehensive well-being. To address these challenges, future efforts should focus on:

Educational programs: Implementing initiatives to raise awareness about the importance of regular, balanced meals and the health risks associated with harmful habits.

School interventions: Ensuring access to healthy food options in school cafeterias and creating environments that promote mindful eating.

Targeted research: Conduct detailed studies to explore the reasons behind irregular eating patterns, preferences for unhealthy foods, and the relationship between physical activity and nutrition.

Motivational strategies: Develop programs to foster intrinsic motivation for adopting and maintaining a healthy lifestyle among students.

Teacher training: Equipping educators with effective methods to teach health-related topics and instill lifelong healthy habits in students.

CONCLUSION

The study on attitudes toward a healthy lifestyle among students from two schools in Varna highlights a mixture of positive and concerning trends. While certain practices reflect awareness of healthful habits, others underscore significant gaps that require targeted interventions to encourage healthier behaviors.

Positive trends among the students include:

✓ A high percentage of students (82%) actively participate in physical education and sports, demonstrating an understanding of the importance of physical activity for their well-being.

✓ Daily consumption of vegetables (67.2%) and fruits (45.6%) by many students shows a basic awareness of the role of these foods in a healthy diet.

✓ A majority of students (56.8%) prefer chicken, reflecting some inclination toward healthier protein choices.

Areas of concern involve:

✓ *Unhealthy dietary habits*: Many students regularly consume unhealthy foods such as sweets (56.8% daily) and carbonated drinks (68.8% daily), while only 19.2% consume fish weekly. Additionally, 34.4% of students eat fewer than three meals a day, and only 39.2% have breakfast regularly, highlighting irregular and unbalanced eating patterns.

✓ *Social and structural barriers*: The lack of healthy food options in school cafeterias and the preference for fast food hinder efforts to establish better habits. Furthermore, only half of the students take adequate time to eat, often opting for quick and less mindful meals.

✓ *Harmful habits:* High levels of alcohol consumption (43.2%) and smoking (21.6%) among students emphasize the need for comprehensive health education and prevention programs.

Studies involving a larger, more representative sample of adolescents are necessary to draw more definitive conclusions. The findings from this study were used to develop project-based, non-formal education programs focused on promoting a healthy lifestyle among the participating students. The effectiveness of these programs will be the subject of future research.

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REFERENCES

1. P. Wennberg, T. Andersson, M. Bohman, *Psychol Addict Behav*. **14,** 303 (2000).
2. World Health Organization Prevention of Cardiovascular Disease. [on August 2024)], (2007) Geneva, Switzerland. Available onlinettp://www.who.int/cardiovascular_diseases/guidelines/en/
3. I. Vucenik, J.P. Stains, *Ann. N.Y. Acad. Sci.*, **1271,** 37 (2012). doi: 10.1111/j.1749-6632.2012.06750.x.
4. A. LLewellyn, M. Simmonds, C.G. Owen, *Obes. Rev*., **17,** 56 (2016). doi: 10.1111/obr.12316.
5. L. Li, A. Pérez, L.T. Wu, N. Ranjit, H.S. Brown, S.H. Kelder, *Child Obes*., **12**: 12 (2016). doi: 10.1089/chi.2015.0136]
6. C.L. Ogden, M.D. Carroll, B.K. Kit, K.M. Flegal, *JAMA*, **311,** 8064 (2014). doi: 10.1001/jama.2014.732.
7. A. Suder, A. Gomula, S. Koziel, *Eur. J. Pediatr.*, **176,** 909 (2017). doi: 10.1007/s00431-017-2938-4
8. World Health Organization Global Nutrition Monitoring Framework: Operational guidance for tracking progress in meeting targets for 2025. [(access on 23 August 2024)], (2017) Geneva, Switzerland. Available online: https://www.who.int/publications/i/item/9789241513609
9. A.A. Lake, J.C. Mathers, A.J Rugg-Gunn., *J Public Health*.; **28**:10 (2006).
10. R.M. Viner, D. Ross, R. Hardy, D. Kuh, C. Power, A. Johnson, et al. *J Epidemiol Community Health*,.**69**: 719 (2015).
11. WHO. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. *WHO Technical Report Series* 854. Geneva: World lth Organization, (1995)
12. World Health Organization. Healthy diet. *World Health Organization*. https://www.who.int/news-room/fact-sheets/detail/healthy-diet
13. B. Popov, Hygiene, diet and professional diseases (in Bulgarian), 5th edn., 2021.
14. D. Duan, C. Gu, V. Y. Polotsky, J. C. Jun, L. V. Pham *Nature and Science of Sleep*, **13**, 601 (2021). https://doi.org/10.2147/NSS.S301113
15. World Health Organization. Who updates guidelines on fats and carbohydrates. *World Health Organization*. https://www.who.int/news/item/17-07-2023-who-updates-guidelines-on-fats-and-carbohydrates
16. Bint-E-Zahra, S, Shoaib, R.K. Iqbal, *Nat J Health Sci* (2020) Dec.22 4(2):80-4. Available from: https://ojs.njhsciences.com/index.php/njhs/article/view/52\

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Assessing ChatGPT's effectiveness in designing personalized diet plans for secondary stroke prevention: preliminary reports

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Stroke is one of the leading causes of morbidity and mortality worldwide, and secondary prevention, including dietary modifications and a healthy lifestyle, is essential to reduce the risk of recurrence. This study evaluates the effectiveness of ChatGPT, an artificial intelligence model, in creating personalized dietary plans for secondary stroke prevention. Patient data, including vital signs and biomarkers, belong to the ambulatory patients, who have been treated at the First Neurology Clinic at the University Hospital St. Marina in Varna, Bulgaria. Five patients with a history of ischemic stroke were selected based on age (less than 65 years), minimal comorbidity, and no prior stroke incidents. Based on data from their scans, ChatGPT generated personalized seven-day, five-meal-a-day diet plans for each patient, providing accurate data on the amount of food, calories, protein, carbohydrates, fat, and fiber in each meal. The plans were evaluated against the principles of the Mediterranean diet, DASH, and the European Food Pyramid using the Alternative Healthy Eating Index 2010, which emphasizes food groups associated with reducing the risk of chronic diseases. ChatGPT-generated diets are tailored to patients' medical needs, but further studies with larger numbers of participants are needed for full personalization. The plans are healthy and provide an adequate intake of energy (1800-2000 kcal), protein (70-90 g), carbohydrates (200-250 g), fat (60-70 g), fiber (25-35 g), vitamins, and minerals.

Keywords: stroke, meal plans, secondary prevention, ChatGPT, personalized nutrition

INTRODUCTION

Stroke is one of the leading causes of morbidity, mortality, and disability worldwide [1-4]. According to the World Health Organization (WHO), about 15 million people have a stroke each year; five million die, and another five million are left permanently disabled [5]. The Stroke Action Plan for Europe (SAP-E) 2018-2030 has been developed for stroke prevention [6]. Stroke survivors are a high-risk group, and for them, a healthy lifestyle is a key strategy for effective secondary prevention. It is recommended that they follow these basic principles: healthy eating, regular physical activity, spiritual relaxation, and adequate rest. Their diet should include low salt, potassium-rich foods, plenty of vegetables and fruits, whole grains, unsaturated fats, moderate fish consumption, and a low intake of lean meats [7, 8].

In such cases, the Mediterranean diet [9], Dietary Approaches to Stop Hypertension (DASH) [10], and diets that emphasize the intake of more fruits and vegetables, whole grains, plant-based proteins, and lean meats are strongly recommended [11, 12]. The Healthy Eating Pyramid also organizes the principles of a balanced diet. These three approaches are used to assess the overall quality of a diet [10, 12, 13]. Another assessment approach is the Healthy Eating Index (HEI) [14] and the Alternative Healthy Eating Index (AHEI) [15], both developed to evaluate the nutritional quality of diets in American citizens. AHEI emphasizes food groups and ingredients directly linked to reducing the risk of chronic diseases such as heart disease, type 2 diabetes, and cancer. Studies indicate that the impact of diet on disease occurrence can also vary based on gender [15-17].

Stroke is a life-threatening condition, and the role of diet in its prevention is the focus of extensive research [18-20]. For effective secondary prevention, national stroke plans are essential. These plans should include patient follow-up, access to key preventive strategies for a healthy lifestyle, and prescription medications such as antihypertensives, lipid-lowering agents, antiplatelets, anticoagulants, oral hypoglycemic agents, and insulin. Secondary prevention strategies also encompass physical activity, a healthy diet, limited or safe alcohol consumption, smoking cessation, and management of mood and psychological stress [21].

The integration of artificial intelligence (AI) into our daily lives enables consultations on a variety of

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issues, including health. AI has the potential to provide relevant information related to nutrition, identify causes of diseases such as cardiovascular conditions, diabetes, cancer, and obesity, and offer guidance on potential treatments [22]. However, it always recommends consulting a doctor. Numerous studies [23-27] have evaluated the capabilities of ChatGPT and other AI models in medicine, although research in this area is still developing. Based on this, we aim to investigate and assess ChatGPT-4's ability to create personalized dietary plans for secondary stroke prevention.

MATERIALS AND METHODS

The study began with a review of published research on the impact of healthy diets on the secondary prevention of stroke. We searched Scopus, Web of Science, PubMed, and Google Scholar databases using the keywords "healthy diet and stroke." The review indicated that a healthy diet positively affects stroke survivors in secondary prevention. We then explored the role of artificial intelligence in nutrition using the keywords *nutrition* and *AI*. We found that AI is primarily used to evaluate dietary plans, while areas such as designing personalized plans, predicting malnutrition, and understanding the relationship between nutrition and disease are less developed. The evaluation of customized diet plans by ChatGPT is still in progress. To appropriately assess the generated diets, we continued our study using the keywords *dietary plans* and *assessment*. We selected the Mediterranean diet, Dietary Approaches to Stop Hypertension (DASH), the European Food Pyramid, and the Alternative Healthy Eating Index (AHEI) for comparison and evaluation.

For the study, we selected data from five ambulatory patients with a history of ischemic stroke, who have been treated at the First Neurology Clinic at the University Hospital St. Marina in Varna, Bulgaria. We selected patients based on the following criteria:

- Age under 65 years;
- No gender discrimination – both men and women;
- History of ischemic stroke;
- Minimal number of concomitant diseases;
- First stroke for each patient.

ChatGPT was used to generate personalized seven-day diet plans by inputting the data and test results (Table 1) for each patient and requesting a seven-day healthy menu with five meals per day.

For each food item included in the menu, we required the following information: quantity in grams or number of servings, energy contribution in calories, and the amounts of protein, carbohydrates, fat, and fiber in grams. The menu was tabulated, and a brief analysis of the nutritional content of the diet plans was conducted to ensure they met the patients' nutritional needs. The AI-generated menus are presented in Table 2. They were compared against the criteria of healthy diets – Mediterranean, DASH, and the European Food Pyramid. For clarity, the menus were evaluated according to the AHEI 2010 criteria (Table 3). The AHEI provides a more precise and current assessment of dietary patterns, emphasizing food groups and ingredients that directly reduce the risk of chronic diseases such as heart disease, type 2 diabetes, and cancer. The meal plans were assessed for energy intake, nutrient accuracy, and meal variability. Each food item was scored from 0 to 10 based on its quantity and servings. The scores were statistically analyzed using Microsoft Excel, and the results are illustrated in graphs (Figs. 1, 2).

DISCUSSION

In ChatGPT, we input some of the patients' health status indicators (Table 1) and requested a seven-day healthy diet plan, including five meals per day. As shown in the table, the patients range in age from 45 to 63 years, with three men and two women. All patients have experienced an ischemic stroke. Patient 2 has an accompanying condition of Type 2 diabetes, and Patient 4 is a heavy smoker; the remaining three patients have no reported accompanying diseases. All patients have higher-than-normal blood pressure. Heart rate data is available only for patients 1 and 2, while the data for the other patients are not reported. One indicator, CRP (C-reactive protein), as shown in Table 1, was within the normal range only for patient 3, while in the other patients, it was elevated. Elevated CRP levels can indicate an increased risk of cardiovascular disease [29], inflammatory conditions such as rheumatoid arthritis and Crohn's disease, and various infections. These levels can often be regulated through diet and exercise if the patient is able [30-32]. Foods with antioxidant properties, such as fruits and vegetables, and those rich in vitamin C, such as citrus fruits, can help reduce and prevent inflammation and lower CRP levels.

Additionally, foods high in omega-3 fatty acids, such as fatty fish (e.g., salmon, mackerel, sardines), certain vegetable fats found in avocados and olive oil, nuts (e.g., almonds, walnuts), and seeds (e.g., chia, flaxseed) also help reduce inflammation [33].

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Table 1. Patient information entered into the ChatGPT

Patient №	1	2	3	4	5	Normal Range
Age	63	52	49	45	54	-
Gender	Female	Female	Female	Male	Male	-
Final Diagnosis	Ischemic stroke in the vertebrobasilar system	Ischemic stroke in the vertebrobasilar system	Ischemic stroke in the vertebrobasilar system	Ischemic stroke in the territory of the left middle cerebral artery	Ischemic stroke in the vertebrobasilar system	-
Accompanying Diseases	None	Type 2 Diabetes Mellitus	Not reported	Use of one pack of cigarettes per day	Not reported	-
Arterial Pressure	160/95	130/70	130/80	140/90	130/80	90/60 - 120/80 mmHg
Heart Rate	98/min	78/min	Not reported	Not reported	Not reported	60-100 beats/min
CRP	6.25 mg/l	13,9 mg/l	0,60 mg/l	7.72 mg/l	9,3 mg/l	0.0-5.0 mg/l
HDL	1.35 mmol/l	Not reported	Not reported	1.12 mmol/l	1,09 mmol/l	1.0-1.6 mmol/l
LDL	4.53 mmol/l	Not reported	Not reported	3.80 mmol/l	4,46 mmol/l	0-2.6 mmol/l
ALT	98.4 U/l	69 U/l	19,1 U/l	14.0 U/l	90 U/l	10-49 U/l
AST	74.4 U/l	52 U/l	21 U/l	19.8 U/l	92 U/l	0-34 U/l
Glucose	6.4 mmol/l	8,6 mmol/l	9,3 mmol/l	6.0 mmol/l	6,9 mmol/l	4.1-5.9 mmol/l
Creatinine	72 mcmol/l	56 mcmol/l	61 mcmol/l	99 mcmol/l	65 mcmol/l	62-115 μmol/l
Triglycerides	2.62 mmol/l	4,49 mmol/l	3,38 mmol/l	1.35 mmol/l	3,02 mmol/l	0-1.7 mmol/l
Urea	12.7 mmol/l	4,7 mmol/l	5,1 mmol/l	5.6 mmol/l	4,9 mmol/l	3.2-8.2 mmol/l
Cholesterol	7.07 mmol/l	5,30 mmol/l	7,69 mmol/l	5.53 mmol/l	6,94 mmol/l	2.70-5.20 mmol/l
Sodium	141 mmol/l	142 mmol/l	140 mmol/l	138 mmol/l	138 mmol/l	132-146 mmol/l
Potassium	3.8 mmol/l	4,5 mmol/l	4,0 mmol/l	3.6 mmol/l	4,3 mmol/l	3.50-5.50 mmol/l
Chloride	99 mmol/l	104 mmol/l	100 mmol/l	100 mmol/l	99 mmol/l	99-109 mmol/l

Fiber and plant-protein-rich legumes (e.g., lentils, beans, chickpeas) and whole grains (e.g., oats, brown rice, quinoa) contribute to reducing inflammatory processes. Due to their beneficial effects, these foods are recommended by doctors and are included daily in the generated diets, suggesting that ChatGPT has adhered to this criterion.

Another important indicator is HDL (high-density lipoprotein), often referred to as "good" cholesterol. HDL's role is to transport cholesterol from the blood to the liver, where it is broken down and eliminated from the body. This process helps remove accumulated plaques from the arterial walls in atherosclerosis, thereby reducing the risk of heart attacks and strokes. Consequently, HDL is considered a "cleaner" of the blood vessels. Foods that help increase HDL levels include omega-3 fatty acids found in fish, flaxseeds, chia seeds, nuts, and olive oil. These foods are an essential part of the menu recommended by ChatGPT. For patients 2 and 3, there are no data on HDL levels, but for the other patients, HDL levels are within the normal range, though close to the lower limit (Table 1). The diet includes the aforementioned foods, which contribute to increasing HDL levels.

LDL (low-density lipoprotein), often referred to as "bad" cholesterol, also transports cholesterol in the blood. However, high levels of LDL lead to cholesterol buildup in artery walls, forming plaques that narrow the arteries (atherosclerosis) and increase the risk of heart disease and stroke. Table 1 indicates that LDL data are missing for two patients, while for patients 1, 4, and 5, LDL levels are above the permissible values. To regulate LDL, it is important to avoid foods high in saturated fats (such as red meat, full-fat dairy products, and processed foods), increase the consumption of foods rich in omega-3 fatty acids, and eat more fiber-rich foods (such as oats, legumes, fruits, and vegetables). Avoiding smoking, maintaining a normal weight, engaging in aerobic exercise (such as walking, running, or swimming), and managing stress through methods like meditation, yoga, or deep breathing can also be beneficial. Table 2 shows that fish, which is high in omega-3 fatty acids, is frequently recommended in the dietary plan, likely due to the elevated LDL levels.

ALT (alanine aminotransferase) is an enzyme primarily found in the liver and plays a crucial role in amino acid metabolism. Elevated levels of ALT in the blood can indicate liver damage or disease, such as hepatitis, cirrhosis, alcohol-related damage, use of hepatotoxic drugs, muscle damage, or other conditions. Normal ALT levels in adults range from 7 to 56 U/L (units per liter). To regulate ALT levels, a healthy diet is essential, including avoiding alcohol, consuming foods rich in antioxidants, steering clear of saturated fats and processed foods, achieving weight loss, and engaging in physical activity. Nutritional supplements such as vitamin E and silymarin (milk thistle extract) are known for their liver-protective properties and may help lower ALT levels. Table 1 shows that ALT levels were within the normal range for patients 3 and 4, while the remaining three patients had elevated levels. These patients require a healthy diet and appropriate physical activity to manage their condition.

Another liver indicator used in the study is AST (aspartate aminotransferase), an enzyme found in various tissues of the body, including the liver, heart, muscles, kidneys, and brain. Its primary role is to support amino acid metabolism. Normal levels of AST in adults range from 10 to 40 U/L (units per liter). Table 1 shows that AST levels are within the normal range for patients 3 and 4, while the levels are elevated in the other patients. Regulating AST levels involves maintaining a healthy diet that includes antioxidant-rich foods, omega-3 fatty acids, and limiting alcohol intake. It is also important to avoid hepatotoxic drugs, manage chronic conditions such as diabetes or metabolic syndrome, and consider antioxidant supplements like silymarin (under medical supervision). Increasing physical activity can also aid in regulating AST levels.

Glucose levels were elevated in all patients. Normal glucose levels range from 4.1 to 5.9 mmol/L. Patients should avoid simple carbohydrates, such as sugar, sweets, and carbonated drinks. Table 2 indicates that these items are not included in the diet plans. Instead, the diet should focus on complex carbohydrates, such as whole grains, vegetables, and legumes, which are broken down more slowly and help maintain stable blood glucose levels. Eating smaller portions more frequently, as in a five-meal-a-day plan, is beneficial. Additionally, it is important to consume an adequate amount of water to support normal metabolic processes, which is currently lacking in the diet plans and the analysis [34-41].

Creatinine levels were normal in all patients. Creatinine is a byproduct of normal muscle metabolism and plays a crucial role in energy production within muscle cells. It is synthesized in the liver and kidneys and stored in the muscles. During muscle activity, creatinine breaks down into various metabolites, which are then excreted through urine. Therefore, creatinine levels in the blood and urine are important indicators of kidney function.

Triglyceride levels were within the normal range only for patient 4; in all other patients, they were elevated. Triglycerides are a type of fat (lipid) found in the blood and serve as a primary energy source. After eating, excess calories are converted into triglycerides and stored in fat cells. These triglycerides are used as an energy reserve to support basic bodily functions and the absorption of fat-soluble vitamins (A, D, E, and K). To manage elevated triglyceride levels, it is essential to follow a healthy diet, avoid sugar and sugary products, engage in physical activity, and maintain a healthy weight.

Urea levels were above normal only for patient 1. Urea is a chemical compound produced in the liver from ammonia, which is released during protein breakdown. It is transported *via* the blood to the kidneys, where it is filtered and excreted in urine. Normal urea levels range from 3.2 to 8.2 mmol/L. Elevated urea levels can indicate kidney disease, dehydration, or excessive protein intake, while low levels may suggest liver disease or malnutrition. Urea plays a crucial role in detoxifying the body by helping to eliminate waste products from nitrogen metabolism.

All patients exhibited elevated cholesterol levels, indicating hypercholesterolemia (high cholesterol). Cholesterol is a type of lipid (fat) that plays a crucial role in various bodily processes. Produced in the liver, cholesterol is present in cell membranes, where it helps maintain their structural integrity and is essential for synthesizing hormones (such as estrogen and testosterone), vitamin D, and bile acids that aid in fat digestion. While the body produces sufficient cholesterol, it is also obtained from dietary sources, particularly animal products like meat, eggs, and dairy. High cholesterol levels can be managed through dietary changes and, if necessary, cholesterol-lowering medications. In the recommended diets, red meat, dairy products, and eggs are included very cautiously (Table 2).

The levels of sodium, potassium, and chloride are within the normal range for all patients. Sodium is crucial for fluid regulation and maintaining normal nerve and muscle function. Potassium supports proper heart, muscle, and nervous system function. Chloride helps maintain acid-base balance and proper body hydration. Normal values of these electrolytes indicate a well-balanced electrolyte status in the patients.

The analysis of health indicators highlights the need for a healthy lifestyle among stroke survivors. Recommended diets for stroke prevention emphasize low salt intake and include more fruits, vegetables, whole grains, legumes, seeds, nuts, low-fat dairy products, and lean white meats. Conversely, red and processed meats, sweets, and alcohol are not recommended. Following a stroke, dietary intervention should be viewed as a crucial strategy for preventing recurrence. Table 2 presents the diet plans generated by ChatGPT, and it can be concluded that these plans meet the criteria for stroke prevention.

Table 2. Diet plans generated by ChatGPT.

Days	Meals	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
1	Breakfast	Oatmeal (100g), fresh berries (50g), ground flaxseed (15g), herbal tea (unsweetened)	Whole grain cereal (50g) with low-fat milk (200ml), a small apple (100g)	Oatmeal (100g), fresh strawberries (100g), chia seeds (1 tbsp), green tea (unsweetened)	Oatmeal (100g), fresh strawberries (50g), chia seeds (1 tbsp), herbal tea (unsweetened)	Oatmeal (100g), fresh blueberries (50g), ground flaxseed (1 tbsp), herbal tea (unsweetened)
	Morning snack	Apple (150g), almonds (15g)	Carrot sticks (100g), hummus (50g)	Orange (150g)	Apple (150g), walnuts (10, 15g)	Orange (150g), almonds (10, 15g)
	Lunch	Grilled chicken breast (100g), quinoa salad with mixed vegetables (200g), whole grain bread (30g)	Grilled chicken breast (120g), quinoa salad with mixed vegetables (200g)	Grilled chicken breast (120g), mixed vegetable salad (200g), quinoa (100g)	Grilled chicken breast (100g), quinoa salad with mixed vegetables (200g), whole grain bread (1 slice, 30g)	Grilled chicken breast (100g), quinoa salad with mixed vegetables (200g), whole grain bread (1 slice, 30g)
	Afternoon snack	Low-fat yogurt (150g), walnuts (20g)	Greek yogurt (150g), mixed berries (50g)	Greek yogurt (150g), a handful of almonds (15g)	Greek yogurt (150g), mixed berries (50g)	Greek yogurt (150g), mixed berries (50g)
	Dinner	Baked salmon (120g) with lemon and dill, steamed broccoli and carrots (200g), brown rice (100g)	Baked salmon (120g) with steamed broccoli and carrots (200g), brown rice (100g)	Baked cod (150g), roasted Brussels sprouts (150g), brown rice (100g)	Baked salmon (120g) with lemon and dill, steamed green beans and carrots (200g), brown rice (100g)	Baked salmon (120g) with lemon and dill, steamed green beans and carrots (200g), brown rice (100g)
2	Breakfast	Whole grain toast (30g) with avocado spread (50g), boiled egg (1), herbal tea (unsweetened)	Whole grain toast (30g) with avocado spread (50g), boiled egg (1)	Whole grain toast (30g) with avocado spread (50g), boiled egg (1)	Whole grain toast (30g) with avocado spread (50g), boiled egg (1), herbal tea (unsweetened)	Whole grain toast (30g) with avocado spread (50g), boiled egg (1), herbal tea (unsweetened)
	Morning snack	Carrot sticks (100g), hummus (50g)	Apple (150g), almonds (15g)	Apple (150g)	Carrot sticks (100g), hummus (50g)	Carrot sticks (100g), hummus (50g)
	Lunch	Lentil soup (250ml), mixed green salad with olive oil and lemon dressing (200g)	Lentil soup (250ml), mixed green salad with olive oil and lemon dressing (200g)	Lentil soup (250ml), mixed green salad with olive oil dressing (200g)	Lentil soup (250ml), mixed green salad with olive oil and lemon dressing (200g)	Lentil soup (250ml), mixed green salad with olive oil and lemon dressing (200g)
	Afternoon snack	Blueberries (100g), hazelnuts (15g)	Blueberries (100g), hazelnuts (15g)	Carrot sticks (100g), hummus (50g)	Apple (150g), almonds (10, 15g)	Apple (150g), cashews (10, 15g)
	Dinner	Grilled turkey breast (120g), quinoa (100g) with sautéed spinach and garlic (200g), steamed green beans (150g)	Grilled turkey breast (120g), quinoa (100g) with sautéed spinach and garlic (200g)	Grilled turkey breast (120g), baked sweet potato (150g), steamed green beans (150g)	Grilled turkey breast (120g), quinoa (100g) with sautéed spinach and garlic (200g), steamed green beans (150g)	Grilled turkey breast (120g), quinoa (100g) with sautéed spinach and garlic (200g), steamed green beans (150g)
3	Breakfast	Smoothie (200ml almond milk, 1 banana, 50g spinach), chia seeds (15g)	Smoothie (200ml almond milk, 1 banana, 50g spinach), chia seeds (15g)	Smoothie (200ml almond milk, 1 banana, 50g spinach), flaxseeds (1 tbsp)	Smoothie (200ml almond milk, 1 banana, 50g spinach), chia seeds (1 tbsp)	Smoothie (200ml almond milk, 1 banana, 50g spinach), chia seeds (1 tbsp)
	Morning snack	Celery sticks (100g), peanut butter (20g)	Celery sticks (100g), peanut butter (20g)	Celery sticks (100g), peanut butter (20g)	Celery sticks (100g), peanut butter (20g)	Celery sticks (100g), peanut butter (20g)
	Lunch	Baked cod (120g) with tomato and basil sauce, barley salad with cherry tomatoes and parsley (200g)	Baked cod (120g) with tomato and basil sauce, barley salad with cherry tomatoes and parsley (200g)	Baked salmon (120g), quinoa salad with cherry tomatoes and cucumbers (200g)	Baked cod (120g) with tomato and basil sauce, barley salad with cherry tomatoes and parsley (200g)	Baked cod (120g) with tomato and basil sauce, barley salad with cherry tomatoes and parsley (200g)
	Afternoon snack	Greek yogurt (150g), mixed berries (50g)	Greek yogurt (150g), mixed berries (50g)	Mixed berries (100g), walnuts (20g)	Greek yogurt (150g), mixed berries (50g)	Greek yogurt (150g), mixed berries (50g)
	Dinner	Stuffed bell peppers (200g) with quinoa, vegetables, and lean ground beef, mixed green salad (150g) with balsamic vinaigrette	Stuffed bell peppers (200g) with quinoa, vegetables, and lean ground beef, mixed green salad (150g)	Stuffed bell peppers (200g), with quinoa and lean ground beef, mixed green salad (150g)	Stuffed bell peppers (200g) with quinoa, vegetables, and lean ground beef, mixed green salad (150g) with balsamic vinaigrette	Stuffed bell peppers (200g) with quinoa, vegetables, and lean ground beef, mixed green salad (150g) with balsamic vinaigrette
4	Breakfast	Greek yogurt (150g), oats (30g), honey (10g)	Greek yogurt (150g), oats (30g), honey (10g)	Greek yogurt (150g), oats (30g), honey (1	Greek yogurt (150g), oats (30g), honey (1 tsp), herbal tea (unsweetened)	Greek yogurt (150g), oats (30g), honey (1 tsp), herbal tea (unsweetened)

				tsp), green tea (unsweetened)		
	Morning snack	Kiwi (100g), almonds (15g)	Kiwi (100g), almonds (15g)	Kiwi (100g), almonds (15g)	Kiwi (100g), walnuts (10, 15g)	Kiwi (100g), walnuts (10, 15g)
	Lunch	Grilled chicken salad (250g) with mixed greens, tomatoes, cucumbers, olive oil dressing, whole grain bread (30g)	Grilled chicken salad (250g) with mixed greens, tomatoes, cucumbers, olive oil dressing	Grilled chicken breast salad (200g) with mixed greens, cherry tomatoes, and olive oil dressing	Grilled chicken salad (250g) with mixed greens, tomatoes, cucumbers, olive oil dressing, whole grain bread (1 slice, 30g)	Grilled chicken salad (250g) with mixed greens, tomatoes, cucumbers, olive oil dressing, whole grain bread (1 slice, 30g)
	Afternoon snack	Cottage cheese (100g) with pineapple chunks (50g)	Cottage cheese (100g) with pineapple chunks (50g)	Cottage cheese (100g) with pineapple chunks (50g)	Cottage cheese (100g) with pineapple chunks (50g)	Cottage cheese (100g) with pineapple chunks (50g)
	Dinner	Baked trout (120g) with lemon and herbs, steamed asparagus (150g), wild rice (100g)	Baked trout (120g) with lemon and herbs, steamed asparagus (150g), wild rice (100g)	Baked trout (120g) with lemon, steamed asparagus (150g), wild rice (100g)	Baked trout (120g) with lemon and herbs, steamed asparagus (150g), wild rice (100g)	Baked trout (120g) with lemon and herbs, steamed asparagus (150g), wild rice (100g)
5	Breakfast	Whole grain toast (30g) with almond butter (15g), banana (100g), herbal tea (unsweetened)	Whole grain toast (30g) with almond butter (15g), banana (100g)	Whole grain toast (30g) with almond butter (15g), a small banana (100g)	Whole grain toast (30g) with almond butter (15g), banana (1 small, 100g), herbal tea (unsweetened)	Whole grain toast (30g) with almond butter (15g), banana (1 small, 100g), herbal tea (unsweetened)
	Morning snack	Cucumber slices (100g), tzatziki dip (50g)	Cucumber slices (100g), tzatziki dip (50g)	Cucumber slices (100g), tzatziki dip (50g)	Cucumber slices (100g), tzatziki dip (50g)	Cucumber slices (100g), tzatziki dip (50g)
	Lunch	Chickpea and vegetable stew (250ml), mixed green salad (150g)	Chickpea and vegetable stew (250ml), mixed green salad (150g)	Chickpea and vegetable stew (250ml), mixed green salad (150g)	Chickpea and vegetable stew (250ml), mixed green salad (150g)	Chickpea and vegetable stew (250ml), mixed green salad (150g)
	Afternoon snack	Strawberries (100g), cashews (15g)	Strawberries (100g), cashews (15g)	Strawberries (100g), cashews (15g)	Strawberries (100g), cashews (10, 15g)	Strawberries (100g), cashews (10, 15g)
	Dinner	Grilled turkey breast (120g), sweet potato mash (150g), steamed broccoli (150g)	Grilled turkey breast (120g), sweet potato mash (150g), steamed broccoli (150g)	Grilled turkey breast (120g), sweet potato mash (150g), steamed broccoli (150g)	Grilled turkey breast (120g), sweet potato mash (150g), steamed broccoli (150g)	Grilled turkey breast (120g), sweet potato mash (150g), steamed broccoli (150g)
6	Breakfast	Smoothie (200ml almond milk, 50g berries, 50g spinach, 15g flaxseed)	Smoothie (200ml almond milk, 50g berries, 50g spinach, 1 tbsp flaxseed)	Smoothie (200ml almond milk, 50g berries, 50g spinach, 1 tbsp flaxseeds)	Smoothie (200ml almond milk, 50g berries, 50g spinach, 1 tbsp flaxseed), chia seeds (1 tbsp)	Smoothie (200ml almond milk, 50g berries, 50g spinach, 1 tbsp flaxseed), chia seeds (1 tbsp)
	Morning snack	Bell pepper slices (100g), hummus (50g)	Bell pepper slices (100g), hummus (50g)	Bell pepper slices (100g), hummus (50g)	Bell pepper slices (100g), hummus (50g)	Bell pepper slices (100g), hummus (50g)
	Lunch	Baked chicken breast (120g), quinoa and vegetable stir-fry (200g)	Baked chicken breast (120g), quinoa and vegetable stir-fry (200g)	Baked chicken breast (120g), quinoa and vegetable stir-fry (200g)	Baked chicken breast (120g), quinoa and vegetable stir-fry (200g)	Baked chicken breast (120g), quinoa and vegetable stir-fry (200g)
	Afternoon snack	Greek yogurt (150g), sliced strawberries (50g)	Greek yogurt (150g), sliced strawberries (50g)	Greek yogurt (150g), sliced strawberries (50g)	Greek yogurt (150g), sliced strawberries (50g)	Greek yogurt (150g), sliced strawberries (50g)
	Dinner	Baked mackerel (120g) with herbs, steamed Brussels sprouts and carrots (200g), brown rice (100g)	Baked mackerel (120g) with herbs, steamed Brussels sprouts and carrots (200g), brown rice (100g)	Baked mackerel (120g), steamed Brussels sprouts and carrots (200g), brown rice (100g)	Baked mackerel (120g) with herbs, steamed Brussels sprouts and carrots (200g), brown rice (100g)	Baked mackerel (120g) with herbs, steamed Brussels sprouts and carrots (200g), brown rice (100g)
7	Breakfast	Whole grain cereal (50g) with low-fat milk (200ml), small apple (100g)	Whole grain cereal (50g) with low-fat milk (200ml), a small apple (100g)	Whole grain cereal (50g) with low-fat milk (200ml), small apple (100g)	Whole grain cereal (50g) with low-fat milk (200ml), small apple (100g)	Whole grain cereal (50g) with low-fat milk (200ml), small apple (100g)
	Morning snack	Radishes (100g), low-fat cheese (50g)	Radishes (100g), low-fat cheese (50g)	Radishes (100g), low-fat cheese (50g)	Radishes (100g), low-fat cheese (50g)	Radishes (100g), low-fat cheese (50g)
	Lunch	Lentil and vegetable stew (250ml), mixed green salad (150g)	Lentil and vegetable stew (250ml), mixed green salad (150g)	Lentil and vegetable stew (250ml), mixed green salad (150g)	Lentil and vegetable stew (250ml), mixed green salad (150g)	Lentil and vegetable stew (250ml), mixed green salad (150g)
	Afternoon snack	Mixed nuts (20g), blueberries (100g)	Mixed nuts (20g), blueberries (100g)	Mixed nuts (20g), blueberries (100g)	Mixed nuts (20g), blueberries (100g)	Mixed nuts (20g), blueberries (100g)
	Dinner	Baked chicken breast (120g), steamed mixed vegetables (200g), whole grain couscous (100g)	Baked chicken breast (120g), steamed mixed vegetables (200g), whole grain couscous (100g)	Baked chicken breast (120g), steamed mixed vegetables (200g), whole grain couscous (100g)	Baked chicken breast (120g), steamed mixed vegetables (200g), whole grain couscous (100g)	Baked chicken breast (120g), steamed mixed vegetables (200g), whole grain couscous (100g)

- One of the most suitable diets for stroke prevention is the Mediterranean diet which emphasizes consuming fresh seasonal foods. Its main principles include: Frequent consumption of dairy products like yogurt and cheese;
- Daily consumption of a large variety of vegetables, antioxidant-rich fruits, legumes, raw nuts, whole grains, and olive oil;
- Weekly intake of fish, eggs, and poultry;
- Limited intake of red meat, refined sugar, and saturated fats;
- Adequate water intake.

Comparing the generated diet plans with the Mediterranean diet shows a significant match. However, there is a difference in the recommended fish consumption. The Mediterranean diet suggests a weekly intake of fish, poultry, and eggs – meaning once a week – whereas the ChatGPT-generated diets recommend fish and white meats more than once a week. This adjustment may align with the elevated indicators noted in the previous studies. Red meat intake is limited to just one occurrence, specifically lean beef. The daily meals include whole grains, nuts (up to 20 grams), seeds, fresh fruits, and vegetables. Additionally, the milk included in the diet is low-fat, which is appropriate for managing elevated triglyceride levels.

Another suitable diet is the DASH diet. While it shares many principles with the Mediterranean diet, it is specifically designed to regulate and prevent high blood pressure. Its main principles include:

- Increased consumption of foods rich in magnesium, potassium, calcium, fiber, and protein;
- Reducing intake of foods high in sugar, saturated and trans fats, and sodium;
- Increased consumption of fruits, vegetables, legumes, nuts, low-fat and skim dairy products, poultry, and fish;
- Limited consumption of processed foods and fatty red meats;
- Restricting daily sodium intake to 2,300 mg, and in some cases, to 1,500 mg per day.

Comparing the ChatGPT-generated diets to the DASH diet criteria also shows a high level of concordance. The recommended sodium intake in the generated diets falls within the range of 1,500-2,000 mg per day. The menu includes foods such as mackerel, salmon, brown rice, oats, chickpeas, lentils, almonds, and flaxseed, which are rich in magnesium. Potassium is provided by bananas, avocados, beans, and lentils, while dairy products, broccoli, and almonds supply calcium. Whole grains, a key component of the DASH diet, are also recommended daily by ChatGPT.

The European food pyramid for a healthy diet emphasizes the importance of consuming a varied and balanced diet. Its main principles include:

- Frequent consumption of foods from the base of the pyramid, such as cereals, vegetables, and fruits, which are rich in vitamins, minerals, fiber, and other essential nutrients;
- Moderate consumption of foods from the middle of the pyramid, including proteins, dairy products, and fats;
- Very limited and cautious consumption of foods from the top of the pyramid, such as processed and refined foods, as well as other high-calorie, low-nutrient items.

Table 2 shows that the menu includes foods from the base of the pyramid, with moderate consumption of those from the middle, and no processed or refined foods are present.

Based on the comparisons made, we can conclude that the diets generated by ChatGPT adhere to the criteria of all three types of healthy eating plans.

Although the dietary plans generated by artificial intelligence follow the established principles, we calculated the health index using the AHEI-2010 criteria for greater accuracy (Table 3) [43-47].

Table 3. The AHEI-2010 scoring method. [43]

Component	Minimum Score (0)	Maximum Score (10)
Vegetables	0 servings/d	≥5 servings/d
Fruits	0 servings/d	≥4 servings/d
Whole grains	0 servings/d	≥4 servings/d
Sugar-sweetened beverages and fruit juice	≥1 servings/d	0 servings/d
Nuts and legumes	0 servings/d	≥1 servings/d
Red/processed meat	≥1.5 servings/d	0 servings/d
Trans fat	≥4% of energy	≤0.5% of energy
Long-chain (n-3) fats (EPA + DHA)	0 mg/d	≥250 mg/d
PUFA	≤2% of energy	≥10% of energy
Sodium	Highest decile	Lowest decile
Alcohol	Women: ≥2.5 drinks/d Men: ≥3.5 drinks/d	Women: 0.5-1.5 drinks/d Men: 0.5-2.0 drinks/d

The AHEI-2010 is a tool designed to assess diets and their association with the risk of chronic diseases. It was developed as an alternative to traditional dietary guidelines to evaluate nutrition quality and its impact on the risk of cardiovascular disease, diabetes, and cancer. In this regard, it provides a more precise assessment of the quality of AI-generated diets.

The AHEI-2010 [43] focuses on the types of foods and food groups linked to better health:

- Encourages increased intake of fresh fruits, which are major sources of vitamins, minerals, and fiber.
- Includes a variety of vegetables, emphasizing leafy greens and avoiding potatoes.
- Assesses consumption of whole grain products such as brown rice, oatmeal, and whole grain bread.
- Encourages regular consumption of nuts and legumes, which are excellent sources of plant-based protein and healthy fats.
- Measures intake of fish and other sources of omega-3 fatty acids, such as flaxseed and walnuts, for their cardiovascular protective effects.
- Promotes replacing saturated and trans fats with polyunsaturated fats found in vegetable oils, nuts, and fish.
- Evaluates the presence of trans fats, long-chain (n-3) fats (EPA + DHA), and polyunsaturated fatty acids (PUFA) in the diet.
- Reduces consumption of red and processed meats, which are associated with higher risks of chronic diseases.
- Limits intake of sweetened beverages and high-sugar fruit juices that contribute to obesity and metabolic diseases.
- Assesses moderate alcohol consumption (up to 1 drink per day for women and 2 for men) for its potential heart-protective effects.
- Recommends limiting sodium intake to reduce the risk of high blood pressure and cardiovascular disease.

Each component of the diet is rated on a scale from 0 to 10 points based on how well it aligns with healthy eating recommendations. The maximum score for a diet according to the AHEI-2010 criteria is 110 points. A diet scoring above 65 points is considered healthy, with higher scores indicating a better alignment with healthy eating practices and a lower risk of chronic diseases. Based on these criteria [43] and the quantities of each food in the diet plans, we calculated the number of servings and points for each component, as well as the total score for each day (Fig. 1). The graph shows that all the diet plans received more than 65 points, indicating that the ChatGPT-generated diets are healthy.

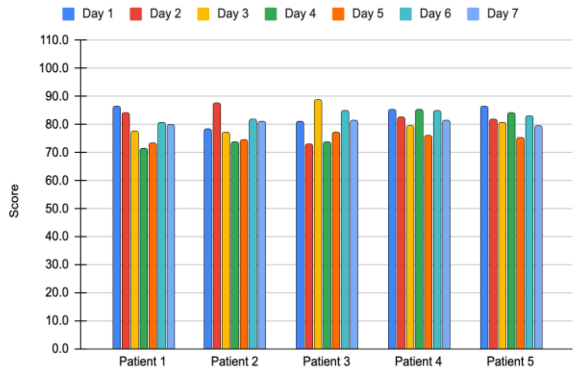

Figure 1. AHEI-2010 scores for daily menus ranged from 71.5 to 88.6 across all five patients

For each diet plan, we calculated the average number of points for each food component and displayed these on a radar chart (Fig. 2) to assess differences in the recommended food quantities for different patients. The chart indicates that there is consistency for some foods (such as whole grains and red meats), while differences exist for others (such as nuts, foods with trans fats, and PUFA). Although fruits and vegetables are included daily, their quantities often fall short of meeting the AHEI-2010 health criteria. The distribution of vegetables and fruits throughout the week is suboptimal, with some days not meeting the minimum recommendations set by the World Health Organization. Fish oils, a major source of polyunsaturated fatty acids (PUFA), are included, but consumption of other PUFA-rich foods (like nuts and flaxseeds) could be higher. Additionally, ChatGPT's diet plans show limited variety, as the same foods are repeated across different meals. While the overall scores for all diet plans exceed 65 points, indicating they are healthy, there is room for improvement in food variety and nutrient distribution.

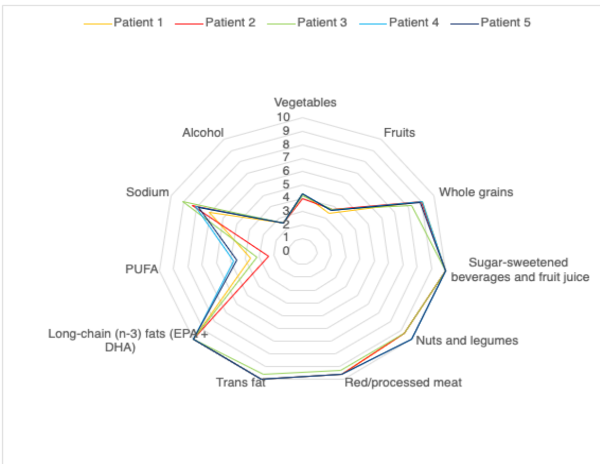

Figure 2. Average distribution of AHEI-2010 component scores throughout the week for all patients' diets.

CONCLUSIONS

A healthy and balanced diet is crucial for maintaining physical and psychological well-being, particularly for stroke survivors. Large language models like ChatGPT offer a valuable tool for generating dietary plans. This study provides a comprehensive analysis of ChatGPT's potential to create personalized dietary plans aimed at stroke prevention, highlighting both the advantages and limitations of the generated diets.

While databases such as Scopus, PubMed, and Web of Science contain numerous studies on healthy eating recommendations and preventive diets for recurrent stroke, no studies were found specifically addressing personalized stroke prevention dietary plans generated by ChatGPT. Therefore, this study is unique in evaluating ChatGPT's capability to deliver customized dietary plans for secondary stroke prevention.

ChatGPT has successfully generated healthy diet plans that align with patients' nutritional needs and medical conditions. However, to definitively establish these plans as personalized, further research involving larger patient cohorts is necessary. The current plans provide an adequate intake of energy (1800 to 2000 kcal per day), protein (70 to 90 g per day), carbohydrates (200 to 250 g per day), fat (60 to 70 g per day), fiber (25 to 35 g per day), vitamins, and minerals.

The developed plans align with current healthy dietary recommendations; however, additional follow-up is essential to ensure their appropriateness for stroke survivors.

REFERENCES

1. Haim, M. Katson, M. Cohen-Shelly, S. Peretz, D. Aran, S. Shelly, medRxiv, 2024, 2024-01 (2024).
2. D. Lopez, C. D. Mathers, M. Ezzati, D. T. Jamison, C. J. L. Murray, Lancet, 367, 1747 (2006).
3. Sarti, D. Rastenyte, Z. Cepaitis, J. Tuomilehto, Stroke, 31, 1588 (2000).
4. G. J. Hankey, C. P. Warlow, Lancet, 354, 1457 (1999).
5. S. Li, X. Wang, A. Jin, G. Liu, H. Gu, H. Li, B. C. V. Campbell, M. Fisher, Y. Yang, Y. Wei, J. Wang, Y. Wang, X. Zhao, L. Liu, Z. Li, X. Meng, Y. Wang, Stroke, 55, 366 (2024).
6. Norrving, J. Barrick, A. Davalos, M. Dichgans, C. Cordonnier, A. Guekht, K. Kutluk, R. Mikulik, J. Wardlaw, E. Richard, D. Nabavi, C. Molina, P. -M. Bath, K. S. Sunnerhagen, A. Rudd, A. Drummond, A. Planas, V. Caso, Eur. Stroke J., 3, 309 (2018).
7. L. Colarusso, M. Serafini, Y. Trolle Lagerros, O. Nyren, C. La Vecchia, M. Rossi, W. Ye, A. Tavani, H.-O. Adami, A. Grotta, R. Bellocco, Nutrition, 33, 234 (2017).
8. I. Abete, M. A. Zulet, E. Goyenechea, V. Blazquez, A. M. de Arce Borda, A. Lopez de Munain, J. A. Martinez, Nutr. Neurosci., 21, 70 (2016).
9. A. Bicvic, H. Hammer, H. Sarikaya, M. R. Heldner, Ther. Umsch., 78, 259 (2021).
10. L. Chen, W. Tang, X. Wu, R. Zhang, R. Ding, X. Liu, X. Tang, J. Wu, X. Ding, Nutrients, 15, 222 (2023).
11. K.-W. Liang, C.-L. Lee, W.-J. Liu, Nutrients, 14, 3203 (2022).
12. I. Abete, M. A. Zulet, E. Goyenechea, V. Blazquez, A. M. de Arce Borda, A. L. de Munain, J. A. Martinez, Nutr. Neurosci., 21, 70 (2018).
13. A. Aigner, H. Becher, S. Jacobs, L. -R. Wilkens, C. -J. Boushey, L. L. Marchand, C. -A. Haiman, G. Maskarinec, Eur. J. Clin. Nutr., 72, 1035 (2018).
14. T. Kennedy, J. Ohls, S. Carlson, K. Fleming, J. Am. Diet. Assoc., 95, 1103 (1995).
15. M. L. McCullough, D. Feskanich, M. J. Stampfer, E. -L. Giovannucci, E. -B. Rimm, F. -B. Hu, D. Spiegelman, D. -J. Hunter, G. -A. Colditz, W. -C. Willett, Am. J. Clin. Nutr., 76, 1261 (2002).
16. L. A. Kilpatrick, H. M. An, S. Pawar, R. Sood, A. Gupta, Curr. Obes. Rep., 12, 1 (2023).
17. A. Duprez, D. R. Jacobs Jr, P. L. Lutsey, D. Herrington, D. Prime, P. Ouyang, R. G. Barr, D. A. Bluemke, Ethn. Dis., 19, 243 (2009).
18. S. K. Y. Li, M. M. P. Wan, F. P. L. Siu, S. Chung, M. Y. C. Pang, Calcif. Tissue Int., 101, 259 (2017).
19. R. Micha, J. L. Peñalvo, F. Cudhea, F. Imamura, C. D. Rehm, D. Mozaffarian, JAMA, 317, 912 (2017).
20. L. Colarusso, M. Serafini, Y. T. Lagerros, O. Nyren, C. L. Vecchia, M. Rossi, W. Ye, A. Tavani, H. Adami, A. Grotta, R. Bellocco, Nutrition, 33, 234 (2017).
21. M. Lawrence, E. Asaba, E. Duncan, M. Elf, G. Eriksson, J. Faulkner, S. Guidetti, B. Johansson, C. Kruuse, D. Lambrick, C. Longman, L. von Koch, X. Wang, O. Lennon, BMC Res. Notes, 12, 1 (2019).
22. D. Kirk, E. Kok, M. Tufano, B. Tekinerdogan, E. J. M. Feskens, G. Camps, Adv. Nutr., 13, 2573 (2022).
23. M. Côté, B. Lamarche, Appl. Physiol. Nutr. Metab., 46, 1 (2021).
24. Y. Eneva, B. Dogan, J. Lifestyle SDGs Rev., 5, e03545 (2025).
25. S. Liu, A. P. Wright, B. L. Patterson, J. P. Wanderer, R. W. Turer, S. D. Nelson, A. B. McCoy, D. F. Sittig, A. Wright, medRxiv, 2023, 1 (2023).
26. A. Rao, M. Pang, J. Kim, M. Kamineni, W. Lie, A. K. Prasad, A. Landman, K. J. Dreyer, M. D. Succi, medRxiv, 2023, 1 (2023).

27. H. J. Kao, T. W. Chien, W. C. Wang, W. Chou, J. C. Chow, Medicine, 102, e34068 (2023).
28. P. M. Ridker, M. Cushman, M. J. Stampfer, R. P. Tracy, C. H. Hennekens, N. Engl. J. Med., 336, 973 (1997).
29. K. Esposito, D. Giugliano, Eur. Heart J., 25, 1845 (2004).
30. D. E. King, B. M. Egan, R. F. Woolson, J. Am. Coll. Nutr., 22, 368 (2003).
31. L. Galland, Nutr. Clin. Pract., 25, 634 (2010).
32. P. C. Calder, Nutrients, 2, 355 (2010).
33. D. S. Pratt, M. M. Kaplan, N. Engl. J. Med., 342, 1266 (2000).
34. J. B. Buse, S. Caprio, W. T. Cefalu, A. Ceriello, S. Del Prato, S. E. Inzucchi, S. McLaughlin, G. L. Phillips, R. P. Robertson, F. Rubino, R. Kahn, M. S. Kirkman, Diabetes Care, 32, 2133 (2009).
35. W. Geidl, S. Schlesinger, E. Mino, L. Miranda, K. Pfeifer, Eur. J. Prev. Cardiol., 27, 659 (2020).
36. M. A. Cornier, J. P. Després, N. Davis, D. A. Grossniklaus, S. Klein, B. Lamarche, F. Lopez-Jimenez, G. Rao, M. St-Onge, A. Towfighi, P. Poirier, Circulation, 124, 1996 (2011).
37. . Reaven, Diabetes, 37, 1595 (1988).
38. S. E. Chiuve, T. T. Fung, E. B. Rimm, F. B. Hu, M. L. McCullough, M. Wang, M. J. Stampfer, W. C. Willett, J. Nutr., 142, 1009 (2012).
39. M. L. McCullough, W. C. Willett, Public Health Nutr., 9, 152 (2006).
40. T. T. Fung, E. B. Rimm, D. Spiegelman, N. Rifai, G. H. Tofler, W. C. Willett, F. B. Hu, Arch. Intern. Med., 161, 1857 (2001).

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Gastroprotective action of capsaicin: a mini-review

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Capsaicin is a pungent ingredient of chilli peppers that triggers the nociceptive pain pathway by activating the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor. There are stereotypes which claim that capsaicin is one of the phytochemicals that cause gastric injuries. This study aims to explore the recently published research data about the beneficial and protective effects of capsaicin on the gastric mucosa. Research papers and reviews from the period 2005-2024 were thoroughly examined in databases PubMed and Google Scholar. Keywords "gastroprotection" and "capsaicin" were used to identify the articles, directly associated with a gastroprotective effect of capsaicin, including preclinical experiments with animals, and human studies. As a result, the seven main possible mechanisms of its gastroprotective action were reviewed and summarised. Capsaicin leads to a dose-dependent decrease in basal acid output (BAO) and the concentration of ions in the gastric juice also decreases (except for Na^+). Gastric transmucosal potential difference (GTPD) is increased significantly, even after ethanol administration. Indomethacin-induced gastric bleeding is prevented by capsaicin. It also shows anti-inflammatory properties and reduces oxidative stress. Capsaicin-sensitive nerve endings have an important role in gastroprotection: they release calcitonin gene-related peptide (CGRP) or other vasodilators which enhance the microcirculation. The hyperaemic response plays a huge part in ulcer healing. Moderately spicy foods containing low concentrations of capsaicin protect against injurious substances and prevent gastric mucosal damage by stimulating sensory neurons. The secreted neurotransmitters provide gastroprotection by enhancing the blood flow.

Keywords: capsaicin, gastroprotection, capsaicin-sensitive nerve endings

INTRODUCTION

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the component of chilli peppers, responsible for their pungency. It is not distributed evenly inside the chilli pepper: higher concentrations were found in the inner white flesh around the seeds (placenta). In order to cause a spicy sensation, capsaicin excites afferent nociceptive neurons by changing their membrane permeability. All this is possible due to the activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor, a tetrameric structure which allows the influx of sodium and calcium ions [1]. Calcium influx causes mitochondrial function loss and makes the nerve fibers inoperant. The production of mediators from the fibers decreases. One of these mediators is the potent local pain mediator: the substance P [2]. Other activators of the TRPV1 receptor are low extracellular pH, divalent cations (Mg^{2+}, Ba^{2+}), noxious heat, pain-producing exogenous and endogenous substances [1]. The sensation that capsaicin brings is deliberately sought by many people. Spicy foods are a popular staple for many cuisines across the globe. A common belief claims that excessive consumption of chilli peppers has an evident role in gastric ulceration. The main factors implicated in the causation of ulcers are increased acid secretion, abuse of non-steroid anti-inflammatory drugs (NSAIDs) and ethanol, reduced blood flow in the region of the gastric mucosa [3]. Human interventional studies and preclinical experiments with animals have come to controversial conclusions about the harmful effect of capsaicin on the stomach. Few properties of capsaicin, which break all stereotypes, associated with the formation of ulcers, have been discovered.

Our study aims to explore the main mechanisms of the beneficial and protective effects of capsaicin on the gastric mucosa.

MATERIALS AND METHODS

In order to gather all articles, directly corresponding to the topic, databases PubMed and Google Scholar were used. A period of 2004 – 2025 was set to identify the more recent research papers. Inclusion criterion was the keywords "capsaicin" or "gastroprotection" in the title. Seventeen manuscripts were thoroughly reviewed and many

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experiments both on humans and rodents with low concentrations of capsaicin were explored.

RESULTS AND DISCUSSION

Decrease in basal acid output (BAO)

Gastric juice is produced by gastric glands in the stomach mucosa, containing different types of cells. To notice changes in acid secretion, Mozsik *et al.* [4] suctioned gastric juice every 15 min for 1 hour (basal acid output, BAO) and inserted intragastrically small doses of capsaicin (100-800 μg), dissolved in 100 ml of saline solution. The change in BAO (measured in mEq/h) was dose-dependent and the median effective dose 50 (ED50) value was around 400 μg. A dose of 100 μg capsaicin is responsible for 1.1 mEq/h BAO, while 800 μg for 0.2 mEq/h. Decrease in acid output protects the lining of the digestive tract and prevents ulceration. It was proven that electrical stimulation of the vagal nerve causes maximal secretion. The output (after vagal stimulation and capsaicin application) decreased by 28.1% at 100 mg/ml and by 34.8% at 500 mg/ml. Decrease in gastric juice secretion is explained by H^+ back-diffusion [4-7].

Change in gastric juice composition

Application of capsaicin changes the concentration of different ions and proteins, secreted from the gastric glands [4]. H^+, K^+, Ca^{2+}, Mg^{2+} levels decrease, whereas Na^+ and albumin levels increase. The decrease is associated with the decline in hydrochloric acid secretion. Concentration of H^+ without pretreatment with capsaicin is around 43±3 mEq/L and changes to 25±1 mEq/L after capsaicin is applied. For K^+ the value without capsaicin is 13±1 mEq/L and lowers to 8±0, 6mEq/L. Ca^{2+} goes from 0.98±0.02 to 0.88±0.01. The concentrations of these three ions are measured by photoelectric flame photometry [4]. The rise in Na^+ levels is remarkable: 73±4 mEq/L turns into 89±2 mEq/L after administration of the pungent ingredient. All these changes are statistically significant and have a p value <0.001 [4]. Albumin is the protein component of the gastric juice, found in small amounts. Capsaicin usually increases albumin levels from 1.24 ± 0.001 g/L to 1.63 ± 0.02 g/L [6, 7]. Variations in the “parietal” (Cl^- linked to H^+) and “non-parietal” (Cl^- linked to Na^+) components of the gastric juice can be identified after measurement of cation concentrations and Cl^-, a principle known as Hollander’s method. Due to the increase in H^+ back diffusion, a notable decline in the “parietal” element is noticed, leading to elevation in the “non-parietal” (a buffering part, which can’t be produced in passive metabolic processes) [6, 7].

Effect on the gastric transmucosal potential difference (GTPD)

Gastric transmucosal potential difference is generated due to the ion transportation through the gastric mucosa and its electrical resistance. The gastric mucosal barrier plays an important role in prevention of damage to the stomach lining. Epithelial cells on the surface are part of the barrier due to their ability to transport hydrogen and chloride ions out of the mucosa and sodium ions into the plasma. Many factors destroy the gastric barrier: ethanol, NSAIDs, bile salts [8]. The destruction is followed by H^+ back-diffusion and a decline in potential difference. Capsaicin has the ability to increase GTPD dose-dependently. GTPD is measured endoscopically with one electrode passed through the biopsy force channel of the gastroscope and the other positioned on the forearm. The noted increase reaches maximum within 3-5 min [4, 6, 7].

Ethanol has the ability to induce changes in GTPD. In an experimental study [4], ethanol was administered intragastrically through the biopsy channel of a gastroscope with and without capsaicin. On its own, the alcohol caused a significant decrease in GTPD (Δ 25 (−mV)), which was prevented with capsaicin [4, 6]. Early studies [9] have come to the conclusion that ethanol causes erythema in the gastric mucosa. Reduction in blood flow leads to swift destruction of the stomach walls. This reduction is characterized by venule constriction, accompanied by arteriolar dilatation. Application of capsaicin further dilated the arterioles but also blocked the ethanol-induced constriction by release of calcitonin gene-related peptide [9, 15-17]. Maximum dilatation of the arterioles after capsaicin was reached within 3 min [9]. Ethanol can also induce oxidative damage in rats, but capsaicin manages to inhibit the lipid peroxidation and myeloperoxidase activity, a marker enzyme, released by polymorphonuclear leukocytes (PMNs) [10].

Antioxidant and anti-inflammatory properties

Indomethacin is a nonsteroidal anti-inflammatory drug used to treat moderately acute pain, which is known to change the mitochondrial structure in gastric mucosal cells. Mitochondrial dysfunction is followed by an increase in reactive oxygen species. Superoxide inactivates the enzyme aconitase, iron is released and hydroxyl radical (·OH) is produced. ·OH leads to apoptosis and gastric injuries [11]. The microbleeding after intake of indomethacin can be determined by calculating the concentration of hemoglobin in the gastric juice (Fisher and Hunt method). Before application of indomethacin blood losing was around 2.0 ± 0.02 mL/day for a period of 1 day. After the NSAID was given, the baseline increased to 8.1 ± 0.02 mL/day. Orally given capsaicin in concentrations of 200, 400 and 800 μg prevented the microbleeding and proved the gastroprotective effect of chillies by inhibition of the COX enzymes [4, 6, 7].

Ethanol consumption may lead to serious health conditions such as gastritis, gastric ulcer, gastric cancer. It damages the gastric mucosa, which results in pro-inflammatory cytokine imbalance. Neutrophils and reactive oxygen species (ROS) accumulate in the site of the damage. Experiments *in vitro* [12] show that capsaicin limits the transcription of chemokine receptor 4 (CCR4) which plays the role of a marker for oxidative damage. Src and p47phox expression is also suppressed. Src (a tyrosine protein kinase) regulates the activity of NADPH oxidase, which is a source of ROS and thus acts as a modulator of oxidative stress. P47phox is part of NADPH oxidase, which needs serine phosphorylation in order for the enzyme to be activated. The gastroprotective effect of capsaicin is associated with reduced ROS generation due to suppression of NADPH oxidation [12]. Another damage to the gastric mucosa, caused by ethanol, is increased lipid peroxidation, an index for which is the concentration of malondialdehyde (MDA). Capsaicin inhibits this process by scavenging ROS, responsible for the peroxidation. The inhibition may occur at the level of the membrane by altering oxidoreductase activity. Ethanol induces the myeloperoxidase activity, but a significant decrease is noted (4.6%, 54.1%, 59.4%) with capsaicin doses of 0.5, 5.0, 10 mg/kg BW. The inhibited lipid peroxidation limits the number of PMNs at the site of the damage. Inflammatory processes are associated with the expression of the enzyme COX-2, related to prostaglandins production (arachidonic acid pathway). Capsaicin prevents the ethanol-stimulated expression of the enzyme, again proving its anti-inflammatory properties [10]. Treatment with capsaicin lowers mRNA expression levels not only of COX-2 but other proinflammatory molecules such as TNF-α, IL-1β, IL-6 [13]. Capsaicin also inhibits the production of IL-8 from *Helicobacter pylori* infected gastric epithelial cells. Not only was the secretion of the protein affected but also the IL-8 mRNA expression, which decreased. This interleukin brings neutrophils to the inflamed mucosa. *H. pylori* is a gram-negative bacterium which causes serious harm to the gastric mucosa. In response to the bacterium, gastric cells up-regulate the expression of genes modulated by nuclear factor kappa B (NF-κB), a dimeric transcription factor. The dimers are in an inactive state and are located in the cytoplasm, held by inhibitory kappa B (IκB) proteins. Activation of the factor happens after phosphorylation of IκB and a following proteolysis, mediated by ubiquitin, which sets the dimers free and they translocate in the nucleus. NF-κB is a regulatory element for IL-8 expression so it comes as no surprise that capsaicin suppressed the activation of NF-κB, induced by the bacterium [14].

Effect on ulcer healing

The first layer of protection in the stomach consists of the mucosa-bicarbonate-phospholipid barrier and the epithelial cells, connected with tight and gap junctions. NSAIDs, ethanol and other substances damage the epithelial barrier, which results in an increased H^+ back-diffusion [15]. The barrier's main function is to prevent the contact between hydrochloric acid in the lumen and the gastric mucosa. Without it, serious conditions, such as gastric ulcers, may develop. Another way, in which the mucosa may keep its integrity, is with the help of a neurotransmitter, secreted from capsaicin-sensitive sensory nerves (after activation of the TRPV1 receptor) – calcitonin gene-related protein (CGRP). CGRP is mainly synthesized in the dorsal root ganglion and is transported to the peripheral nerve endings. The main physiological effects of the peptide are vasorelaxation (it is known as the most potent vasodilator) and inotropic actions. There is also evidence that CGRP inhibits gastric acid secretion (in a rat experimental model an estimated 63–78 % inhibition was noticed [16]). CGRP influences the blood flow through adenosine triphosphate sensitive potassium channels (KATP channels), the increase in blood flow helps with the repairment of the gastric mucosa and protects against ulcerations. The peptide is also known to increase nitric oxide (NO) by influencing the inhibitor of nitric oxide synthase: asymmetric dimethylarginine, ADMA [16]. The subserosal injection of acetic acid in the stomach of rats, which leads to chronic gastric ulcers, was used to explore the importance of sensory neurons in gastroprotection. 24 h after the injection necrosis is present and after 1 week the condition reaches a "chronic" state. Daily consumption of capsaicin managed to decrease the size of the ulcer a week later. Rats with ablated sensory nerves showed an increase in the size of the damaged area [17].

CONCLUSIONS

Spicy foods containing low concentrations of capsaicin (50 μg – 800 μg) have the ability to provide gastroprotection. The effect of injurious substances such as ethanol and indomethacin can be significantly reduced with the activation of sensory neurons and the TRPV1 receptor. The secreted neurotransmitters enhance the blood flow and thus repair damages to the gastric mucosa. Reduction of oxidative stress turns capsaicin into an underrated gastroprotective drug.

REFERENCES

1. F. Yang, J. Zheng, *Protein Cell*., **8 (3),** 169 (2017).
2. A. Chang, A. Rosani, J. Quick, *StatPearls - NCBI Bookshelf* (2023).
3. M. Satyanarayana, *Crit. Rev. Food Sci. Nutr.*, **46 (4),** 275 (2006).
4. G. Mózsik, J. Szolcsányi, I. Rácz *World J. Gastroenterol*., **11 (33),** 5180 (2005).
5. K. Imatake, T. Matsui, M. Moriyama, *J Gastroenterol*.; **44 (5),** 396 (2009).
6. G. Mózsik, J. Szolcsányi, A. Dömötör *Inflammopharmacology*, **15 (6),** 232 (2007).
7. G. Mózsik *Prog, Drug Res*., **68,** 209 (2014).
8. V. Nosál'ová, A. Babul'ová, V. Bauer, *Gen. Pharmacol.*, 3**0 (3),** 3259 (1998).
9. T. Saeki, T. Ohno, K. Boku, K. Saigenji, M. Katori, M. Majima, *Alimentary Pharmacology & Therapeutics.*, **14(s1)**, 135 (2000).
10. J. S. Park, M. A. Choi, B. S. Kim, I. S. Han, T. Kurata, R. Yu, *Life Sciences*, **67**(25), 3087 (2000).
11. P. Maity, S. Bindu, S. Dey, M. Goyal, A. Alam, C. Pal, K. Mitra, U. Bandyopadhyay, *Journal of Biological Chemistry*, **284 (5),** 3058 (2009).
12. Z. Yang, H. Guo, P. Zhang, K. Liu, J. Ba, X. Bai, S. Shama, B. Zhang, X. Gao, J. Kang, *Chin. J. Nat. Med*., **22 (0),** 1 (2024).
13. E. J. Mendivil, A. Sandoval-Rodriguez, A. Meza-Ríos, L. Zuñiga-Ramos, A. Dominguez-Rosales, M. V. Mercado, L. Sanchez-Orozco, A. Santos-Garcia, J. Armendariz-Borunda, *Journal of Functional Food*, **59,** 345 (2019).
14. I. O. Lee, K. H. Lee, J. H. Pyo, J. H. Kim, Y. J. Choi, Y. C. Lee, *Helicobacter*, **12 (5),** 510 (2007).
15. J. Szolcsányi, *InTech*. (2014).
16. X. J. Luo, B. Liu, Z. Dai, *Dig. Dis. Sci.*, **58,** 320 (2013).
17. S. Evangelista, *Curr. Pharm. Des*., **12 (23)**, 2977 (2006).

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Lipid content analysis in naval cadets at admission: variations across academic years

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Lipids play a crucial role in human physiology, particularly for maritime professionals who require optimal physical endurance, fatigue resistance, and effective thermoregulation. This study evaluates the lipid content among cadets at the Nikola Vaptsarov Naval Academy to assess their physiological adaptation and preparedness for maritime activities. A total of 113 cadets from various specializations were assessed between 2021 and 2023 using bioimpedance analysis. The results indicate a significant decrease in lipid content from first-year cadets (average: 23.7%) to fifth-year cadets (average: 19.0%), corresponding to a reduction in BMI from 25.1 kg/m² to 22.8 kg/m². These findings underscore the effectiveness of structured physical training and nutritional programs in improving cadets' physical fitness and readiness for maritime duties.

Keywords: lipids, lipid content, maritime professionals, cadets

INTRODUCTION

Lipids are fundamental components of the human body, serving as key elements in energy metabolism, protection of internal organs, and thermoregulation [1]. For individuals engaged in maritime professions, maintaining appropriate lipid levels is vital due to the demanding physical and environmental conditions they encounter. Adequate lipid reserves contribute to buoyancy and effective thermoregulation, which are critical for survival during maritime incidents and exposure to cold water environments [2, 3]. Moreover, optimal lipid content enhances physical endurance and reduces fatigue, directly impacting the performance and safety of maritime personnel [4].

Previous studies have explored the anthropometric and body composition profiles of various athletes and professionals, emphasizing the importance of tailored physical conditioning [5, 6]. However, there is limited research specifically focusing on the lipid content of naval cadets during their training period. Understanding these parameters can inform the development of targeted interventions to improve the health and operational readiness of future maritime professionals.

This study aims to evaluate the lipid content among cadets at admission to the Nikola Vaptsarov Naval Academy, analyzing variations across different academic years and providing insights into their physical preparedness for maritime duties.

MATERIALS AND METHODS

Study design and participants

This study was conducted between 2021 and 2023 and included 113 cadets from the Nikola Vaptsarov Naval Academy in Varna, Bulgaria. All participants voluntarily took part in the study across different academic years, from the first to the fifth year, allowing for a comprehensive assessment of lipid content changes throughout their training.

The inclusion criteria required cadets to be enrolled at the Naval Academy during the study period, to have provided informed consent for participation, and to have no known medical conditions affecting lipid metabolism.

Data collection

Data were collected during scheduled physical assessments at the Academy's facilities. The following parameters were measured and recorded for each participant:

- Age
- Gender
- Height (cm)
- Weight (kg)
- Body mass index (BMI)
- Body fat percentage (lipid content)

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Measurement techniques

- *Anthropometric measurements*

Height and weight were measured using standardized equipment with participants barefoot and wearing light clothing. BMI was calculated using the formula: BMI = weight (kg) / [height (m)]².

- *Bioimpedance analysis (BIA)*

Lipid content (body fat percentage) was assessed using bioimpedance analysis, a non-invasive and reliable method for estimating body composition [8]. Measurements were conducted using standardized bioimpedance scales. Participants were instructed to avoid food and intense physical activity for at least 2 hours prior to assessment to ensure accuracy.

- *Data analysis*

Comparative analyses were performed to identify differences in lipid content across academic years and between genders.

RESULTS AND DISCUSSION

Overall findings

The study revealed that the average BMI among cadets across all academic years was within the normal range (18.5–24.9 kg/m²) as defined by the World Health Organization. However, variations in lipid content were observed, with certain groups exhibiting higher than optimal levels.

First-year cadets

The first-year cadets demonstrated the highest average lipid content and BMI, with values slightly exceeding the normal range. This may be attributed to the transition from civilian to military lifestyle, decreased physical activity prior to admission, and lack of established fitness routines. These findings suggest the need for early intervention programs focusing on physical conditioning and nutritional education upon admission.

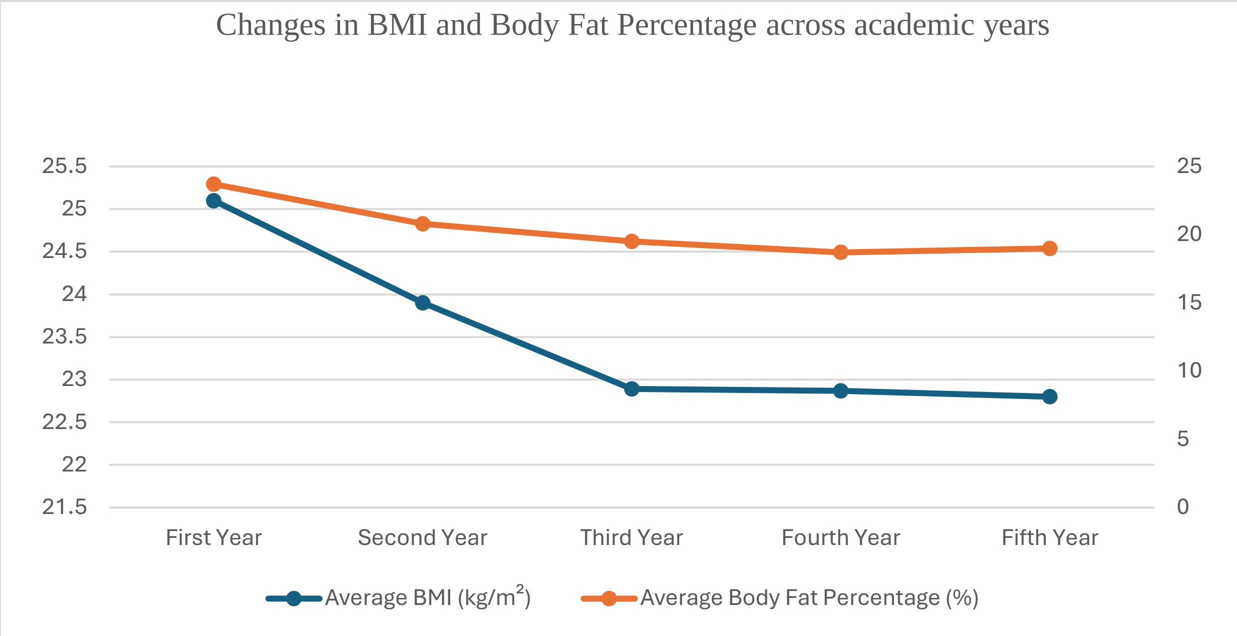
Upper-year cadets

As cadets progress through their training, they demonstrate the effectiveness of structured physical activity and nutritional guidance. The second year marks a notable decrease in BMI and lipid content compared to the first year, reflecting early adaptation to the rigorous training regimen. This trend continues into the third year, where cadets maintain a stable BMI of 23.9 kg/m² while further reducing their lipid levels, reinforcing the impact of sustained physical engagement.

By the fourth year, cadets exhibit a further reduction in lipid content, achieving an average BMI of 22.9 kg/m², indicating a stable physical condition suitable for the demands of their training and future maritime responsibilities. Some isolated cases of slightly elevated BMI are observed, potentially due to reduced physical activity caused by increased academic workload. In the fifth year, these trends remain consistent, with cadets reaching an average BMI of 22.8 kg/m². This stability suggests that they have achieved physical maturity and conditioning that aligns with the requirements of their maritime careers.

Table 1. Summary of cadet measurements across academic years

Academic Year	Number of Participants	Gender (M/F)	Age (Min/Avg/Max)	Height (cm) (Min/Avg/Max)	Weight (kg) (Min/Avg/Max)	BMI (Min/Avg/Max)	Body Fat % (Min/Avg/Max)
First Year	25	22M / 3F	18 / 19 / 20	165 / 176 / 191	50 / 79.5 / 105.9	20.7 / 25.1 / 30.2	12.9 / 23.7 / 34.1
Second Year	22	19M / 3F	19 / 20 / 21	160 / 174 / 182	50 / 76 / 100	18.8 / 24.5 / 30.2	12.6 / 20.8 / 34.5
Third Year	21	18M / 3F	20 / 21 / 22	160 / 173 / 182	53 / 74 / 100	18.9 / 23.9 / 30.2	12.9 / 19.5 / 29.1
Fourth Year	23	19M / 4F	21 / 22 / 23	154 / 174 / 192	45 / 74 / 105	18.6 / 22.9 / 31.6	15.0 / 18.7 / 41.1
Fifth Year	22	17M / 5F	22 / 23 / 24	152 / 175 / 188	49 / 73 / 100	17.9 / 22.8 / 30.0	16.9 / 19.0 / 39.6



Graph 1. Changes in BMI and body fat percentage across academic years

The comprehensive analysis presented in Graph 1 illustrates a steady decline in BMI and body fat percentage across academic years, indicating the impact of naval training on cadets' body composition.

BMI decreases from 25.1 kg/m² in first-year cadets to 22.8 kg/m² in fifth-year cadets, suggesting gradual weight regulation and adaptation to physical training.

Body fat percentage follows a similar trend, dropping from 23.7% to 18.7% by the fourth year, before slightly increasing to 19.0% in the fifth year.

The sharpest decline occurs between the first and third years, likely due to the initial impact of structured physical activity.

The small increase in body fat in the final year may be linked to reduced physical training as academic demands rise.

Overall, the data reflect effective physical conditioning, with early-year cadets experiencing the most significant changes in BMI and body fat.

Gender differences

Analysis across genders revealed that male cadets generally had higher lipid content compared to female cadets, although both remained within acceptable ranges. These differences highlight the importance of gender-specific training and nutrition programs to ensure optimal health outcomes.

CONCLUSION

This study demonstrates a progressive decrease in lipid content and BMI among naval cadets from their first to final academic year, reinforcing the effectiveness of the Academy's structured physical training and nutritional programs. The transition from civilian life to a regimented training schedule significantly impacts body composition, with first-year cadets displaying the highest BMI and lipid content. The gradual decrease in these metrics throughout the training period suggests that continuous physical conditioning and lifestyle modifications play a crucial role in achieving optimal body composition. Maintaining appropriate lipid levels is essential for buoyancy, thermoregulation, and overall endurance in maritime operations. These findings highlight the necessity of tailored fitness and nutrition programs throughout cadets' academic tenure, ensuring their preparedness for the physically demanding conditions of maritime service.

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REFERENCES

1. J. Bourgois, A. L.Claessens, M. Janssens, et al. *Journal of Sports Sciences*, **19**(3), 191 (2001).
2. D. Barbieri, L. Zaccagni, V. Babic, M. Rakovac, M. Misigoj-Durakovic, E. Gualdi-Russo, *Journal of Sports Medicine and Physical Fitness*, **57**(9), 1132 (2017).
3. D. Stavrev, Water traumatism at sea. Projections for Bulgaria. Medical University of Varna, 2022 (in Bulgarian).
4. J. M. Cortell-Tormo, J. A. Perez-Turpin, R. Cejuela-Anta, J. J. Chinchilla-Mira, M. J. Marfell-Jones, *Journal of Human Kinetics*, **23**, 95 (2010).
5. A. J. Sanchez-Oliver, I. Caraballo, A. Perez-Bey, et al., *Journal of Exercise Science & Fitness*, **21**, 125 (2023).
6. H. D. McCarthy, et al., *International Journal of Obesity*, **30**, 1340 (2006).
7. D. Stavrev, Compendium of Algorithms for the Examination of Marine Professionals. Part 1, Medical University of Varna, 2023 (in Bulgarian).
8. D. Gallagher, et al., *American Journal of Clinical Nutrition*, **72**(3), 694 (2000).
9. M. J. Tipton, *Journal of Applied Physiology*, **120**(5), 493 (2016).

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Prevention and management of cow's milk allergy in infants

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This review explores current strategies for the prevention and management of cow's milk allergy (CMA) in infants, emphasizing the role of breastfeeding, hydrolyzed formulas, and early allergen exposure. Exclusive breastfeeding is the preferred approach to reduce allergy risk, offering various protective immune mechanisms. The use of partially hydrolyzed formulas (pHF) has been investigated for CMA prevention, with mixed results; while some studies suggest benefits in reducing atopic dermatitis and respiratory symptoms, recent evidence questions its efficacy. Extensively hydrolyzed formulas (eHF) are recommended as the first-line treatment for infants with CMA, but variations in peptide profiles necessitate stricter standards and better testing. Amino acid-based formulas (AAF) are utilized in severe CMA cases where eHF are ineffective, though their long-term use poses risks such as hypophosphatemic bone disease. The potential of early exposure to cow's milk proteins (CMP) to promote tolerance highlights that timing and individual factors might significantly influence outcomes. Emerging evidence points to the importance of personalized approaches in CMA management, recognizing that dietary and environmental factors play critical roles in allergy development.

Keywords: cow's milk allergy, breastfeeding, extensively hydrolyzed formulas, partially hydrolyzed formulas, amino

INTRODUCTION

Food allergies (FAs) have become a significant health issue over the past decades since they have been increasing in prevalence worldwide, particularly among infants and young children. [1] The allergic reaction occurs due to pathological immune reaction of hypersensitivity, triggered by specific food protein allergens. Cow's milk allergy (CMA) is one of the most prevalent FAs in infants, affecting up to 3.8% of young children in developed countries [2]. The allergy usually occurs in the first two years of life and is frequently one of the earliest food allergies identified in infants. CMP are commonly among the first food proteins they encounter, as it is the primary source of nutrition either through breastfeeding (with CMP passed through maternal diet) or formula feeding [3]. CMA is a multifactorial condition that can be presented with both IgE-mediated and non-IgE-mediated mechanisms. It is characterized by a consistent immune reaction to one or more CMP, typically casein or serum β-lactoglobulin [4].

It's crucial to differentiate CMA from other adverse reactions to CM, lactose intolerance in particular, as they involve distinct underlying mechanisms and require different management approaches. CMA is an immune-mediated response to proteins found in milk, leading to allergic reactions, whereas lactose intolerance is not immune-mediated and results from the body's inability to digest lactose, due to a deficiency of the enzyme lactase [5].

Our aim is to identify, elucidate and analyze the mechanism laying behind CMA and the contemporary strategies for its prevention and management.

PATHOGENESIS OF COW'S MILK ALLERGY

Cow's milk proteins

Cow's milk contains about 32 g of protein per liter, which is divided into two main fractions: caseins (~80%) and whey proteins (~20%). [6, 7] (Table 1). Cow's milk allergens are labeled "Bos d," based on the genus and species name (*Bos domesticus*), followed by an identification number [9]. For instance, Bos d 8 refers to all caseins, which are further categorized: Bos d 9 for αs1-casein (~32% of total casein), Bos d 10 for αs2-casein (~10%), Bos d 11 for β-casein (~28%), and Bos d 12 for κ-casein (~10%) [10]. These caseins are classified as secreted calcium-binding phosphoproteins with a loose structure, forming quaternary structures known as casein micelles. These micelles have a hydrophobic core of αs1, αs2, and β-caseins interacting with calcium phosphate, and a hydrophilic surface layer of κ-casein [11]. The

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structure of casein micelles is dynamic, changing with pH, temperature, and pressure. For example, rennet treatment causes micelles to lose solubility and form aggregates. Caseins are major allergens, implicated in over 50% of IgE-mediated cow's milk allergy (CMA) cases [12].

In the whey protein fraction, the most abundant protein is β-lactoglobulin (Bos d 5, ~10% of total proteins), followed by α-lactalbumin (Bos d 4, ~5%), immunoglobulins (Bos d 7, ~3%), bovine serum albumin (Bos d 6, ~1%), and lactoferrin (<1%). β-lactoglobulin and α-lactalbumin are major allergens, characterized by globular structures stabilized by disulfide bridges [13-15]. Although bovine serum albumin is present in small amounts, it is a common allergen, with up to 50% of CMA patients producing IgE against it. [16, 17] Along with lactoferrin, bovine serum albumin has a high number of disulphide bridges, making its structure very stable even under denaturing conditions. [18] While lactoferrin is not registered as an allergen, studies indicate its potential to trigger allergic reactions. [9, 19-21]

CMA usually involves reactivity to multiple CMP, both caseins and whey proteins [22]. IgE antibodies target specific regions within these proteins, known as epitopes, which can be linear (continuous amino acid sequences) or conformational (discontinuous sequences formed by protein folding). Both types of epitopes are present in cow's milk allergens [23-25].

Pathophysiologic mechanisms of cow's milk allergy

Food allergies can be classified into IgE-mediated and non-IgE-mediated types based on their underlying mechanisms [26]. IgE-mediated food allergies are the most well-known and studied type, consisting of two phases: sensitization and elicitation [27-29]. During the initial exposure to food proteins, sensitization may occur when the immune system first encounters the antigens. Antigen-presenting cells (APCs), primarily dendritic cells (DCs), process the food proteins into smaller peptides and present them on their surface main histocompatibility complex (MHC) II molecules to T-cell receptors (TCRs) on naïve T cells specific to the peptide. This triggers T cell activation, facilitated by the interaction of CD28 on T cells with CD80 and CD86 on DCs, along with co-stimulatory signals from pro-inflammatory cytokines like IL-4, IL-25, IL-33, and TSLP [30, 31] . These signals cause naïve T cells to differentiate into CD4+ Th2 cells [32, 33].

The activated Th2 cells then engage with naïve B cells through their TCRs and MHC II-bound allergens on the B cells, alongside signals from IL-4 and IL-13 secreted by Th2 cells. This interaction promotes the maturation of B cells into plasma cells that secrete IgE specific to the food allergen. These IgE molecules bind to high-affinity FcεRI receptors on the surface of tissue mast cells or blood basophils, completing the sensitization phase [27, 34].

The elicitation phase occurs with subsequent exposure to the same or cross-reactive food allergens, where these allergens cross-link FcεRI-bound IgEs on mast cells and basophils, leading to their degranulation and the release of mediators such as histamine. These mediators cause the symptoms of a food allergic reaction, which can affect multiple organs, leading to gastrointestinal issues, respiratory inflammation, skin and eye itching and swelling, and in severe cases, life-threatening anaphylaxis [27].

Non-IgE-mediated cow's milk allergy engages distinct immunological pathways, predominantly involving T cell-mediated mechanisms rather than the classical IgE antibody-driven responses. The strongest evidence points to the involvement of food allergen-specific suppressor CD8 T cells in patients with food protein-induced enteropathy (FPE). The presence of food-specific IgE antibodies locally in the gut, despite their absence in the bloodstream, suggests a role for local mucosal IgE in these conditions [33]. Food protein-induced enterocolitis syndrome (FPIES) is often thought to be mediated by T cells, although research on T cells in FPIES patients is limited. Some studies have shown T-cell proliferation in response to food antigens, but the stimulation index is not consistently different from that of nonallergic controls [31]. Increased levels of intestinal interferon-gamma (IFN-g) have been associated with villous damage, and in FPIES patients, an imbalance between intestinal tumor necrosis factor-alpha (TNF-a) levels and reduced transforming growth factor-beta (TGF-b) expression has been observed [35]. It is suggested that T-cell activation by food allergens might trigger local intestinal inflammation by releasing proinflammatory cytokines, such as TNF-a and IFN-g, leading to increased intestinal permeability and fluid shift [35, 36]. The clinical manifestation of symptoms usually appears several hours to days following the ingestion of cow's milk. Unlike IgE-mediated allergies, non-IgE-mediated food hypersensitivities predominantly target the gastrointestinal system, with minimal involvement of the cutaneous or respiratory systems [37]. The symptoms include diarrhea, constipation, vomiting, abdominal pain, gastroesophageal reflux disease (GERD).

PROPHYLAXIS AND MANAGEMENT OF COW'S MILK ALLERGY

The literature reveals diverse strategies aimed at reducing the incidence and managing CMA in infants. Primary prevention strategies should be implemented in infants considered at high risk for developing CMA. This group includes infants reported with first-degree relatives with a history of FAs, history of atopic dermatitis or other allergic conditions and delayed introduction of common allergenic food during infancy [38].

The role of maternal diet in cow's milk allergy prophylaxis

There is limited evidence to support altering the maternal diet during pregnancy or lactation to prevent CMA [39]. Kramer and Kakuma analyzed five trials and concluded that avoiding allergens during pregnancy and lactation is unlikely to reduce the risk of atopy in children [40]. Studies have shown that avoiding specific foods, like cow's milk or eggs, during pregnancy and lactation does not decrease the incidence of CMA or egg allergy in infants [41, 42]. This is because the relationship between allergen exposure and allergy risk follows a bell-shaped curve: high exposure fosters tolerance, very low exposure fails to elicit a response, and mid-range exposure increases the risk of sensitization. Avoidance merely shifts this curve, leading to no significant change in the overall population, with some individuals moving from high-dose tolerance to mid-range sensitization, and others from mid-range to low-dose without sensitization.

High-dose allergen exposure during pregnancy is an emerging approach in CMA prophylaxis. Animal studies suggest potential benefits, for instance, administering oral ovalbumin during pregnancy induced tolerance in infant BALBc mice, a protective effect disrupted by inhibiting placental IgG transfer or infant memory T-cell IFN-γ production [43]. Additionally, a study of 1.277 mother-child pairs in the USA found that high peanut consumption in the first trimester reduced the risk of peanut allergy, while high milk intake in the first trimester lowered the odds of developing asthma and rhinitis. High wheat intake in the second trimester was also associated with a reduced risk of eczema [44].

Breastfeeding as a primary option in cow's milk allergy prophylaxis

The composition of breastmilk is complex and consists of many biologically active substances, immunomodulating proteins, growth factors and antioxidants. (Figure 1). These mechanisms include the anti-allergic properties found in breast milk, the possibility that extended breastfeeding might delay allergen introduction, and the presence of antibodies in the milk that may bind with food antigens to promote tolerance [45]. In animal studies, it has been shown that antigen-immunoglobulin G immune complexes from sensitized mothers can be transferred to their newborns *via* the neonatal Fc receptor, leading to the development of antigen-specific FoxP3(+) CD25(+) regulatory T cells [46]. This transfer may induce tolerance and support the primary prevention of CMA [47].

Recent research has highlighted the role of short-chain fatty acids (SCFAs), particularly butyrate, in enhancing oral tolerance in offspring. [48, 49] Additionally, human milk oligosaccharides (HMOs) in breast milk, as well as prebiotics like galactooligosaccharides or specific HMOs such as 2-fucosyllactose included in infant nutrition, can stimulate SCFA production, suggesting a potential role in preventing CMA [50]. Breast milk also contains a variety of bioactive components, including immune-active peptides (cytokines), fatty acids, HMOs, microbial content, and micronutrients, all of which have been shown to modulate the immune system [51].

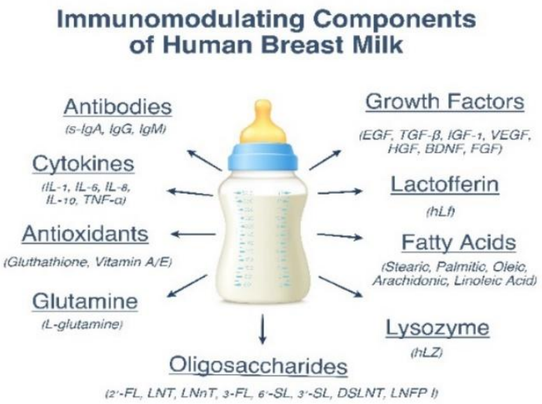

Figure 1. Molecules in the human milk with potential immune-modulating role. Abbreviations: s-IgA - soluble Immunoglobulin A; IgG - Immunoglobulin G; IgM - Immunoglobulin M; IL-1 - Interleukin 1; IL-6 - Interleukin 6; IL-8 - Interleukin 8; IL-10 - Interleukin 10; TNF-α - Tumor Necrosis Factor-Alpha; EGF - Epidermal Growth Factor; TGF-β - Transforming Growth Factor-beta; IGF-1 - Insulin-like Growth Factor 1;VEGF - Vascular Endothelial Growth Factor; HGF - Hepatocyte Growth Factor ; BDNF - Brain-Derived Neurotrophic Factor; FGF - Fibroblast Growth Factor; hLf - Human Lactoferrin; hLz - Human Lysozyme; 2'-FL - 2'-Fucosyllactose; LNT - Lacto-N-tetraose; LNnT - Lacto-N-neotetraose; 3-FL - 3-Fucosyllactose; 6'-SL - 6'-Sialyllactose; 3'-SL - 3'-Sialyllactose; DSLNT - Disialyllacto-N-tetraose; LNFP I - Lacto-N-fucopentaose I.

In a study of non-high-risk newborns followed up to 17 years of age, breastfeeding was associated with lower rates of food allergy at 1 and 3 years, as well as a lower "score of respiratory allergies" up to 17 years of age compared with cows' milk formula fed individuals [52, 53]. Exclusive breastfeeding is recommended by the World Health Organization (WHO) as the optimal feeding practice for the first 6 months of the infant's life [54]. Breast milk promotes the development of oral tolerance to allergens as it contains various immunologically active components. A multidisciplinary systematic review concluded that exclusive breastfeeding has a protective effect against CMA in early childhood, particularly in infants considered to be at high risk [55]. However exclusive breastfeeding does not totally eliminate the risk as statistics show that approximately 0,5% of exclusively breastfed infants still develop CMA [56].

The PROBIT trial, one of the longest cohort studies, recently reported that infants who were breastfed had a lower risk of developing flexural dermatitis when 18 years old. However, the study found no significant impact on lung function or the incidence of CMA and asthma [57].

Secondary alternatives in cow's milk allergy prophylaxis and management

If breastfeeding is insufficient or not possible, infants at high-risk can be recommended a hypoallergenic formula as a primary prevention strategy [58]. The most common hypoallergenic alternatives include infant formulas based on CMP. Enzymatic hydrolysis is a key biochemical process in reducing the allergenicity of CMP and providing a safe nutritional option for high-risk infants or those already diagnosed with CMA. By breaking down proteins into smaller fragments with low immunogenic characteristics, the immune system is less likely to trigger an allergic response but at the same time the exposure to small protein fragments helps in inducing oral tolerance to CMP. A social-metric study conducted among infants with atopy signs shows that hydrolyzed formulas can minimize the clinical signs and prevent severe allergic reactions [59]. Other less common alternatives include plant-based formulas or formulas based on mammalian milk proteins (goat, sheep, camel, horse, donkey).

✓ *Partially hydrolyzed formulae for non-exclusively breastfed infants.* Partially hydrolyzed formulae (pHF) typically contain peptides with molecular weights under 5,000 Da, reducing allergenicity by removing some sensitizing epitopes while retaining peptide sizes that promote oral tolerance. [60-62] The role of pHF in preventing CMA has been debated [63]. The GINI study, a long-term, large-scale research project, found that whey pHF significantly reduced atopic dermatitis (AD) and respiratory symptoms up to age 15. However, more recent studies, including those with prebiotic-enriched pHF, have shown no preventive effect [64-68]. There is no consensus on the impact of early exposure to intact CMP on CMA risk later in life. Some studies suggest that early introduction of CMP within the first 14 days of life may lower IgE-mediated CMA rates, while delayed introduction is associated with higher atopy risk by age of 2 [69].

✓ *Continuous early CMP exposure* might reduce CMA risk, while intermittent exposure followed by avoidance could increase it, with family history possibly influencing outcomes [70].

✓ *Extensively hydrolyzed formulae* Whey- or casein-based extensively hydrolyzed formulas (eHF) are considered the primary treatment for formula-fed infants with CMA [71]. These formulas contain short peptides derived from cow's milk, created through enzymatic breakdown and ultrafiltration of intact proteins. There are notable differences in the peptide molecular weights and profiles within eHF products [72, 73]. Due to this variation, the European Academy of Allergy and Clinical Immunology (EAACI) has advocated for stricter standards for eHF in Europe, including preclinical testing, quality assurance, and specific labeling requirements [74]. In 2016, DRACMA expanded their recommendations to include rice hydrolysate as a first-line option in regions where it is available [75]. According to ESPGHAN, eHF should be recommended as a first choice for CMA management for infants when breastfeeding is insufficient or not possible [76].

✓ *Amino acid-based formulae* Amino acid-based formula (AAF) is a synthetic, nutritionally complete formula free of cow's milk antigens, used to treat infants with severe CMA. Its cost-effectiveness varies depending on the healthcare system and formula costs in different countries [77, 78]. AAF is not a first-line treatment but is recommended for infants who do not respond to eHF or have very severe symptoms like cow's milk anaphylaxis or multiple food intolerances [79, 80]. Since tolerance development is believed to be driven by exposure to antigens, AAF is unlikely to promote tolerance, although a recent study found that adding synbiotics to AAF improved gut microbiota in non-IgE-mediated allergic infants [81, 82]. However, prolonged use of AAF for more than six months has been linked to hypophosphatemic bone disease, particularly in children with complex gastrointestinal conditions, with calcium and phosphorus levels normalizing after switching to eHF [83, 84]. Studies on healthy volunteers showed no differences in calcium and phosphorus bioavailability from various types of AAF, even when used with acid-suppressant medication [85].

CONCLUSION

CMA is the most common and most studied FA in early childhood particularly in infants considered as high-risk. Despite recent advances in the understanding of CMA, it still represents a challenging condition with many uncertainties regarding its prophylaxis and treatment. A significant body of evidence highlights the important role of exclusive breastfeeding as a gold standard for infant nutrition and a primary preventive measure against CMA. For non-breastfed infants at risk of developing CMA, pHF or eHF are considered a safe and successful alternative for both prevention and management among high-risk infants with a family history of allergy. Additionally, infants with confirmed CMA who show severe allergic reactions, such as anaphylaxis, are recommended the use of a non-allergenic infant formula, where AAF is considered an alternative. The allergenicity of proteins in AAF is completely reduced in order to avoid elicitation of allergic reactions, and is thus suitable for the management of CMA. Overall, CMA management requires a personalized approach, ongoing research, and refinement of dietary and formula-based interventions to better prevent and treat food allergies in infants.

List of abbreviations

CMA	Cow's milk allergy
pHF	Partially hydrolyzed formulas
eHF	Extensively hydrolyzed formulas
AAF	Amino acid-based formulas
FAs	Food allergies
IgE-mediated	Immunoglobulin E-mediated
non-IgE-mediated	Non-immunoglobulin E-mediated
CM	Cow's milk
CMP	Cow's milk proteins
APCs	Antigen-presenting cells
DCs	Dendritic cells
MHC	Major histocompatibility complex
TCRs	T-cell receptors
TSLP	Thymic stromal lymphopoietin
IL	Interleukin
FPE	Food protein-induced enteropathy
FPIES	Food protein-induced enterocolitis syndrome
IFN-g	Interferon-gamma
TNF-α	Tumor Necrosis Factor-alpha
TGF-β	Transforming Growth Factor-beta
GERD	Gastroesophageal reflux disease
Ig	Immunoglobulin
EGF	Epidermal Growth Factor
IGF-1	Insulin-like Growth Factor 1
VEGF	Vascular Endothelial Growth Factor
HGF	Hepatocyte Growth Factor
BDNF	Brain-Derived Neurotrophic Factor
FGF	Fibroblast Growth Factor
hLf	Human Lactoferrin
hLz	Human Lysozyme
2'-FL	2'-Fucosyllactose
LNT	Lacto-N-tetraose
LNnT	Lacto-N-neotetraose
3-FL	3-Fucosyllactose
6'-SL	6'-Sialyllactose
3'-SL	3'-Sialyllactose
DSLNT	Disialyllacto-N-tetraose
LNFP I	Lacto-N-fucopentaose I
WHO	World Health Organization
AAP	American Academy of Pediatrics
AD	Atopic dermatitis
EACCI	European Academy of Allergy and Clinical Immunology

REFERENCES

1. J. D. Flom, S. H. Sicherer, *Nutrients*, **11**, 1051 (2019**)**.
2. W. Loh, M. L. K. Tang, *Int. J. Environ. Res. Public Health*, **15**(9), 2043 (2018). doi: 10.3390/ijerph15092043.
3. J. Savage, S. Sicherer, R. Wood, *J. Allergy Clin. Immunol. Pract.*, **4**, 196 (2016**)**.
4. A. Burks, M. Tang, S. Sicherer, A. Muraro, P. A. Eigenmann, M. Ebisawa, A. Fiocchi, C Whiang, K. Beyer, R. Wood, *et al.*, *J. Allergy Clin. Immunol.*, ***129***, 129906 (2012).
5. Walsh J, Meyer R, Shah N, Quekett J, Fox AT. *Br. J. Gen. Pract.* **66**(649), e609-11 (2016). doi: 10.3399/bjgp16X686521.
6. Blanchard, E.; Zhu, P.; Schuck, P. Infant formula powders, in: Handbook of Food Powders: Processes and Properties, Woodhead Publishing Limited: Sawston, UK, 2013.
7. Malacarne, M.; Martuzzi, F.; Summer, A.; Mariani, P. *Int. Dairy J.*, **12**, 869 (2002).
8. Bøgh, K.L.; Larsen, J.M. Chapter 19: Reducing allergenicity by proteolysis, in: Agents of Change: Enzymes in Milk and Dairy Products. Springer: Berlin/Heidelberg, Germany, 2021; p. 499.
9. Pomés, A.; Davies, J.M.; Gaderm aier, G.; Hilger, C.; Holzhauser, T.; Lidholm, J.; Lopata, A.L.; Mueller, G.A.; Nandy, A.; Radauer, C.; et al. *Mol. Immunol.*, **100**, 3 (2018).
10. WHO/IUIS. Allergen Nomenclature Sub-Committee. Available online: http://allergen.org/ (accessed on 5 January 2022).
11. Smyth, E.; Clegg, R.A.; Holt, C. *Int. J. Dairy Technol.*, **57**, 121 (2004).

12. Holt, C.; Carver, J.A.; Ecroyd, H.; Thorn, D.C. *J. Dairy Sci.*, **96**, 6127 (2013).
13. Holzmüller, W.; Gmach, O.; Griebel, A.; Kulozik, U. *Int. Dairy J*, **63**, 115. (2016).
14. Corredig, M.; Dalgleish, D.G. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.*, **9**, 233 (1999).
15. Chapman, M. *Clin. Allergy Immunol.*, **21**, 47 (2008).
16. Monaci, L.; Tregoat, V.; van Hengel, A.J.; Anklam, E. *Eur. Food Res. Technol.*, **223**, 149 (2006).
17. Vicente-Serrano, J.; Caballero, M.L.; Rodríguez-Pérez, R.; Carretero, P.; Pérez, R.; Blanco, J.G.; Juste, S.; Moneo, I. *Pediatr. Allergy Immunol.*, **18**, 503 (2007).
18. Villa, C.; Costa, J.; Oliveira, M.B.P.P.; Mafra, I. *Compr. Rev. Food Sci. Food Saf.*, **17**, 137 (2018).
19. Negaoui, H.; El Mecherfi, K.E.; Tadjer, S.A.; Grar, H.; Kheroua, O.; Saidi, D. *Food Agric. Immunol.*, **27**, 711 (2016).
20. Gaudin, J.C.; Rabesona, H.; Choiset, Y.; Yeretssian, G.; Chobert, J.M.; Sakanyan, V.; Drouet, M.; Haertlé, T. *Clin. Exp. Allergy*, **38**, 686 (2008).
21. Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L.P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. *Mol. Nutr. Food Res.*, **48**, 363 (2004).
22. Van Regenmortel, M.H.V. *Methods Mol. Biol.*, **524**, 3 (2009).
23. Arnon, R.; Van Regenmortel, M.H.V. *FASEB J.*, **6**, 3265 (1992).
24. Ansari, H.R.; Raghava, G.P. *Immunome Res.*, **6**, 6 (2010).
25. Yu, W.; Freeland, D.M.H.; Nadeau, K.C. *Nat. Rev. Immunol.*, **16**, 751 (2016). [**Google Scholar**] [**CrossRef**]
26. Czolk, R.; Klueber, J.; Sørensen, M.; Wilmes, P.; Codreanu-Morel, F.; Skov, P.S.; Hilger, C.; Bindslev-Jensen, C.; Ollert, M.; Kuehn, A. *Front. Immunol.*, **11**, 3653 (2021).
27. Broekman, H.C.H.; Eiwegger, T.; Upton, J.; Bøgh, K.L. *Drug Discov. Today Dis. Model*, **17–18**, 37 (2015).
28. Divekar, R.; Kita, H. *Curr. Opin. Allergy Clin. Immunol.*, **15**, 98 (2015).
29. Stark, J.M.; Tibbitt, C.A.; Coquet, J.M. *Front. Immunol.*, **10**, 2318 (2019).
30. P.Humeniuk, P. Dubiela, K Hoffmann-Sommergruber , *Int. J. Mol. Sci.*, **18**, 71491 (2017).
31. S. Anvari, J. Miller, C. Y. Yeh, C.M. Davis, *Clin. Rev. Allergy Immunol.*, **57**, 244 (2019).
32. P. Satitsuksanoa, M. Daanje, M. Akdis, S. D. Boyd, W. van de Veen, *Eur. J. Allergy Clin. Immunol.*, **76**, 1707 (2021).
33. X. P. Lin, J. Magnusson, S. Ahlstedt, A. Dahlman-Hoglund, L. L. Hanson, O, Magnusson *et al.* J. Allergy Clin. Immunol. **109**, 879 (2002).
34. H. Morita, I, Nomura K. Orihara, K. Yoshida, A. Akasawa, H. Tachimoto, *et al. J. Allergy Clin. Immunol*., **131**,:590-2, e1-6 (2013).
35. Chung HL, Hwang JB, Park JJ, Kim SG. *J Allergy Clin Immunol* 2002;109:150-4.
36. Caubet JC, Nowak-Wegrzyn A. *Exp Rev Clin Immunol* 2011;7:317-27.u W, Freeland DMH, Nadeau KC. Food allergy: immune mechanisms, diagnosis and immunotherapy. *Nat Rev Immunol.* 2016 Dec;16(12):751-765. doi: 10.1038/nri.2016.111. Epub 2016 Oct 31. PMID: 27795547; PMCID: PMC5123910.
37. YuW, Freeland DMH, Nadeau KC. *Nat Rev Immunol.* 2016 Dec;16(12):751-765. doi: 10.1038/nri.2016.111. Epub 2016 Oct 31. PMID: 27795547; PMCID: PMC5123910.
38. Von Berg, A.; Filipiak-Pittroff, B.; Krämer, U.; Link, E.; Heinrich, J.; Koletzko, S.; Grübl, A.; Hoffmann, U.; Beckmann, C.; Reinhardt, D.; et al. *Allergol. Sel.* **2017**, *1*, 28–39.
39. Venter C, Agostoni C, Arshad SH, Ben-Abdallah M, Du Toit G *Pediatr Allergy Immunol*, Fleischer DM, et al. (2020) 31(8):889–912. doi: 10.1111/pai.13303.
40. Kramer MS, Kakuma R. *Cochrane Database Syst Rev* (2012) 2012(9): Cd000133. doi: 10.1002/14651858.CD000133.pub3
41. Khakoo A, Lack G. *Curr Allergy Asthma Rep* (2004) 4(1):36–42. doi: 10.1007/s11882-004-0041-1
42. Vance GH, Lewis SA, Grimshaw KE, Wood PJ, Briggs RA, Thornton CA, et al. *Clin Exp Allergy* (2005) 35(10):1318–26. doi: 10.1111/j.1365-2222.2005.02346.x
43. Polte T, Hansen G. *Clin Exp Allergy* 2008) 38(12):1950–8. doi: 10.1111/j.1365-2222.2008.03096.x
44. Bunyavanich S, Rifas-Shiman SL, Platts-Mills TA, Workman L, Sordillo JE, Camargo CAJr., et al. *J Allergy Clin Immunol* (2014) 133(5):1373–82. doi: 10.1016/j.jaci.2013.11.040
45. du Toit G, Tsakok T, Lack S, Lack G. *J Allergy Clin Immunol* (2016) 137(4):998–1010. doi: 10.1016/j.jaci.2016.02.005
46. Mosconi E, Reki A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, et al. *Mucosal Immunol* (2010) 3 (5):461–74. doi: 10.1038/mi.2010.23
47. Ohsaki A, Venturelli N, Buccigrosso TM, Osganian SK, Lee J, Blumberg RS, et al. *J Exp Med* (2018) 215(1):91–113. doi: 10.1084/jem.20171163
48. Vonk MM, Blokhuis BRJ, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, et al. *Mediators Inflammation* (2019) 2019:9062537. doi: 10.1155/2019/9062537

49. Folkerts J, Redegeld F, Folkerts G, Blokhuis B, van den Berg MPM, de Bruijn MJW, et al.. *Allergy* (2020) 75(8):1962–74. doi: 10.1111/all.14254
50. Seppo AE, Savilahti EM, Berin MC, Sampson HA, Jarvinen KM. *Clin Exp Allergy* (2017) 47 (10):1275–84. doi: 10.1111/cea.12945
51. Munblit D, Peroni DG, Boix-Amorós A, Hsu PS, Van't Land B, Gay MCL, et al. *Nutrients* (2017) 9(8):843. doi: 10.3390/nu9080894
52. Høst A, Koletzko B, Dreborg S, Muraro A, Wahn U, Aggett P, Bresson JL, Hernell O, Lafeber H, Michaelsen KF, Micheli JL, Rigo J, Weaver L, Heymans H, Strobel S, Vandenplas Y. Dietary products used in infants for treatment and prevention of food allergy. Joint Statement of the European Society for Paediatric Allergology and Clinical Immunology (ESPACI) Committee on Hypoallergenic Formulas and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition. Arch Dis Child. 1999 Jul;81(1):80-4. doi: 10.1136/adc.81.1.80. PMID: 10373144; PMCID: PMC1717972.
53. Saarinen UM, Kajosaari M. *Lancet*. 1995 Oct 21;346(8982):1065-9. doi: 10.1016/s0140-6736(95)91742-x. PMID: 7564787.
54. WHO. In Proceedings of the Fifty Fifth World Health Assembly, Geneva, Switzerland, 13–18 May 2002; Volume 53, pp. 1–18.
55. van Odijk J, Kull I, Borres MP, Brandtzaeg P, Edberg U, Hanson LA, Høst A, Kuitunen M, Olsen SF, Skerfving S, Sundell J, Wille S. *Allergy*. 2003 Sep;58(9):833-43. doi: 10.1034/j.1398-9995.2003.00264.x. PMID: 12911410.
56. Vandenplas Y, Koletzko S, Isolauri E, Hill D, Oranje AP, Brueton M, Staiano A, Dupont C. *Arch Dis Child*. 2007 Oct;92(10):902-8. doi: 10.1136/adc.2006.110999. Erratum in: Arch Dis Child. 2007 Oct;92(10):following 908. Erratum in: Arch Dis Child. 2008 Jan;93(1):93. PMID: 17895338; PMCID: PMC2083222.
57. Flohr C, Henderson AJ, Kramer MS, Patel R, Thompson J, Rifas-Shiman SL, et al. *JAMA Pediatr* (2018) 172(1):e174064. doi: 10.1001/jamapediatrics.2017.4064
58. Muraro A, Halken S, Arshad SH, Beyer K, Dubois AE, Du Toit G, Eigenmann PA, Grimshaw KE, Hoest A, Lack G, O'Mahony L, Papadopoulos NG, Panesar S, Prescott S, Roberts G, de Silva D, Venter C, Verhasselt V, Akdis AC, Sheikh A; *Allergy*. 2014 May;69(5):590-601. doi: 10.1111/all.12398. Epub 2014 Apr 3. PMID: 24697491.
59. Bezruk VV, Godovanets OS, Buriak OH, Voytkevich NI, Makarova OV, Yurkiv OI, Sheremet MI, Bilookyi OV, Hresko MM, Velia MI, Yurniuk SV, Hresko MD, Bulyk TS, Rynzhuk LV, Maksymiv OO, Shkrobanets ID. *J Med Life*. 2022 Dec;15(12):1536-1539. doi 10.25122/jml-2022-0254. PMID: 36762331; PMCID: PMC9884356.
60. Vandenplas Y, Abuabat A, Al-Hammadi S, Aly GS, Miqdady MS, Shaaban SY, et al. *Pediatr Gastroenterol Hepatol Nutr* (2014) 17(2):61–73. doi: 10.5223/pghn.2014.17.2.61
61. Fritsché R, Pahud JJ, Pecquet S, Pfeifer A. *J Allergy Clin Immunol* (1997) 100(2):266–73. doi: 10.1016/S0091-6749(97)70235-5
62. Inuo C, Tanaka K, Nakajima Y, Yamawaki K, Matsubara T, Iwamoto H, et al. *Asia Pac J Clin Nutr* (2019) 28(1):49–56. doi: 10.6133/apjcn.201903_28(1).0008
63. Szajewska H, Horvath A. *World Allergy Organ J* (2017) 10(1):27. doi: 10.1186/s40413-017-0158-z
64. von Berg A, Koletzko S, Grubl A, Filipiak-Pittroff B, Wichmann HE, Bauer CP, et al. *J Allergy Clin Immunol* (2003) 111(3):533–40. doi: 10.1067/mai.2003.101
65. von Berg A, Koletzko S, Filipiak-Pittroff B, Laubereau B, Grubl A, Wichmann HE, et al. *J Allergy Clin Immunol* (2007) 119(3):718–25. doi: 10.1016/j.jaci.2006.11.017
66. von Berg A, Filipiak-Pittroff B, Kramer U, Link E, Bollrath C, Brockow I, et al. *J Allergy Clin Immunol* (2008) 121(6):1442–7. doi: 10.1016/ j.jaci.2008.04.021
67. von Berg A, Filipiak-Pittroff B, Kramer U, Hoffmann B, Link E, Beckmann C, et al. *J Allergy Clin Immunol* (2013) 131 (6):1565–73. doi: 10.1016/j.jaci.2013.01.006
68. von Berg A, Filipiak-Pittroff B, Schulz H, Hoffmann U, Link E, Sussmann M, et al. *Allergy* (2016) 71(2):210–9. doi: 10.1111/all.12790
69. Katz Y, Rajuan N, Goldberg MR, Eisenberg E, Heyman E, Cohen A, et al. *J Allergy Clin Immunol* (2010) 126(1):77–82 e1. doi: 10.1016/j.jaci.2010.04.020
70. Urashima M, Mezawa H, Okuyama M, Urashima T, Hirano D, Gocho N, et al. *JAMA Pediatr* (2019) 173(12):1137–45. doi: 10.1001/jamapediatrics.2019.3544
71. Koletzko S, Niggemann B, Arato A, Dias JA, Heuschkel R, Husby S, et al., *J Pediatr Gastroenterol Nutr* (2012) 55(2):221–9. doi: 10.1097/MPG.0b013e31825c9482
72. Lambers TT, Gloerich J, van Hoffen E, Alkema W, Hondmann DH, van Tol EA. *Food Sci Nutr* (2015) 3(1):81–90. doi: 10.1002/fsn3.196
73. Chauveau A, Nguyen-Grosjean VM, Jacquenet S, Richard C, Mouton-Faivre C. *Pediatr Allergy Immunol* (2016) 27(5):541–3. doi: 10.1111/pai.12556
74. Heine RG. *Ann Nutr Metab* (2018) 72(Suppl 3):33–45. doi: 10.1159/000487380
75. Fiocchi A, Dahda L, Dupont C, Campoy C, Fierro V, *Nieto World Allergy Organ J* (2016) 9(1):35. doi: 10.1186/s40413-016-0125-0
76. Vandenplas Y et al. *J Pediatr Gastroenterol Nutr*. 2023 Jul 26. doi: 10.1097/MPG.0000000000003897.
77. Taylor RR, Sladkevicius E, Panca M, Lack G, *Pediatr Allergy Immunol* (2012) 23(3):240–9. doi: 10.1111/j.1399-3038.2011.01262.x

78. Sladkevicius E, Nagy E, Lack G, Guest JF. *J Med Econ* (2010) 13 (1):119–28. doi: 10.3111/13696990903543242
79. Fiocchi A, Schunemann HJ, Brozek J, Restani P, Beyer K, Troncone R, et al. *J Allergy Clin Immunol* (2010) 126(6):1119–28.e12. doi: 10.1016/j.jaci.2010.10.011
80. Hill DJ, Heine RG, Cameron DJ, Francis DE, Bines JE. *J Pediatr* (1999) 135(1):118–21. doi: 10.1016/S0022-3476(99)70341-0
81. Nowak-Wegrzyn A, Chatchatee P. *Ann Nutr Metab* (2017) 70 Suppl 2:7–24. doi: 10.1159/000457915
82. Candy DCA, Van Ampting MTJ, Oude Nijhuis MM, Wopereis H, Butt AM, Peroni DG, et al. *Pediatr Res* (2018) 83(3):677–86. doi: 10.1038/pr.2017.270
83. Gonzalez Ballesteros LF, Ma NS, Gordon RJ, Ward L, Backeljauw P, Wasserman H, et al. *Bone* (2017) 97:287–92. doi: 10.1016/j.bone.2017.02.003
84. Akhtar Ali S, Mathalikunnel A, Bhardwaj V, Braskett M, Pitukcheewanont P. *Osteoporosis Int* (2019) 30(9):1887–91. doi: 10.1007/s00198-019-04836-8
85. Bergwitz C, Eussen S, Janssens P, Visser M, Carpenter TO, van Helvoort A. *Nutr Res* (2021) 85:71–83. doi: 10.1016/j.nutres.2020.11.0

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Comfrey (*Symphylus officinale* L.) roots – a source of polyphenols and fructans

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The aim of this research was to evaluate the content of polyphenols, fructans and the antioxidant activity of water extracts of comfrey roots obtained using ultrasound-assisted extraction. Isolation of inulin from the roots using microwave-assisted extraction and chemical characterization was also performed. The total contents of phenols and flavonoids were evaluated using spectrophotometric methods. Antioxidant potential was evaluated using DPPH and FRAP methods. Sugars and fructans were determined by HPLC-RID method. Isolated inulin was characterized for yield, fructose content, degree of polymerization and molecular weight. Its structure was elucidated using FTIR spectroscopy. From the conducted research it was found that comfrey roots were rich sources of total flavonoids (64.86 mg quercetin equivalents/g dry weight) and fructans (13.43 g/100 g dry weight). Higher values of the antioxidant potential were found using FRAP method. Polysaccharide isolated by microwave-assisted extraction had fructose content of 64.1 %, high degree of polymerization (29–33) and molecular weight of 5.15-5.36 kDa. FTIR spectroscopy confirmed that isolated polysaccharide was inulin-type fructan with characteristic bands for $\beta(2\rightarrow1)$ bonds. To the best of our knowledge this is the first detailed analysis of inulin-type fructan isolated from comfrey (*Symphylus officinale* L.) roots. As sources of flavonoids and inulin, comfrey roots can find further application in cosmetics and pharmacy.

INTRODUCTION

Comfrey (*Symphylus officinale* L.) is a medicinal plant from *Boraginaceae* family that occurs mainly in Europe, Asia and South America. Its roots are traditionally used for curing wounds, joint disorders, and musculoskeletal injuries [1-3]. Due to the rich content of phytochemical compounds in comfrey roots as allantoin, rosmarinic acid, and other hydroxycinnamic acid derivatives, as well as muco-polysaccharides [4], inulin [5] A, B and C vitamins, triterpenoid saponins, tannins, calcium, potassium, and selenium [1,2] these roots find various applications in phytoterapy. Moreover, the phenolic acids (e.g., rosmarinic, p-hydroxybenzoic, caffeic, chlorogenic and p-coumaric) display remarkable antioxidant effects, besides their positive impact on human skin fibroblasts [6]. Extraction techniques using different solvents were the most important tools for obtaining biologically active compounds from comfrey roots. Many researches deal with different extraction approaches for obtaining mainly polyphenolic compounds [1, 3, 4] – conventional extraction [2, 5], pressure-liquid extraction [1], supercritical fluid extraction [1]. Less attention was paid to the extraction of carbohydrate and evaluation of the polysaccharide structure of comfrey roots. In our previous study the carbohydrate composition was partially revealed, especially inulin [5]. It was reported that polysaccharides from comfrey roots possess a strong antioxidant effect due to the presence of uronic acid group [7], as well as hypoglycemic activity [7-9]. Polysaccharides from comfrey roots were isolated by other researchers and the optimum extraction conditions were found. In most of the studies water was used as a sovent in combination with enzyme and or ultrasonic waves [8, 9]. In some studies, microwaves and ultrasonic irradiation were mentioned as prospective approaches for isolation of polysaccharides, especially inulin [8-10]. However, the analysis of bioactive compounds in water extracts, as well as the elucidation of the characteristics of inulin obtained from comfrey roots still remains a challenge. Little is known about the structure of inulin-type fructan from comfrey roots. The aim of this research was to determine the content of polyphenols and inulin-type fructans, as well as the antioxidant activity of water extracts of comfrey roots obtained using ultrasound-assisted extraction. As an additional task, inulin was isolated by microwave-assisted extraction and its chemical characterization was performed.

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EXPERIMENTAL

Plant material

Dried roots of *Symphylus officinale* (Radix Symphyti) were produced by Thalloderma (Bulgaria). The dry plant material was finely ground and passed through a 0.5 mm sieve. The ground samples were kept in a screwed capped container at room temperature for further analysis. For the isolation of fructan from comfrey roots, successive extraction with hexane, chloroform, and ethyl acetate was performed by maceration for 24 h at 25°C, at solid-to-liquid ratio (1:5 w/v), as previously described [10] to remove fatty and polyphenolic compounds.

Ultrasound-assisted extraction of comfrey roots

The extraction procedure was performed in 50 ml plastic tubes at solid-to-liquid ratio of 1:10 (w/v) in an ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) operating at 36 kHz frequency and 240 W ultrasonic power. The extraction was done with distilled water at 75 °C for 20 min in duplicate [11]. The water extracts were filtered and combined for further analysis of total phenols, flavonoids, antioxidant activity and carbohydrate composition.

Microwave-assisted isolation of inulin from S. officinalis

The residual comfrey root sample after washing with hexane, chloroform, and ethyl acetate was dried at room temperature and then extracted with distilled water (30 g roots with 300 ml of boiling water) in duplicate using a microwave device Crown (2452 kW), with microwave power of 700 W for 5 min [10]. The hot water extract was filtered through nylon cloth. The cooled filtrate was precipitated with acetone (1:4 v/v). The precipitate was separated by centrifugation and then purified by recrystallization in boiling water and precipitated with acetone (1:5 v/v). The obtained polysaccharide was filtered and dried at 40 °C.

Evaluation of total phenols, flavonoids and antioxidant activity

The evaluation of total phenols was done using Folin–Ciocalteu's reagent. The total flavonoids were determined using $Al(NO_3)_3$ reagent at 415 nm [12]. The results were expressed as mg equivalents quercetin (QE)/ g sample [13].

DPPH radical scavenging assay was performed with DPPH (2,2-diphenyl-1-picrylhydrazyl radical) reagent as previously described [14]. Ferric-reducing antioxidant power (FRAP) assay was performed according to [14]. The absorbance of the reaction mixture was recorded at 593 nm. The results for the antioxidant activity were expressed as mM Trolox equivalent (TE)/g dw.

Carbohydrate analysis

The fructans content was determined by resorcinol-thiourea spectrophotometric method at 480 nm [10]. The individual sugars and inulin were determined by HPLC-RID method [5].

Characterization of fructan from comfrey roots obtained by microwave-assisted extraction

The melting point was measured with a Kofler apparatus. The reducing groups were determined by the PAHBAH method at 410 nm [10], while total fructose content - by resorcinol-thiourea reagent at 480 nm [11]. The purity of fructan was assessed on an HPLC instrument Elite Chrome Hitachi with a Shodex® Sugar SP0810 column (300mm × 8.0mm i.d.) (Shodex Co., Tokyo, Japan) at 85°C, flow rate of 1.0 ml/min and injection volume of 20 µl [10]. High-performance size-exclusion chromatography (HPLC-SEC) was used for the determination of number average molecular weight (Mn), and weight average molecular weight (Mw). The analysis of comfrey inulin was performed on HPLC chromatograph ELITE LaChrome (VWR Hitachi, Japan) using a column Shodex OH-pack 806 M (300mm × 8.0mm) at 30°C and an RI detector (VWR Hitachi Chromaster, 5450, Japan) with 0.1M $NaNO_3$ [15]. Polydispersity index (X) of inulin was calculated as the ratio of the two molecular weights (Mw/Mn).

FTIR spectroscopy

The isolated fructan (2 mg) was pressed into a KBr tablet and FTIR spectrum was collected on a FTIR Avatar Nicolet (Thermo Scientific, USA) spectrometer in the wavelength range of 4000–400 cm^{-1} after 128 scans at a resolution of 2 cm^{-1}.

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation.

RESULTS AND DISCUSSION

Biologically active compounds and antioxidant activity in comfrey root water extracts

Most studies of comfrey roots using the acoustic cavitation effect induced by the ultrasound waves lead to sonolysis facilitating solvent migration into the cell. The extraction of intracellular compounds was connected with extraction of polysaccharides, polyphenols, allantoin and pyrrolizidine alkaloid

[16]. The results for total polyphenols, flavonoids, antioxidant activity and fructan content in water extracts obtained by ultrasound-assisted extraction of comfrey roots are presented in Table 1.

Table 1. Total polyphenols, flavonoids, fructans and antioxidant activity in water extracts of comfrey roots

Characteristics	Comfrey root water extract
Total polyphenols, mg GAE/g dw	0.72±0.07
Total flavonoids, mg QE/g dw	64.86±0.53
Antioxidant activity, mM TE/g dw	
DPPH	2.60±0.32
FRAP	4.31±0.35
Total fructans, g/100 g dw	13.41±0.53
Inulin, g/100 g dw	10.10±0.53
Nystose, g/100 g dw	Not detected
1-kestose , g/100 g dw	Not detected
Sucrose, g/100 g dw	0.80±0.01
Glucose, g/100 g dw	1.21±0.03
Fructose, g/100 g dw	1.32±0.05

It was found that the highest amount of total flavonoids was 64.86 mg QE/g dw. This confirmed our earlier finding that the comfrey root extracts showed higher content of flavonoids than phenolic acids [5]. The total phenolic content (0.72 mg GAE/g dw) in this study was between 10-100 times lower than the values for other extracts reported previously [5, 17, 18]. The antioxidant potential was evaluated by two methods based on different mechanisms, and FRAP showed the highest values 4.31 mM TE/g dw. The radical scavenging ability of the water extract obtained by ultrasonic extraction evaluated by DPPH method was 2.60 mM TE/g dw. Our results were higher than the literature data for the antioxidant potential of *S. officinale* root ethanol/water extracts evaluated by DPPH method (0.985 mM TE/g [2]. The antioxidant potential of comfrey water extracts was comparable with previous reports for comfrey extracts [5, 17]. In previous studies comfrey roots hydroalcoholic extracts demonstrated antioxidant activity by FRAP method between 14.42–82.42 mg TE/g extract [3,16], while that of the dichloromethane fraction was 6.67 mg TE/g extract [16].

The presence of fructans (or polyfructans) in *Symphylus* sp., especially comfrey *Symphylus officinale* was reported previously [19-21], as fructan content varied between 27-47% dw depending of time of harvest and development of plant [21]. Our previous study clearly demonstrated that comfrey roots contained fructans (27-32 g/100 g dw) [5]. In the present research the HPLC-RID chromatogram of the carbohydrate profile of comfrey root water extracts obtained by ultrasound-assisted extraction is shown (Fig. 1). In the water extracts inulin, sucrose and two monosacharides - glucose and fructose are clearly seen. The detected total fructans in the water extract of comfrey root obtained by ultrasound-assisted extraction were 13.43 g/100 g, while inulin was 10.10 g/100 g dw. However, fructooligosacchrides nystose and 1-kestose were not detected. Only small amounts of sucrose, glucose and fructose were found (Table 1). These results were almost two times lower in comparison with our previous report for fructan and inulin (27.6 and 24.9 g/100 g dw) in comfrey roots obtained by classical sequential ethanol and water extraction [5]. The levels of sucrose and glucose were also two times lower, while fructose was more than 4 times lower. The difference in composition of sugars and inulin can be related to harvest time of roots, as well as used extraction technique.

Isolation and characterization of inulin-type fructan from comfrey roots

The physicochemical characteristics of inulin from comfrey roots obtained after microwave-assisted extraction are summarized in Table 2.

Table 2. Physicochemical characteristics of inulin isolated form comfrey roots obtained by microwave-assisted extraction

Characteristics	Comfrey inulin	Inulin raftiline HP
Yield, %	15.4±1.4	-
Purity, %	36.6±0.5	85.5±0.4
Melting point, °C	183-184	175-179
Fructose content, %	64.1±0.5	75.3±0.4
Reducing groups, %	2.3±0.5	2.3±0.4
DP by spectrophotometric method	29	34
DP by HPLC-SEC	33	26
Mw, kDa	5.36	4.02
Mn, kDa	5.15	4.19
Polydispersity index	1.04	1.03

The results for the obtained comfrey inulin-type fructan were compared with those for commercial chicory high-molecular inulin with degree of polymerization 26. The yield of isolated comfrey polysaccharide was 15.4 % with low purity (36.6%). In this fructan the fructose content was 64.1 % and that of reducing groups was 2.3 %.

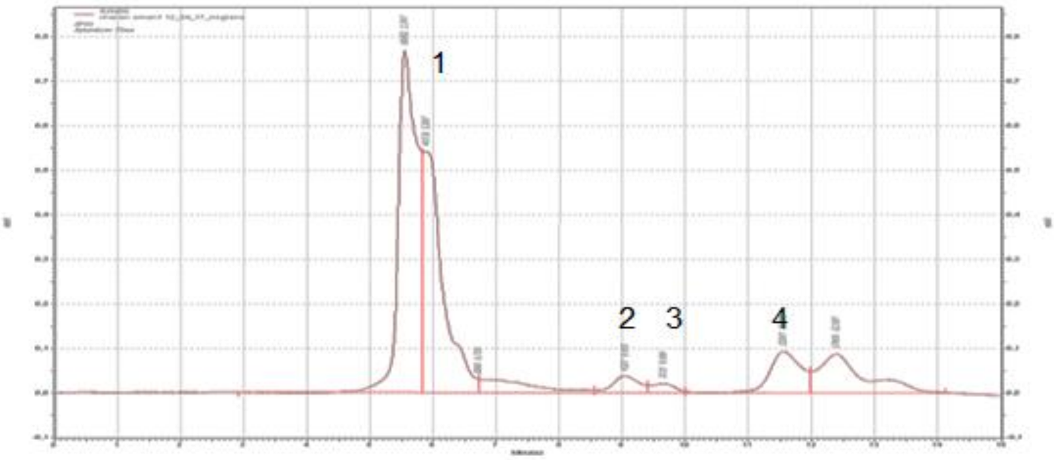

Fig. 1. HPLC-RID chromatogram of water extract from comfrey roots, where 1-inulin, 2-sucrose, 3-glucose and 4-fructose

It was the first detailed study about inulin isolated from the comfrey roots. Inulin isolated from its roots possessed high degree of polymerization (29–33) and molecular weight of 5.15-5.36 kDa. Vasfilova and Vorob'eva [21] reported on the presence of glucofructan in *Symphylus officinale* roots with low molecular weight at the beginning of the vegetation period and high molecular weight at the end of the vegetation - 45-47%. According to these authors the low-molecular fructans reached 11%, while high molecular ones - 29.5% with index of polymerization 0.7-1.0. It was reported that comfrey polysaccharide fractions (comprising 78.01% to 85.70% of total polysaccharides) were recovered with yields varying from 7.39 to 24.51% [8, 25]. Moreover, it was reported that polyfructans in roots and rhizomes of *Symphytum caucasicum* can reach 22-25%, as fructose containing carbohydrates are 44-51% with degree of polymerization of fructans between 0.49-0.50 [22]. It was also found that in a water-soluble high-molecular preparation of *Symphytum grandiflorum* the main components of mucilage are glucofructans (67 %) [24]. In addition, Haaβ *et al*. [25] reported for inulin type fructan from root and leaves of *Symphylus officinale* with degree of polymerization 33, that coincided with the degree of polymerization of inulin from comfrey roots obtained by microwave-assisted extraction found in this study. In general, the isolated inulin-type fructan is characterized with high molecular mass and degree of polymerization higher than chicory, dahlia and burdock inulin [10, 15]. The degree of polymerization is an important feature that brings about functional and biological activity of fructans [25]. The isolated comfrey inulin with DP 29-33 could be a good immunomodualtor and taste enhancer, but additional studies are required.

HPLC-SEC chromatogram of polysaccharide from comfrey isolated by microwave-assisted extraction is presented (Fig. 2). Four peaks are seen, the first broad peak at 9.6 min due to polysaccharide with high molecular mass (Mw 1158 kDa and Mn 955 kDa) and polydispersity index of 1.21. The second symmetric peak at 12.7 min is fructan with high molecular weight (Mw 5.1 kDa and Mn 5.3 kDa). The polydispersity index of the isolated comfrey fructan was 1.04, that was near to chicory inulin, as well as burdock, echinacea and dahlia inulin-type fructans [10, 15-23]. In addition, comfrey polysacchrides with molecular weight distribution from 2.8 to 3420 kDa have been reported [8, 9]. It was explained that the size distribution depended on the drying conditions or extraction procedures [26, 27]. Acording to Chen *et al.* [8] when comfrey root polysaccharides were extracted with hot water, there was a distinct group with a molecular weight of 5811.42 Da, that was close to our finding for fructan from comfrey with molecular weight of 5.3 kDa.

It was previously reported that comfrey polysaccharides are non-starch polysaccharides and are primarily composed of galactose, arabinose, glucose, and galacturonic acid, indicating that they may pass to the end of the intestinal tract of monogastric animals and be fermented by intestinal microflora [26].

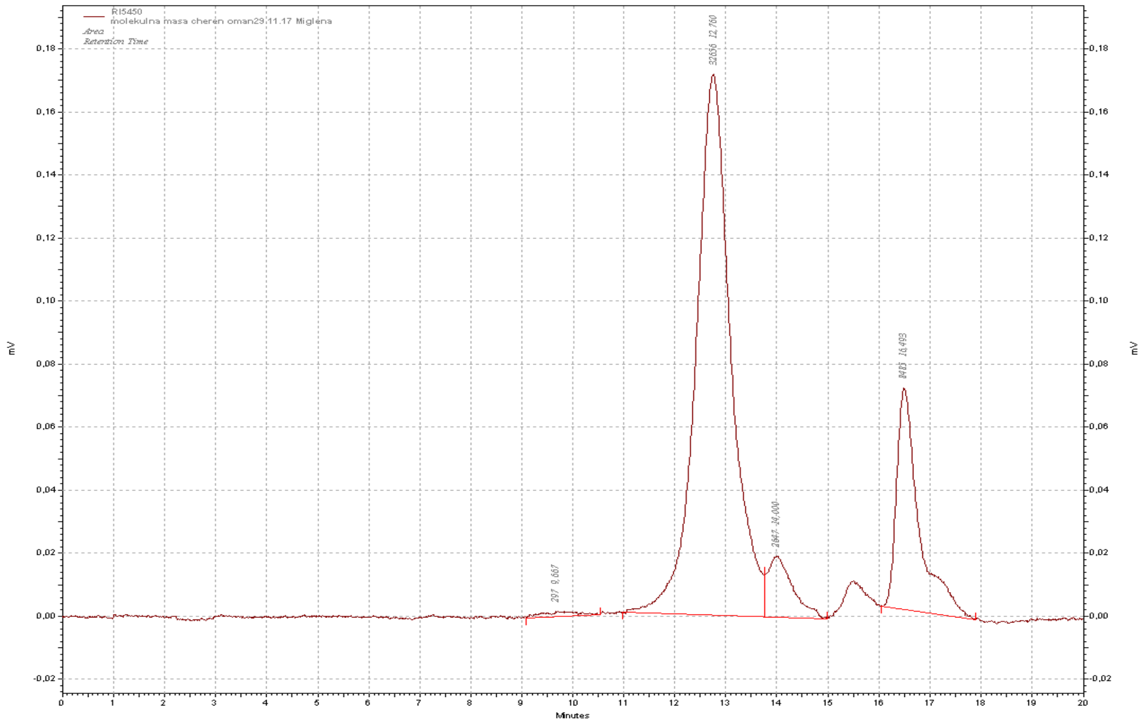

Fig. 2. HPLC-SEC chromatogram of inulin from comfrey isolated by microwave–assisted extraction

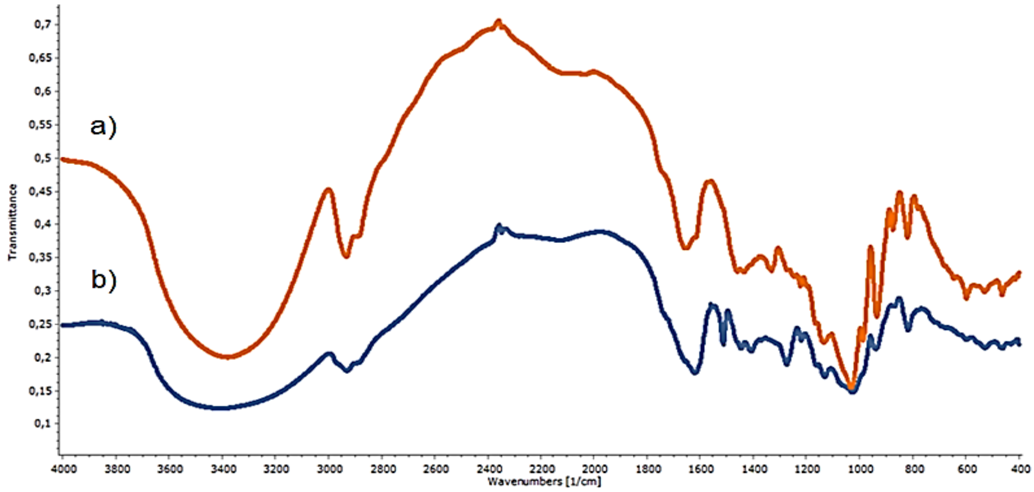

Fig. 3. FTIR spectra of inulin: a) chicory inulin Raftiline HPX DP=25, b) isolated inulin from comfrey roots by microwave-assisted extraction with DP 29-33

FTIR specroscopy

The structure of comfrey inulin isolated by microwave-assisted water extraction was confirmed by FTIR spectroscopy (Fig. 3). The comfrey inulin spectrum was compared with the spectrum of chicory inulin raftiline HPX DP=25. The assigned bands are summarized in Table 3. These spectra demostrated all typical bands for inulin-type fructans [28]. It was found that a broad band at 3396 cm^{-1} was due to O–H stretching vibrations associated with inter- and intramolecular hydrogen bonds in the inulin structure. The bands at 2930–2932 cm^{-1} were due to C–H asymmetric stretching vibrations. The bands at 2889 cm^{-1} were characteristic of symmetric stretching vibrations of C–H from CH_2. The bands at 1445 cm^{-1} were due to symmetric stretching vibrations of C–H in pyranosyl ring and β_{O-H} (OH). The bands at 1129 cm^{-1} were assigned to C–O–C ring stretching vibrations from glycoside linkage. The bands at 1024 cm^{-1} were assigned to C–O stretching vibrations. Similar bands at 3377 cm^{-1}, 2937 cm^{-1}, 1419 cm^{-1} and 1140 cm^{-1} were reported in the FTIR spectrum of comfrey root polysaccharide with molecular weight of 3812.39 Da and monosaccharide composition including galacturonic acid (GalA), arabinose (Ara), glucose

(Glc), and galactose (Gal) in a molar ratio of GalA, Ara, Glc, and Gal of 1.00:0.88:2.28:1.13, respectively [9]. The band at 937 cm^{-1} showed the presence of α-D-glucopyranosyl residue in the carbohydrate chain. The band for 2-ketofuranose and β-anomer bendings in C1–H was detected at 873 cm^{-1} and the occurence of a typical band at 816 cm^{-1} confirmed the presence of 2-ketose in a pyranosyl or furanosyl ring (Table 3).

Table 3. Assignment of bands in the FTIR spectrum of inulin isolated from comfrey roots

Band intervals	Observed bands	Assignment
3200 - 3400	3396	O–H stretching vibrations, intramolecular H-bonds
2933 - 2981	2930	νC–Has(CH_2)
2850 - 2904	2889	νC–Hs(CH_2)
1455 - 1470	1445	νC–Hs(CH_2) in pyranosyl ring, βo–н (OH)
1225 - 1235	1218	βo–н (OH)
1125 - 1162	1129	νC-O-Cas (C–O–C), glycoside linkage
1015 - 1060	1024	νC–O (C–O)
925 - 930	937	α-D- Glcp residue in polymer chain
867	873	ρ CH_2 in ring, β-anomer bendings C1-H, β-anomer, 2-ketofuranose
817	816	2-ketose in pyranosyl or furanosyl ring

These bands are in accordance with a previous report [27]. The last bands in the fingerprint region are typical for inulin and inulin-type fructans. Similar bands in the FTIR spectra were reported earlier for inulin-type fructan, especially the bands at 935, 873, and 818 cm^{-1} were typical for inulin from different plant sources such as burdock, echinacea, dahlia, and chicory [10, 15, 23, 27].

CONCLUSION

The current research enlarges the knowledge about the phytochemical composition of comfrey roots emphasizing on carbohydrate profile and flavonoids content in the extracts obtained after ulrasonic irradiation. This is the first detailed report on the isolation and chemical characterization of high- molecular long-chain inulin-type fructan with degree of polymerisation of 29-33 from defatted comfrey (*Symphylus officinale* L.) roots by microwave-assisted extraction. The comfrey roots were evaluated as a promising source of inulin – 10-15 g/100 g dw, depending on the applied extraction technique. Further studies about the health impract of comfrey fructan are needed. In general, it may be concluded that comfrey roots are sources of flavonoids, antioxidants and inulin, and may thus find application in cosmetics and pharmacy.

REFERENCES

1. N. Nastić, I. Borrás-Linares, J. Lozano-Sánchez, J. Švarc-Gajić, A. Segura-Carretero, A. *Molecules*, **25**, 837 (2020).
2. B. Salehi, F. Sharopov, T. Boyunegmez Tumer, A. Ozleyen, C. Rodríguez-Pérez, S., M. Ezzat, E. Azzini, T. Hosseinabadi, M. Butnariu, I. Sarac, C. Bostan, *Molecules*, **24**, 2272 (2019).
3. S.V. Luca, G. Zengin, Ł. Kulinowski, K. Sinan, K. Skalicka-Woźniak, A. Trifan, *J. Sci. Food Agric.*, **104,** 3971 (2024).
4. V.L. Savić, S. Savić, V. Nikolić, L. Nikolić, S. Najman, J. Lazarević, A. Đorđević, *Hem. Ind*., **69**, 1 (2015).
5. N. Petkova, K. Stefanov, M. Ognyanov, D. Mihaylova, D. Vassilev, *ETR*, **1,** 295 (2024).
6. I. Tahirovic, Z. Rimpapa, S. Cavar, S. Huseinovic, S. Muradic, M. Salihovic, E. Sofic, *Planta Med.,* **76**, 293 (2010).
7. I. Sowa, R. Paduch, M. Strzemski, S. Zielińska, E. Rydzik-Strzemska, J. Sawicki, R. Kocjan, J. Polkowski, A. Matkowski, M. Latalski, M. Wójciak-Kosior, *Nat. Prod. Res*., **32**, 605 (2018).
8. S. Chen, H. Shang, J. Yang, R. Li, H. Wu, *Ind. Crop. Prod*., **121**, 18 (2018).
9. H. Shang, J. Zhao, Y. Guo, H. Zhang, M. Duan, H. Wu, *Ind. Crop. Prod.*, **146**, 112183 (2020).
10. N. Petkova, I. Hambarlyiska, Y. Tumbarski, R. Vrancheva, M. Raeva, I. Ivanov, *Biointerface Res. Appl. Chem*, **12**, 2826 (2022).
11. N. Petkova, I. Ivanov, D. Mihaylova, A. Lante, *Acta Sci. Pol. Hortorum Cultus*., **3**, 125 (2020).
12. I.F.Benzie, J. Strain, *Methods Enzymol*., **299**, 15 (1999).
13. I. Kivrak, M. Duru, M. Öztürk, N. Mercan, M. Harmandar, G. Topçu, *Food Chem.,* **116**, 470 (2009).
14. I. Ivanov, R. Vrancheva, A. Marchev, N. Petkova, I. Aneva, P. Denev, V. Georgiev, A. Pavlov, *Int. J. Curr. Microbiol. Appl. Sci*., **3,** 296 (2014).
15. N. Petkova, G. Sherova, P. Denev, *Int. Food Res. J.*, ***25***, 1876 (2018).
16. A.Trifan, E. Wolfram, K. Skalicka-Woźniak, S. Luca, *Phytochem. Rev.*, **1**, 58 (2024).
17. E. Neagu, G. Roman, G. Radu, *Rom. Biotechnol. Lett.*, **15**, 5505 (2010).
18. F. U. Alkan, C. Anlas, O. Ustuner, T. Bakırel, A Sari, *Asian J. Plant Sci. Res.*, **4**, 62 (2014).
19. T.L. Housley, C.J. Pollock, in: Science and Technology of Fructans, M. Suzuki, N.J. Chatterton (eds.), CRC Press, Florida, 1993, p. 191.
20. A. van Laere, W. van den Ende, *Plant Cell Environ.,* **25**, 803 (2002).

21. T. Vasfilova, T. Vorob'eva, *Chem. Plant Raw Mater.*, **1**, 71 (2022) (in Russian).
22. E. Vasfilova, R. Bagautdinova, T. Okoneshnikova, *Tomsk State Univ. J.. Biology*, **2,** 30 (2015). (in Russian).
23. N. Petkova, A. Petrova, I. Ivanov, I. Hambarlyiska, Y. Tumbarski, I. Dincheva, M. Ognyanov, P. Denev, *ChemEng.*,**7**, 94 (2023).
24. S. Gokadze, L. Gogilashvili, L. Amiranashvili, V. Barbakadze, M. Merlani, A. Bakuridze, A. Salgado, B. Chankvetadze, *Bull. Georg. Natl. Acad. Sci*., **11**, 115 (2017).
25. D. Haaβ, G. Stadler, G. Franz, A. Abou-Mandour, F. Czygan, *Planta Medica*, **52**, 511 (1986).
26. M. Duan, H. Shang, S. Chen, R. Li, H Wu, *Int. J. Biol. Macromol*., **115**, 876 (2018).
27. Z. Cao, Y. Guo, Z. Liu, H. Zhang, H. Zhou, H. Shang, *Process Biochem.*, **112**, 223 (2022).
28. M. Grube, M. Bekers, D. Upite, E. Kaminska, *Spectrosc.*, **16,** 289 (2002).

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The abstract should be self-explanatory and intelligible without any references to the text and containing up to 250 words. It should be followed by keywords (up to six).

References should be numbered sequentially in the order, in which they are cited in the text. The numbers in the text should be enclosed in brackets [2], [5, 6], [9−12], etc., set on the text line. References are to be listed in numerical order on a separate sheet. All references are to be given in Latin letters. The names of the authors are given without inversion. Titles of journals must be abbreviated according to Chemical Abstracts and given in italics, the volume is typed in bold, the initial page is given and the year in parentheses. Attention is drawn to the following conventions: a) The names of all authors of a certain publications should be given. The use of "et al." in the list of references is not acceptable; b) Only the initials of the first and middle names should be given. In the manuscripts, the reference to author(s) of cited works should be made without giving initials, e.g. "Bush and Smith [7] pioneered...". If the reference carries the names of three or more authors it should be quoted as "Bush et al. [7]", if Bush is the first author, or as "Bush and co-workers [7]", if Bush is the senior author.

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EXAMPLES FOR PRESENTATION OF REFERENCES

REFERENCES

1. D. S. Newsome, Catal. Rev.–Sci. Eng., 21, 275 (1980).
2. C.-H. Lin, C.-Y. Hsu, J. Chem. Soc. Chem. Commun., 1479 (1992).
3. R. G. Parr, W. Yang, Density Functional Theory of Atoms and Molecules, Oxford Univ. Press, New York, 1989.
4. V. Ponec, G. C. Bond, Catalysis by Metals and Alloys (Stud. Surf. Sci. Catal., vol. 95), Elsevier, Amsterdam, 1995.
5. G. Kadinov, S. Todorova, A. Palazov, in: New Frontiers in Catalysis (Proc. 10th Int. Congr. Catal., Budapest (1992), L. Guczi, F. Solymosi, P. Tetenyi (eds.), Akademiai Kiado, Budapest, 1993, Part C, p. 2817.
6. G. L. C. Maire, F. Garin, in: Catalysis. Science and Technology, J. R. Anderson, M. Boudart (eds.), vol. 6, Springer Verlag, Berlin, 1984, p. 161.
7. D. Pocknell, GB Patent 2 207 355 (1949).
8. G. Angelov, PhD Thesis, UCTM, Sofia, 2001, pp. 121-126.
9. JCPDS International Center for Diffraction Data, Power Diffraction File, Swarthmore, PA, 1991.
10. CA 127, 184 762q (1998).
11. P. Hou, H. Wise, J. Catal., in press.
12. M. Sinev, private communication.
13. http://www.chemweb.com/alchem/articles/1051611477211.html.

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