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IN MEMORIAM

To the memory of Prof. Dr. Natasha Vaklieva-Bancheva (1951-2022)

This year we lost our dear colleague Prof. Natasha Vaklieva-Bancheva. She was born in 1951 in Breznik. She was graduated from Moscow Institute of Chemical Technology "D. Mendeleev" with a degree in Chemical Cybernetics in 1976. After graduating, she was almost immediately employed as an engineer and then as an assistant professor at Central Laboratory of Theoretical Foundations of Chemical Engineering (which in 1986 was transformed into Institute of Chemical Engineering) at Bulgarian Academy of Sciences. Her research career took place in Process Systems Engineering laboratory of the same institute. She defended a PhD thesis in 1992 on the subject of "On the optimization of multi-assortment chemical technological systems" under the supervision of Prof. DSc Christo Boaydjiev. She was promoted as associate professor in 1999 and was elected as Full Professor in 2012. During the years of her scientific career, Prof. Vaklieva-Bancheva has had successful international collaborations with various European universities such as Pannon University, Veszprem, Hungary; Universitat Politecnica de Catalunya, Barcelona, Spain; Vrije Universiteit Brussel, Brussels, Belgium; Imperial College, London, UK. She was also a member of scientific councils like Bulgarian Society of Chemical Engineering and CAPE WP at European Federation of Chemical Engineering. She has significant contributions in development of methods for modeling and optimization of chemical and biochemical production systems and production complexes for the purpose of their energy efficiency and sustainability improvement. It includes mathematical methods of heat integration and environmental impact minimization of production systems with batch and continuous processes, optimum scheduling of batch production systems as well as the development of methods for optimal design of sustainable supply chains considering all aspects of sustainability – economic, environmental and social with application in food industry and biofuels production. Prof. Vaklieva-Bancheva also had a significant contribution to the creation of special methods for modeling and optimization in the field of artificial intelligence. She was at the heart of developing a special genetic algorithm called BASIC (**B**ulgarian **A**cademy of **S**ciences **I**nstitute of **C**hemical Engineering), which has been successfully applied to solve a number of complex chemical engineering problems. She also developed static and dynamic neural networks for modeling complex biotechnological processes like wastewater treatment and biotransformation of crude glycerol from biodiesel production. Prof. Vaklieva-Bancheva had developed a two-stage stochastic optimization approach for capturing parameters uncertainty in an autothermal thermophilic aerobic digestion system for wastewater treatment. She was an author and co-author of 100 papers, published in prestigious international journals, like Computers & Chemical Engineering, Journal of Cleaner Production, Energy, Clean Technologies and Environmental Policy, Energies, etc. She was an author of different chapters in specialized issues published by Elsevier and Springer.

Prof. Natasha Vaklieva-Bancheva delivered lectures on "Synthesis and optimization of chemical production systems - Modeling, optimal schedules and optimal design of chemical-technological objects and systems with batch processes" in University "Prof. D-r Assen Zlatarov" – Burgas.

She was also the supervisor of two PhD students who successfully defended their theses and continued their scientific careers in the Institute of Chemical Engineering at Bulgarian Academy of Sciences.

Her high professionalism, dedication and broad scientific interests will be remembered and appreciated by her colleagues and friends.

We shall remember Prof. Natasha Vaklieva-Bancheva as a remarkable Bulgarian scientist in the field of chemical engineering and as a best colleague and friend.

From the team of the
Institute of Chemical Engineering at
Bulgarian Academy of Sciences.

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Effects of weight ratio of novel *Calotropis Procera* seed fiber on PLA polymer composite

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The current study presents a new bio-composite of PLA reinforced with *Calotropis Procera* seed fiber. The effect of *Calotropis Procera* seed fiber on the mechanical behaviour of polylactic acid (PLA) polymer was investigated. The composite was prepared by varying the percent weight of *Calotropis Procera* seeds with PLA as the matrix and then the impact, tensile and microstructural properties were evaluated using Charpy impact test, ultimate tensile machine and scanning electron microscope (SEM), respectively. Also, the thermal behaviour of *Calotropis Procera* seed fiber was investigated using a differential scanning calorimeter (DSC). The results showed that the impact strength decreases with the increase in fiber content from 17 kJ/m^2 for the pure PLA to 11.198 kJ/m^2 at 20%. There was no significant change in the impact strength for more than 20% fiber content. Tensile strength was found to decrease with the increase in fiber content from 54.2 MPa for pure PLA to an average strength of 47 MPa at 30% fiber in the composite. Tensile modulus, however, was found to increase with fiber content from 2.14 GPa for pure PLA to 2.45 GPa at 30% wt. fiber in the composite. The obtained result suggests that the *Calotropis Procera* seed fiber has a potential to be used as a reinforcement in polymer composites. Furthermore, this fiber could assist in cost reduction of PLA and thus increase the application of PLA.

Keywords: *Calotropis Procera*; composites; PLA; DSC; mechanical properties; microstructure

INTRODUCTION

Composites are heterogeneous materials which are derived from two or more constituents with different properties to obtain a material with better properties than those of the individual components [1]. Composites have been in use in a wide range of applications mainly in the fields such as biomedicine, aerospace, automotive and emerging consumer and construction industries, and others [2]. Their adoption in various fields has been attributed to their high strength-to-weight ratio, ease in manufacturability, good corrosion resistance, etc. [3]. However, most of the commercial composite materials, which are extensively used, are fabricated from synthetic materials [4]. Environmental issues associated with the disposal of these materials, cost and diminishing fossil fuels together with the new regulations has forced most industries to adopt the use and continuous search for eco-friendly materials to substitute the fossil fuel-based polymeric materials [5]. Natural fiber-reinforced polymer composites have been found to be neutral to the environment, resistant to chemical corrosion, offering good electrical resistance and acoustic insulation [6]. These composites find applications in the design and manufacturing of automotive interiors such as dashboard parts, cabin linings, door panels and center console among others [7]. Natural fibers possess good and desirable properties such as low density, abundancy, biodegradability, eco-friendliness, low carbon emissions and non-toxicity when compared to the synthetic fibers (Kevlar, glass and carbon) [8] and are most commonly used for the production of paper or packaging materials [9] [10]. These properties elevate lignocellulosic fibers as a potential substitute to the synthetic fibers.

In the search for sustainable fibers for polymer reinforcement, *Calotropis Procera* is identified, it is a member of the plant family, *Asclepiadaceae* and grows at an altitude of up to 1300 m with a mean annual rainfall of between 300-400 mm, in a preferably alkaline sandy soil [11]. It is a native plant in Kenya and other tropical countries amongst Afghanistan, Algeria, Burkina Faso and others. The latex and leaves of *Calotropis Procera* trees have antiviral, antifungal, and insecticidal functions [12]. Ushar seed fibers, which are composed of strong white and silky fibers with the length of 2–3.5 cm, and diameter between 28-33 μm, have been used as a stuffing material for mattresses and pillows, as well as a weaving into a strong cloth and a substitute for cotton wool for surgical uses [13]. *Calotropis Procera* grows well in drylands and is being domesticated in Kenya for textile uses [14]. Rhim *et al*. [15] found out that *Calotropis Procera* seed fiber

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contained 45.07% α-cellulose, 35.07% hemicellulose, 11.57% lignin, and 8.57% extractives which are comparable to those of wood and higher than those of some non-wood/vegetable fibers like kenaf, coir, rice straw, corn stalks and wheat straw, although the composition is said to depend on the geography of growth. *Calotropis Procera* seed fiber has not or insignificantly been investigated as a possible reinforcement or filler in bio-composite production [16].

In an attempt to evaluate its applicability as a natural fiber for bio-composites, this study seeks to evaluate the physical and thermal properties of *Calotropis Procera* fruit fiber, as sufficient information could not be found in the literature. Then, a composite was fabricated from seed fiber as a reinforcement at percentage weight ratios of 5%, 10%, 20% and 30% of the total fiber mass and PLA matrix combined. The resulting composites were characterized for mechanical and morphological properties. The study is an advancement towards the application of *Calotropis Procera* seed fiber as a natural fiber for reinforcing polymer and bioplastics - PLA for advanced applications.

EXPERIMENTAL

In this study, polylactic acid (PLA), which was used as the matrix, was delivered from Nature Works with the trade name Nature Works® Biopolymer 2003D and exhibited the following properties: residual monomer of 0.27%, relative viscosity of 4.04 P.s and color, yellowness index – 33.3. The PLA was oven-dried at 105℃ for 24 hours to reduce the amount of water suspension in the materials. The *Calotropis Procera* seed fiber used as matrix reinforcement in this study was delivered from World Agroforestry Center Nairobi - Kenya. Prior to use, impurities, including seed, were manually picked and the selected clean glossy fibers were dried at 105 ℃ for 3 hours.

The composite sample was prepared by mixing *Calotropis Procera* seed fibers and PLA as the matrix. The formulation of the constituents was aimed at determining the maximum amount of fiber that can be blended with PLA and the effects on its properties. The process consisted of two steps, namely: 1. Mixing the components on a Brabender measuring mixer, and 2. Pressing the mixture on a Servitec laboratory press Polystat 400 S. The raw material PLA was loaded through the top opening into the heated (160℃) mixer bowl where it was melted and homogenized by mixing blades. After the PLA was melted, the fibers were added, progressively from 5, 10, 20 and 30 percent wt of mixture as shown in Table 1. After the whole fiber amount was added, the blends were mixed for another 15 min at 160℃ and then all blends were pressed in a mold 200 × 200 × 4 mm on a laboratory press at a temperature of 160℃.

A scanning electron microscope (SEM) (JEOL JSM-6010 LV, TH Wildau - Germany) was used to analyze and collect microscopy images of the fiber and the fractured surface of the tensile test samples. The samples for microscopy were prepared by spraying with a 50 nm thick gold layer at pressure of 10-2 bar and a current of 50 mA. The machine used during this process was a high vacuum sputter coater (LEICA EM SCD 500, Germany).

The thermal phase transformation of the fiber was estimated using differential scanning calorimetry (DSC) 204 Phoenix, NETZSCH, Germany. The fiber sample weighed accurately in aluminium pan, the weight of the DSC samples was between 5 and 10 mg and heated in, from -100 to 300 ℃ temperature range with heating rate 10 °C/min under nitrogen. DSC curves showing exothermic and endothermic reaction peaks were recorded from the analysis.

The mechanical properties of the fabricated composites were studied through tensile and impact tests. Measurements were performed with a Zwick Z 020 (Zwick GmbH & Co. KG, Ulm, Germany) tensile testing machine according DIN EN ISO 527-1. For the determination of the Young's modulus an initial speed of 1 mm/min was chosen. After 0.25% elongation, the speed was increased to 55 mm/min. The resulting stress-strain curves show values such as the Young's modulus, the yield strength, the breaking strength, as well as the elongation at yield and at break. The ambient air temperature during the tensile test was 23°C. For each sample, five measurements were taken and the average was computed for statistical accuracy. The samples were cut and prepared according to EN ISO 527-1:1995 [17] [18].

Table 1. Sample formulation

	Composite				
Constituents	Sample $_1$	Sample $_2$	Sample $_3$	Sample $_4$	Sample $_5$
PLA (%)	100	95	90	80	70
Calotropis Procera (%)	0	5	10	20	30

Charpy impact test was carried with PSW 4J impact tester according to DIN EN ISO 179 [19], using 10 unnotched samples as standards. In each case a standard deviation of < 15% (weight drop) was used to calculate the Charpy impact strength. The Charpy impact strength of the unnotched specimen a_{cU} expressed in kilojoules per square meter, was calculated for each sample as follows:

$$a_{cU} = \frac{E_c \times 10^3}{h \times b}$$

where: *Ec = corrected stored energy in Joules, h = thickness in mm of the test sample, b = width in mm of the test sample.*

RESULTS AND DISCUSSION

Fig. 1 presents the thermal properties of the fiber. The fiber showed an endothermic reaction between 25°C and approx. 125°C. This peak was associated with the evaporation of moisture absorbed by the fiber. This evaporation occurred at 68°C. Between 125°C - 260°C, there was no endothermic or exothermic reaction, signifying that the fiber was thermally stable between these temperatures. The exothermic peak experienced at 327°C was associated with the degradation of lignin and hemicellulose present in the fiber. Above this temperature, an endothermic peak was observed signifying the degradation of cellulosic matter in the fiber. The behavior observed is comparable to those of other lignocellulosic fibers [20]. Figs. 2(a) and 2(c) show SEM images of *Calotropis Procera* fiber at different magnifications (×150, ×1200 and ×30000), respectively. Fig. 2(a) shows the broken fibers cross-section which was observed to be hollow. An approximate diameter was measured as shown in Fig. 2(c) and found to be about 102 nm. Fig. 2 (b) at a magnification of ×150 shows feather-like appearance crisscrossing one another with some bent and macerated.

Scanning electron microscope images were taken from a broken surface of a tensile test specimen and presented in Figs. 3 and 4. Higher magnification of ×550 was used to study the interface between the matrix and the fiber while low magnification of ×150 was used to study the fiber dispersion in the matrix, as well as the failure modes during the tensile test. Figs. 3(a) and (b) show SEM images of a composite with 5 % fiber content. Fig. 3(a) shows a good interface between the matrix and the fiber. Breakage of the latter, as well as delamination was observed. The dispersion of fibers in the matrix was noted to be uniform as shown in Fig. 3(b). Figs. 3 (c) and 3 (d) show SEM images at 10% fiber weight, voids were observed which could be attributed to vaporization of moisture in the mixture. Uniform fiber distribution in the matrix was noted. The interface was also found to be good, delamination was also noted. The figures also indicate the matrix failure and fiber debonding due to the compressive loading on the specimen. At 20 % as in Figs. 4 (a) and (b), there was uniform fiber dispersion in the matrix with little physical appearance of the matrix; this could lead to poor wetting of the fiber resulting in poor interface between the matrix and fiber and hence to constant deterioration of mechanical properties. Fiber breakage was intense at 20% and could be attributed to the low density of fiber which occupied large volume and hence longer time to load to the mixer.

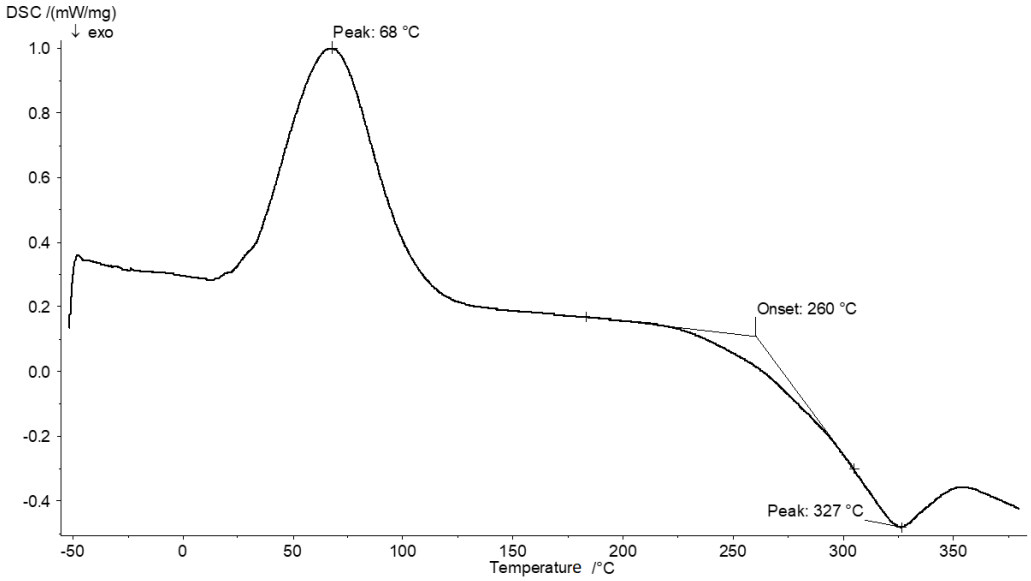

Figure 1. DSC analysis of the *Calotropis Procera* seed fiber

This destruction process of the fiber explains the decrease in the mechanical properties of the composites. SEM images at 30 % as in Figs. 4 (c) and (d) show fiber breakage and uniform fiber distribution in the matrix with no physical appearance of the matrix. Furthermore, they show that the fibers collapsed. This could mean that more than 30% weight could be used. Poor wetting of the fibers was also noted.

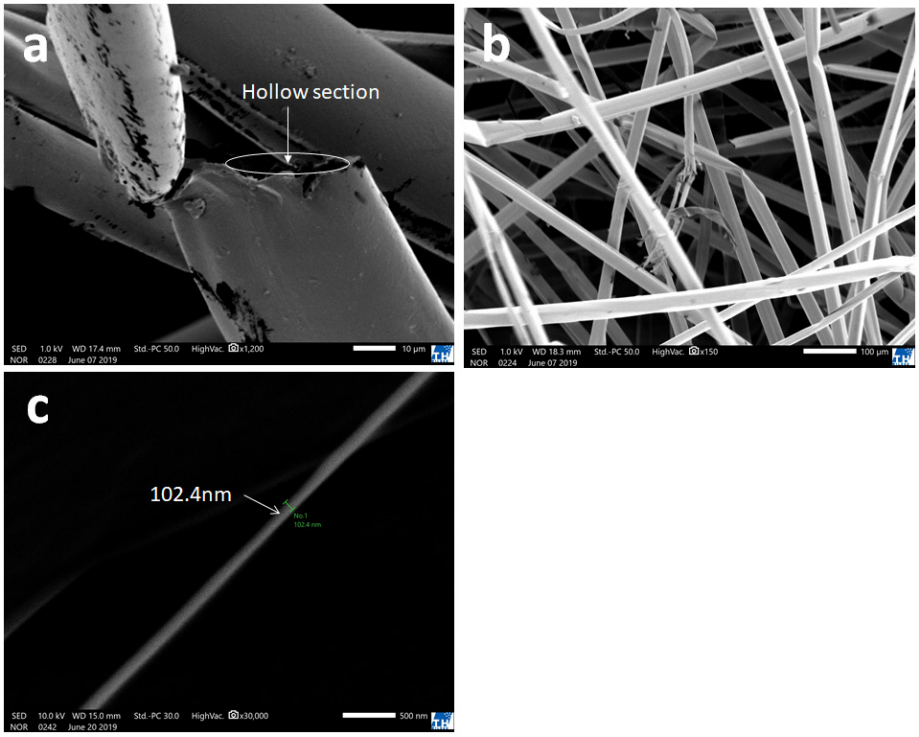

Figure 2. SEM micrographs of *Calotropis Procera* seed fiber at magnification (a)×1200, (b) ×150 and (c) ×30000

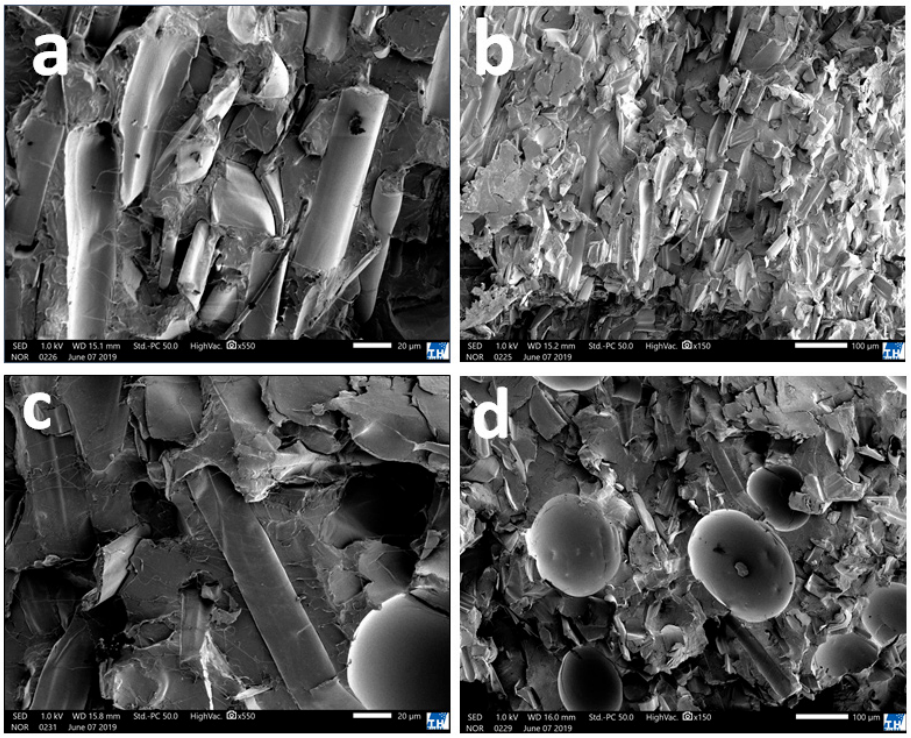

Figure 3. SEM micrographs of PLA reinforced with 5% and 10% *Calotropis Procera* seed fiber at magnifications of ×550 ((a, c) and ×150 (b, d)

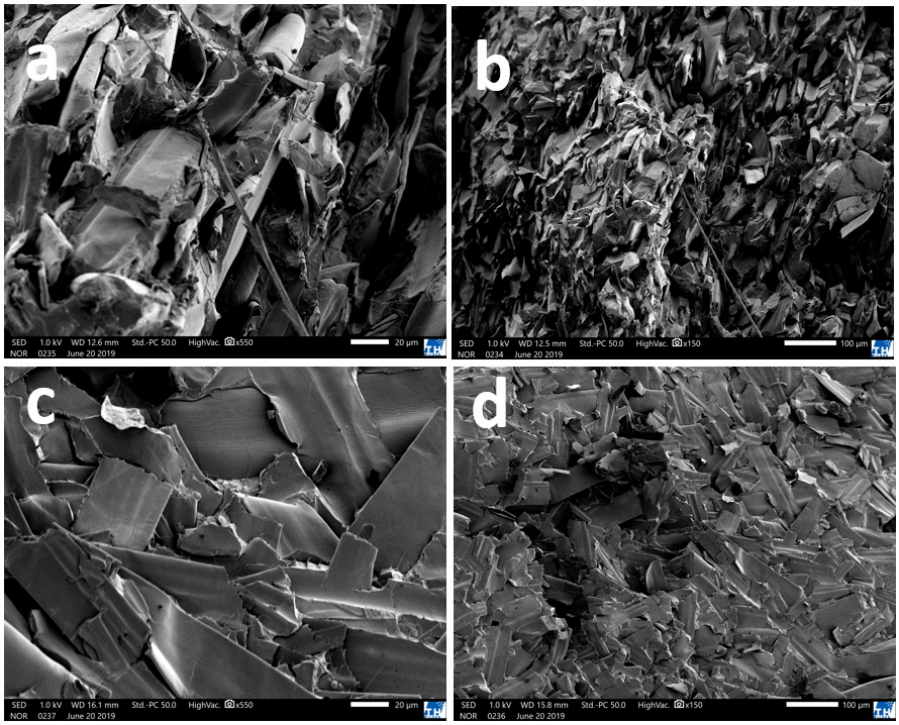

Figure 4. SEM micrographs of PLA reinforced with 20% and 30% *Calotropis Procera* seed fiber at magnifications of ×550(a, c) and ×150 (b, d)

The impact strength of the composite samples tested using 10 unnotched samples showed a reduction in impact strength with fiber loading. The impact strength for PLA reinforced with *Calotropis Procera* fibers at 5% 10% 20% and 30% was 15, 11.7, 11.1 and 11.1 kJ/m^2, respectively, which was found to be less than that of pure PLA at 17 kJ/m^2. The reduction in the strength could be a result of fiber breakage owed to the long time required to load the fiber into the extrusion machine and milling of the fibers as portrayed in the SEM images.

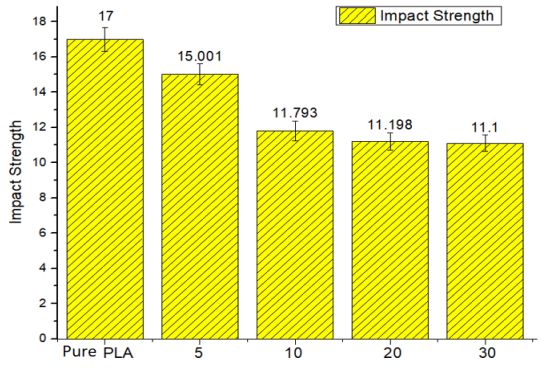

Figure 5. Impact strength of composites for pure PLA, 5%, 10%, 20% and 30% fiber content

The results of the tensile tests conducted on the *Calotropis procera* fiber-reinforced PLA composite are shown in Figure 6. The tensile strength of the composite at 5% is 47.4 MPa which is lower than that of PLA at 54.2 MPa and on further addition of the fiber there is no significant change. From these results, it is clear that there was no significant enhancement of the mechanical properties, however, there was a reduction in the amount of PLA used and this means a reduction in cost of producing PLA composites. The U.S. export price of PLA was 1,910 U.S. dollars per ton in 2018. The reduction of 5 % means a cost reduction of 100 U.S. dollars per ton. The reduction in tensile strength could be due to the short fiber resulting from milling of fibers during loading. The higher the fiber content the longer the time it takes to completely load the fiber in the mixing chamber owing to the large volume per kilogram of the fiber.

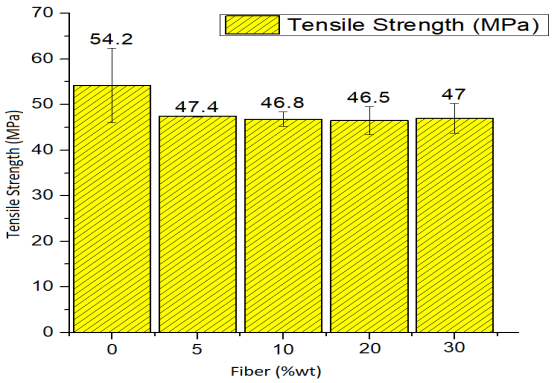

Figure 6. Tensile strength of composites for pure PLA, 5%, 10%, 20% and 30% fiber content

Tensile modulus was used to measure the stiffness of the material. Figure 7 shows an increase in the tensile modulus of the composite on increasing the fiber amount. This means the stiffness of the composite was maximum at 30% fiber loading at 2450 MPa. This is slightly higher than that of pure PLA at 2140 MPa. These results are important for the future choice of application of the composite materials. On the one hand, the tensile strength decreased with increasing the amount of the fiber but the stiffness of the materials increased. There is a vast array of applications for polylactic acid. Some of the most common uses include plastic films, bottles, and biodegradable medical devices (e.g. screws, pins, rods, and plates that are expected to biodegrade within 6-12 months). The use of *Calotropis Procera* as a reinforcement in synthetic polymer composites could reduce the amount of plastics released to the environment. On the other hand, when blended with biopolymers, the degradability is enhanced.

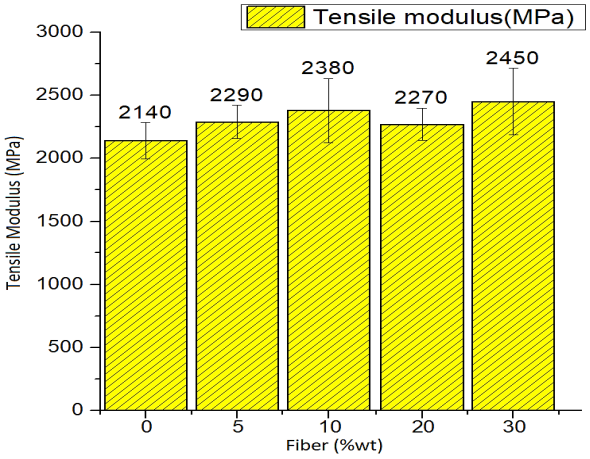

Figure 5. Tensile modulus of the composites for pure PLA, 5%, 10%, 20% and 30% fiber content

CONCLUSION

The physical and thermal properties of the novel *Calotropis Procera* fiber were evaluated. The effects of this fiber as a reinforcement of a PLA matrix were also determined. The result obtained from the SEM analysis revealed that the fiber had hollow cross-section which makes it lighter, with a diameter of approximately 102.4 nm. DSC analysis showed that the fiber was thermally stable between 125°C - 260°C. This temperature allows for easy blending with most thermoplastics without deterioration of properties. Results obtained from tensile test and impact test showed a marked decrease in strength with an increase in fiber loading. This could have been due to the nature of the fiber surface. SEM images revealed a smooth surface, further the non-cellulosic components present in the fiber may have led to deterioration in the properties. Narayana *et al.* [21] reported on surface treatments as a way of enhancing the surface properties of natural fibers. The treatments accelerate the creation of interfibrillar spaces which promote the anchoring mechanism of the matrix to the fiber. Therefore, a further investigation on the effect of surface properties of this fiber on the properties of the composite is recommended.

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REFERENCES

1. F. Campbell, Structural Composite materials, USA, ASM International, 2010.
2. G. Gupta, A. Kumar, R. Tyagi, S. Kumar, *International Journal of Innovative Research in Science Engineering and Technology,* **5** (5), 6907 (2016).
3. C. Unterweger, O. Bruggemann, C. Furst, *Polymer Composites,* **35**, 227 (2014).
4. M. Sanjay, P. Madhu, M. Jawaid, P. Senthamaraikannan, S. Senthil, S. Pradeep, *Journal of Cleaner Production,* **172**, 566 (2018).
5. T. Väisänen, O. Das, L. Tomppo, *Journal of Cleaner Production,* **149**, 582 (2017).
6. H. Wang, M. Ho, J. Lee, T. Lau, J. Leng, D. Hui, *Composite Part B: Engineering,* **43** (8), 3549 (2012).
7. K. L. Pickering, M. A. Efendy, T. M. Le, *Composite Part A: Applied Science and Manufacturing,* **112**, 83 (2016).
8. Y. Wu, C. Xia, L. Cai, A. Garcia, S. Shi, *Journal of Cleaner Production,* **184**, 92 (2018).
9. N. Ivanova, D. Todorova, S. Bencheva, *Environmental Engineering and Management Journal,* **3** (3), 527 (2004).
10. D. Todorova, *Journal of Chemical Technology and Metallurgy,* **51** (5), 514 (2016).
11. S. Kareem, I. Akpan, O. Ojo, *Africa Journal of Biomedical Research,* **11** (1), 105 (2008).
12. H. Estabraq, M. Abbas, A. Salam, *IJPSR,* **10**, 1988 (2019).
13. C. Orwa, A. Mutua, R. Kindt, R. Jamnadass, S. Anthony, Agroforestree Database: a tree reference and selection guide version 4.0 (http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp), 2009.

14. A. Muchugi, J. Xu, A. Gachuiri, J. Kimiti, G. Muriira, F. Mutiso,Calotropis procera: A New Investment for African Drylands, in *Tropentag*, Bonn, 2017.
15. J.-W. Rhim, A. A. Oun, *Materials Letters,* **168**, 146 (2016).
16. K. Yoganandam, P. Ganeshan, B. Nagaraja Ganesh, K. Raja, *Journal of Natural Fibers,* 1 (2019).
17. T. Grätzl, Y. v. Dijk, N. Schramm, L. Kroll, *Composite Structures,* **208**, 557 (2019).
18. B. ISO, Plastics-determination of tensile properties, British Standard, Germany, 1997.
19. I. ISO, Plastics-determination of charpy impact properties, Part 1: Non-Instrumented Impact Test, International Organization for Standardization, Germany, 2010.
20. A. L. F. S. d'Almeida, D. W. Barreto, V. Calado, J. R. M. d'Almeida, *Journal of Thermal Analysis and Calorimetry,* **91** (2), 405 (2008).
21. L. V. Narayana, L. B. Rao, *Materials Today: Proceedings,* **44**, 1988 (2020).

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Textile dyeing of cotton and wool textile material with natural dyes extracted from bluish purple grapes

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Fruits and vegetables contain considerable amounts of natural dyes which could serve in textile dyeing operations. A natural dye was extracted from fresh bluish purple grapes by aqueous extraction method. To get the maximum yield of natural dye from bluish grape, variation in temperatures and time was explored. The dyeing was carried out on wool and cotton textile material without, with a single and a mixed mordant system. Color strength; build up properties, CIE Lab coordinates and fastness properties were measured. The results indicated the potential of extractable natural dye on both textile materials, especially on wool. It was observed that mordant dosage and dyeing temperature were the key factors for good dyeing conditions with the natural dye. Regarding fastness, washing was good on all dyeing fabrics but light fastness was not up to the mark with respect to commercial dyeing requirements.

Keywords: Cotton and wool textile material, Dyeing, Bluish purple grapes, Natural dyes, Extraction, Mordant system.

INTRODUCTION

The plants, minerals, and animals are the major source of natural dyes, almost non-substantive and can be used with the help of a mordant (metallic salt). There is an edge to natural dyes as compared to synthetic dyes, as they are soft, shiny and comforting for human eyes [1]. The raw material of synthetic dyes is non-renewable; while the materials for the natural dyes are environmentally friendly, renewable, and biodegradable. The raw material for natural dyes comprises anti-allergy, safety with skin. Its importance is due to medicinal properties on skin especially for kids, and no hazardous elements found for human health [1]. Nature produces different sources of dyes such as insects and plants used since centuries. Plants are the key source of around all natural dyestuffs and sources of different colors - brown, black, yellow, red, and blue, as well as combinations of these [4]. Flowers, wood, seed, root, fruit, leaf, bark, etc. are parts of plants which are the main source of dyes production. For thousands of years, indigo was a very important natural dye in Arab countries [8, 14], the turmeric was the main source plant for natural dyes used in the Egyptian period about four thousand years ago. Indigo dyes can be used only once, due to vat dyes [8]. Insolubility of dyes in water is always a problem, while indigo reduced and converted in the form of white color and it is soluble in water. There are two other types of vat dyes such as biblical blue and Tyrian purple. For reduction of dyes the vat containing fabric/yarn can be applied [9]. Except for few restrictions, there are many advantages of natural dyes. In natural dyes the extraction time is a very important and tedious factor. The dyeing cost by natural dyes is higher as compared to synthetic dyes, due to the color components in the raw material. Among natural dyes some are fugitive and require mordant for improvement of fastness properties. Among these few mordants (metallic) are hazardous [6]. The collection of the plants, lack of the technical expertise for extracting, non-availability of standardization, lack of dyeing techniques by natural dyes is a problem the entire world is facing today. As compared to synthetic dyes the natural dye is less toxic, less polluting, less poisonous, and carcinogenic free. These dyes are soft in use, environmentally friendly and recyclable [6]. Due to mass/public awareness, the use and demand of natural dyes is increasing gradually, including use in food products, due to its lower toxicity and environmentally friendly nature. Many studies have been done for the assessment of benefits, availability of potential for use of natural dyes on pilot plant scale. Some new techniques including biotechnology are needed for the improvement in production of dyes quantity and quality [6]. Most of the natural dyes do not directly combine with the material to be dyed. They require chemical substances known as mordants, which are metallic salts that have an affinity for both fiber and dyestuffs. A mordant is considered equally a

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chemical, which can itself, be stable on the fiber and which also combines with dyestuffs. Mordanting can be carried out in three ways such as, pre-mordanting, simultaneous mordanting, post-mordanting. Mordants (metallic), oil mordant, and tannis are three important types of mordants [13]. The traditional way of extraction of the natural dyes is by soaking the plant or other material containing dye in water and boiling the solution in earthen or metal vessels (preferably copper, stainless steel). Generally, four methods are adopted for extraction such as, aqueous, alkaline, acidic, and fermentation [12].

MATERIAL AND METHODS

Experimental material

The sample 100% free optical brightener, bleached scoured woven fabric (cotton) (150 g/m^2, 1/1 plain weave) and 100% wool yarn was used for dyeing purpose. The chemicals and auxiliaries used such as gallotannic acid, potash alum, acetic acid, sodium chloride, sodium hydroxide, and hydrochloric acid were purchased from Merck. Soaping agent was used of commercial grades purchased from local market.

Equipment

Dyeing machine (AHIBA IR) and Datacolor (SF 650X) was used for the dyeing experiments and data color was used for color match and for colorimetric data evaluation. Light fastness was determined on a Weather-Ometer. Samples were preserved on a curing machine of Rapid. Fastness rating was assessed on a color matching cabinet by comparing with grey scale and blue wool scale for light fastness rating.

Extraction method

A weighed amount of grapes fruit was extracted with distilled water in a beaker. In the standard procedure the mass of grapes / volume of liquid ratio were set at 5:20, i.e. 5 g of grapes was extracted with 20 ml of water. The extraction process was done at different temperatures (60, 95°C) with variation of time (40, 60, 80, 100 min) to get the maximum yield of dyeing extract.

Dyeing experiment

Exhaustion was used for dyeing operations (liquid ratio) 1:30, i.e. for 1 g of textile material a dye-bath of 30 ml was used. As per standard method, the dyeing trials were executed at 95°C. Different dyeing procedures were tested with regard to mordanting and dyeing temperature. Either 10 g of bleached wool yarn or 10 g of bleached cotton fabric was used as textile material. Both cotton and wool textile materials were tried in a sequence.

- Experiment No. 1,8,15: Standard dyeing, without mordant at 60°C, 80°C, 95°C for 60 min, L: R 1:30.
- Experiment No. 2,3,9,10,16,17: Pre-dyeing with mordant (5-15%) gallotannic acid at 60°C, 80°C, 95°C for 60 min, L: R 1:30.
- Experiment No. 4,5,11,12,18,19: Dyeing with mordant used alum (2-4g/l) at 60°C, 80°C, 95°C for 60 min, L: R 1:30.
- Experiment No. 6,7,13,14,20,21: Pre-dyeing with mordant using gallotannic acid and dyeing with alum at 60°C, 80°C, 95°C for 60 min, L: R 1:30.

The alum ($KAl(SO_4)_2.12H_2O$) used as a mordant, was added to the dye bath to a final dye bath concentration 3-4 g/l.

Optimum procedure of pre-mordanting with gallotannic acid ($C_{76}H_{52}O_{46}$) was used. A mass of gallotannic acid corresponding to 5-15 % of the mass of textile material was dissolved in water at 50°C. The textile samples were then impregnated at 80°C for 1h at a liquor ratio of 1:20. The mordanted samples were washed with water, and dried before dyeing. After that, unfixed dyestuff was detached by washing 3 times with cold water.

Measurement of dye fixation

The fixation of the dye in % was calculated first by determining the reflectance (R) of the dyed samples at the wave length of minimum reflectance (maximum absorbance) on an SE-650 spectrophotometer. The color yield (K/S) value was then calculated by using the Kubelka-Munk equation (eq. 1) and the dye fixation (%) was evaluated using eq. 2 [5].

$$K/S = (1-R)^2/2R \quad (1)$$

$$\% \text{ Dye fixation} = \frac{K/S \text{ value of sample after soaping}}{K/S \text{ value of sample before soaping}} \times 100 \quad (2)$$

Fastness testing

The fastness properties were determined according to the international standards. The specific tests used were ISO-105-CO3 [2] (color fastness to washing), ATCC – 16 E [3] option-3 (color fastness to light).

RESULTS AND DISCUSSION

Effect of time and temperature on color extraction

The effect of temperature was observed with different time intervals on the color extraction of grapes as shown in Fig. 1.

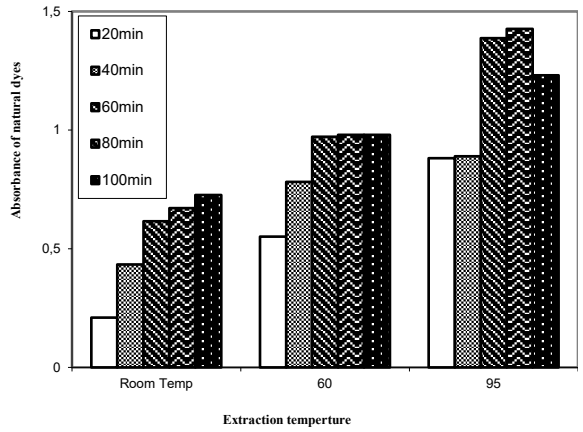

Fig 1. Absorbance of natural dyes at various extraction temperatures as a function of time.

The increase in the absorbance may be regarded as a function of extraction time and temperature [10], absorbance at maximum wavelength (λ_{max}) of the extracted dyes from grapes was found to be at 540 nm at 60°C, the absorbance slightly increased as compared to room temperature. At 95°C the absorbance was found to be maximum (1.388), the majority of the color substances were extracted within the first 60 min of the extraction time. After 60 min there is no significant effect on absorbance.

Color strength and dyeing quality

The K/S values calculated from the colorimeter data for the dye applied on cotton and wool using both mordants, i.e. gallotannic acid and alum, are shown in Table 1.

It was observed that with increase in different conditions, i.e. temperature and mordant, the value of K/S increases which shows good build up properties of the dye. At a low temperature 60°C and without mordant the K/S values of cotton and wool were 0.75 and 0.97, respectively, but these values reached a maximum when the best conditions were applied. The difference in color strength of cotton and wool was significant, i.e. at 95°C; it was 1.65 and 3.47, respectively.

Table 1. Results of dyeing experiments on cotton and wool textile material**.** Temperature of dyeing (60, 80, 95°C), type of mordant (gallotannic acid, alum), CIE Lab Coordinates and selected fastness properties (Wet fastness: Color/ bleeding: 1 = poor, 5 = excellent, fastness to light: 1 = poor, 8 = excellent).

Exp No.	Temp. (°C)	Gallo-tannic acid (%)	Alum (g/l)	Textile material	K/S	L	a	b	Fix (%)	Washing CO3 Change in Staining Shade		Light Fast-ness
1.	60	0	0	Cotton	0.75	76.73	4.62	-1.96	66	3/4	3/4	1/2
				Wool	0.97	65.31	5.84	-2.56	72	4	3/4	2
2.	60	0	2	Cotton	0.92	72.21	4.73	-1.98	67	4	3/4	2
				Wool	1.20	64.24	8.12	-2.12	73	4	3/4	2
3.	60	0	4	Cotton	1.16	70.37	5.06	1.92	68	4	3/4	2/3
				Wool	1.34	64.16	8.38	-1.90	74	4	3	2/3
4.	60	5	0	Cotton	1.15	70.24	5.24	-2.16	68	4	3/4	2/3
				Wool	1.34	63.21	8.92	-2.22	75	4/5	3/4	3
5.	60	15	0	Cotton	1.22	70.13	5.47	-2.31	68	4	3/4	½
				Wool	1.76	62.33	9.16	-2.67	75	4/5	3/4	2/3
6.	60	5	2	Cotton	1.22	71.06	5.72	-2.55	70	4/5	3/4	2/3
				Wool	1.75	61.78	9.24	-2.78	78	4	3	3
7.	60	15	4	Cotton	1.42	70.26	5.88	-2.61	72	4/5	3/4	2/3
				Wool	1.96	61.24	9.76	-2.83	80	4	3/4	¾
8.	80	0	0	Cotton	0.92	74.21	5.42	2.77	68	4	4	2
				Wool	1.13	63.42	8.88	-2.68	74	4/5	4	2/3
9.	80	0	2	Cotton	1.34	71.86	6.36	-2.80	68	4	4	2/3
				Wool	1.56	62.54	8.92	-2.81	76	4/5	4	3
10.	80	0	4	Cotton	1.51	69.38	6.72	2.43	70	4/5	3/4	2/3
				Wool	1.80	61.30	9.74	-3.56	78	4/5	4	3
11.	80	5	0	Cotton	1.22	69.83	6.55	-2.46	70	4	3/4	½
				Wool	1.58	61.07	9.12	-2.98	78	4/5	4	2/3
12.	80	15	0	Cotton	1.41	69.76	7.66	-2.73	72	3/4	3/4	2/3

				Wool	1.89	60.35	11.78	-4.23	80	4	3/4	2/3
13.	80	5	2	Cotton	1.55	69.81	7.68	-3.24	76	4	3/4	2/3
				Wool	2.04	60.11	12.37	-7.64	80	4/5	4	3
14.	80	15	4	Cotton	1.73	69.24	7.88	-4.56	78	4	3/4	3
				Wool	2.56	59.96	14.15	-9.77	82	4/5	4	¾
15.	95	0	0	Cotton	1.21	72.63	8.23	-2.81	68	4/5	4	2
				Wool	1.46	62.10	9.21	-3.49	75	4/5	4	2/3
16.	95	0	2	Cotton	1.50	70.38	5.43	-3.12	70	4/5	4	3
				Wool	1.82	61.34	10.22	-4.25	78	4/5	5	¾
17.	95	0	4	Cotton	1.66	68.72	6.21	-4.37	76	4/5	4	3
				Wool	2.21	58.67	13.43	-7.09	80	4/5	4	¾
18.	95	5	0	Cotton	1.64	68.13	6.82	-3.12	76	4/5	3/4	3
				Wool	2.82	57.16	13.76	-4.15	82	4/5	3/4	¾
19.	95	15	0	Cotton	1.67	68.14	6.88	-3.55	76	4/5	4	¾
				Wool	3.18	57.12	14.26	1.28	82	4/5	4	¾
20.	95	5	2	Cotton	1.65	68.27	6.82	-2.21	76	4/5	4	¾
				Wool	3.42	57.15	14.57	-4.56	84	4/5	4	3
21.	95	15	4	Cotton	1.65	68.38	7.21	-2.86	76	4/5	4	3
				Wool	3.47	57.06	15.86	-5.54	86	4/5	4	¾

Effect of temperature

The effect of temperature has been investigated in detail in Table 1. With an increase in temperature; the strength of the color (K/S value) will also increase. This may be a result of the increase in the disaggregation of the dye molecules and/ or increase in the rate of dye penetration into the fiber [7]. With reference to Fig. 2, without mordant the behavior of both textile materials are the same with leading the strength of wool than cotton. While at maximum mordant the maximum K/S of cotton was obtained at 80°C it rapidly increased in wool between 80°C to 95°C, which can be attributed to the optimum temperature for wool dyeing.

Effect of mordant

Table 1 shows that despite the significant role of temperature in color strength, the concentration of mordants plays a vital and major role. The results indicate that the increase in concentration of mordant shifted the reaction towards more dyeing opportunities producing better yield and fixation. The gallotannic acid is very crucial for dyeing purpose and is used for cotton preparation and permanent retaining of color material [11]. Alum has no effect on color, which helps evenness and brightens slightly the textile material.

In this regard, by using both mordants maximum yield and fastness results were observed at all dyeing conditions. At 95°C without mordant the K/S for cotton was 1.21, which increased to 1.65 at maximum mordant while for wool it increased from 1.46 to 3.47.

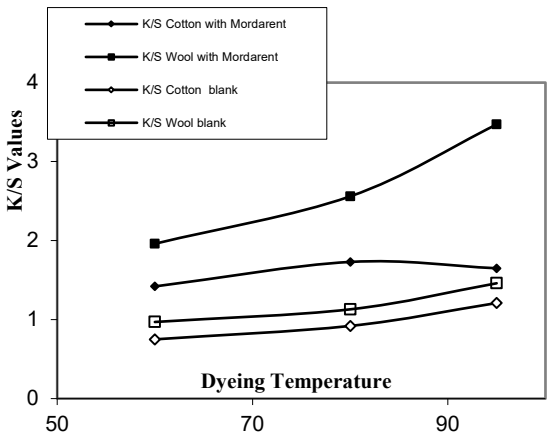

Fig. 2. Color strength at different dyeing temperatures on cotton and wool textile material at optimum mixed mordant system

Fastness properties

Fastness properties of textile material dyed under optimized conditions are shown in Table 1. As can be seen, the dye has fairly good wet fastness properties. As presented, a minor difference in fastness properties was found among the different dyeings of grapes extract. The dyeing experiments carried out without mordant showed no significant difference in light fastness at a higher temperature of 95ºC in the case of cotton. But in the case of wool the light fastness slightly increased from 2 to 2/3 with increasing the dyeing temperature. On dyeing with mordant, the light fastness slightly increased in the case of cotton but for wool textile material it was better and became stable at 3/4 rating. The light

fastness of wool is not up to the mark of textile standards but it is better as compared to the light fastness of cotton dyed with grapes extract. The washing fastness properties in both cotton and wool textile material are better and can be comparable with most of the available commercial direct dyes. However, the washing fastness of wool dyed fabric is better as compared to the cotton dyed fabric. In natural dyeing the mordanting agent directly influences its dyeing properties, as well as fastness properties.

CONCLUSION

The colorimetric data revealed that the bluish purple grape extract is a good and potential natural colorant source for dyeing wool, as well as cotton textile material. The maximum concentration of extractable dye materials was obtained at 95°C with maximum time, which were sufficient for their use as textile dyes. The shades of the dyeing obtained on cotton and wool were beige and pink, respectively, which are interesting in textile dyeing. The dyeing process was performed in the presence of mordant with different concentration and system i.e. gallotannic acid and alum, however the optimal dyeing was carried out using a mixed mordant system consisting of 15% of gallotannic acid and 4 g of alum. Regarding wet fastness both textile materials showed good results but in various cases the fastness to light was insufficient; therefore, research work optimization needs to be explored further. These experiments indicate that the quality of dyeing and fastness of wool is slightly better than of cotton.

REFERENCES

1. A. K. Samanta, A. Konar, Dyeing of Textiles with Natural Dyes, Intech Open Limited, 5 Princes Gate Court, London, SW7 2QJ, UK, 2011.
2. Color fastness to washing, ISO –105-CO3, 2005.
3. Color fastness to light, AATCC – 16E, option-3, 2004.
4. K. V. Chandramouli, Sources of Natural Dyes in India – A Compendium with Regional Names, PPST Foundation, Chennai, 1995.
5. R. Kotek, M. Afshari, S. Gupta, M. H. Kish, D. Jung, *Coloration Technology,* **120**, 26 (2004).
6. R. Siva, *Current science,* **92**(7), 916 (2007).
7. S. Ali, N. Nisar, T. Hussan, *Journal of the Textile Institute,* **98** (6), 559 (2007).
8. S.M. Robertson, Dyes from plants, New York, 1973.
9. R. Singh, A. Jain, S. Panwar, D. Gupta, S. K. Khare, *Dyes and Pigments,* **66** (2), 99 (2005).
10. T. Bechtold, A. Mahmud-Ali, R. Mussak, *Jour. Sci. Food Agric.*, **87**, 2589 (2007).
11. T. Bechtold, A. Turcanu, E. Gangl, S. Geissler, *J. Cleaner Prod.,* **11**, 499 (2003).
12. V. Kumar. B. V. Bharati, *Dyestuff Repr.*, **87**(9) 40 (1998).
13. V. Sundaram, Handbook of cotton in India, Indian society for cotton improvement, Mumbai, 1999, p. 37.
14. J. Balfour-Paul, Indigo, British Museum Press, 2000.

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Cantharidin: A chemical precursor for the development of novel bioinsecticides

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Bioinsecticides based on natural toxins offer an alternative means of pest management with the potential to counter insecticide resistance and reduce our heavy reliance on conventional insecticides. Cantharidin is a natural toxin generally produced by the beetles belonging to the family, Meloidae. It has been a drug of choice in both folk and traditional medicine, especially for the topical treatment of viral skin infections such as warts and molluscum. Historically, it has also been used as an aphrodisiac. Besides, cantharidin is also being used as an insecticide in the form of an emulsifiable concentrate (EC) for the control of lepidopteran pests. Although cantharidin has been proved highly effective against a variety of insect pests, its chemical synthesis and potential toxicity to non-target organisms have been a serious concern. A great deal of research is being carried out to synthesize its bioactive analogues with high bioactivity and improved safety profile to non-target organisms or the environment. Many promising analogues of cantharidin have already been synthesized and their effectiveness to several pest species has been reported. Due to the unique mode of action, these analogues will help to reduce the development of insecticide resistance and may be more cost-effective than cantharidin-based insecticides.

Keywords: Cantharidin, insecticide, emulsifiable concentrate, structural relationship activity, protein serine/threonine phosphatase

INTRODUCTION

Cantharidin (*exo*-1,2-*cis*-dimethyl-3,6-epoxy-hexahydrophthalic anhydride) is a widely distributed compound in the insects belonging to order Coleoptera and family Meloidae, some species of Tenebrionidae, Cerambycidae, and Fulgoridae [1-3]. Some species of the Meloidae, commonly known as blister beetles, secrete a chemical blistering compound, cantharidin as a defensive mean. The beetles belonging to this family are considered cosmopolitan, however, their presence in New Zealand, Antarctic regions, tropical and subtropical savannas has not been reported [3].

Several species belonging to the insect family Meloidae produce a poisonous compound with comparable toxicity to strychnine and cyanide, used by the insect as a defensive tool against predators. Its toxicity has been observed in several organs such as the digestive tract, and kidneys in mammals. However, despite its poisonous effects on several organs, historically cantharidin has been used as a medicine for centuries [4].

In 1810, Robiquet first obtained the crude crystals of cantharidin from *Lytta vesicatoria* in Spain [5]. Later on in 1877, a chemist named Piccard determined the molecular formula of cantharidin $C_{10}H_{12}O_4$ [6] and Gadamer in 1914 identified the molecular structure of cantharidin (Fig. 1) [7]. The structure and chemical properties have been well documented in the past [8, 9].

Cantharidin is a white crystalline compound having a molecular weight of 196.2 g/mol, melting point of 215-216 °C and boiling point of 326.9±35.0 °C at 760 mmHg. Its solubility in organic solvents such as chloroform, acetone, dichloromethane, ethyl acetate is better compared to that in ether [10, 11].

The biosynthesis of cantharidin has been investigated by several scientists, however, its exact biosynthetic pathway has not been entirely understood [12]. Biosynthesis of cantharidin in *Mylabris calida* was investigated through the protein expression at early and advanced stages [13]. The process of cantharidin biosynthesis was also studied [14] while investigating the cantharidin biosynthesis and mevalonate pathway relationship.

Compounds based on natural sources are still important and may be used as precursors for the synthetical development of new bioactive molecules [15]. Compounds of natural origin and their products will play a pivotal role in the development of new compounds [15, 16].

Biopesticides based on cantharidin as an active ingredient have been developed and marketed especially in China for the control of lepidopteran pests in general. The high toxicity of cantharidin has already been documented against various insect pests. Different emulsifiable concentrate (EC) formulations have been developed and successfully used as an insecticide against the different orders of insects.

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The use of cantharidin for large-scale agricultural use has raised environmental concern for its potential non-target effects. Chemical synthesis of cantharidin and its extraction from blister beetle is a tedious job. At present intensive research is being done to synthesize bioactive analogues of cantharidin with an objective of low-cost production and reduced non-target effects. The present review on cantharidin was carried out on its biosynthesis, chemical synthesis, insecticidal use, non-target effects, and structure-activity relationship. Due to the development of insecticide resistance and the pursuit of new chemical molecules, it is very timely to review the body of literature available on various aspects of cantharidin, especially insecticidal use, and to set it in future contexts.

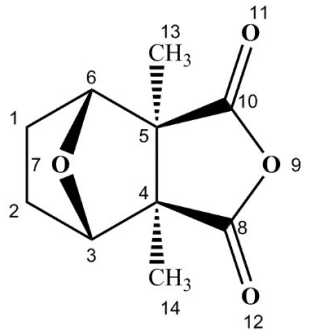


Figure 1. Structural formula of cantharidin and different atomic sites

SYNTHESIS OF CANTHARIDIN

Biosynthesis of cantharidin in insects

Cantharidin is a poisonous defensive compound, widely found in Meloidae beetles [3]. The production of cantharidin is gender-biased and only male insects can secrete it, whereas female insects cannot synthesize cantharidin [17], however, they acquire it by mating with the male. Cantharidin is found in both males and females after mating and its synthesis is continued after mating [18]. The highest quantity of cantharidin is found in the male gonads suggesting that this organ may be involved in the biosynthesis of cantharidin. The idea has been rejected that accessory glands are involved in the biosynthesis of cantharidin [19]. The cantharidin content in salivary glands and digestive tract was higher in the larvae, whereas in adults the level of cantharidin is higher in the hemolymph which is discharged from the leg joints [20].

The previous research shows that cantharidin can be synthesized at various developmental stages, except eggs where cantharidin was detected only on the surface [21]. The content of cantharidin in larvae of *Mylabris cicharii* increased with the development of larvae [22]. Change of cantharidin contents in a different age of *Epicauta chinesis* was assessed by gas chromatography and it was found that there were two peaks of cantharidin synthesis, which were at larval pseudopodae and adult eclosion after 6-8 days [23]. The content of cantharidin in males was increased and the content of cantharidin in females kept in isolation for 60-90 days gradually decreased to a very low level. The above studies indicated that cantharidin could be synthesized before eclosion, and cantharidin could not be produced by a female, it is rather transferred to the female at copulation.

The biosynthesis of cantharidin *in vivo* is a complex process and it is very difficult to determine its exact pathway. However, its biosynthesis through mevalonate pathway is widely accepted [24, 25]. The isotope-labeled acetate and mevalonate in insect feeding experiments suggested the formation of cantharidin by either linkage of two isoprene units in tail-to-tail or head-to-tail configuration. Subsequently, 10 carbon molecules of cantharidin were derived from mevalonate or farnesol precursor by a series of ^{3}H and ^{14}C-labeled farnesol in incorporation experiments [26]. The stable isotope labeling technique was used to determine the transformation of farnesol in the biosynthesis of cantharidin in the male blister beetle *Epicauta pestifera* [19]. In insects, metabolic transformation responsible for the biosynthesis of juvenile hormone oxidizes farnesol to methyl farnesoate in blister beetles suggesting both synthesis of JH and cantharidin sharing the same pathway [27]. Injecting the juvenile hormone synthesis inhibitor, 6-fluoromevalonate (FMVA), can cause a significant reduction in cantharidin production [28]. Recently, a mevalonate pathway gene, 3-hydroxymethyl coenzyme A reductase (HMGR) from the blister beetle *Epicauta mannerheimi* (Maklin) was cloned by RACE technology [29]. The phylogenetic investigation disclosed that EmHMGR has the closest association with HMGRs in chrysomelids. Three genes were identified from *Epicauta chinensis* (methyl farnesoate epoxidase (*EcMFE*), juvenile hormone acid O-methyltransferase (*EcJHAMT*) and juvenile hormone epoxide hydrolase (*EcJHEH*) [30]. It was further demonstrated that interference of *EcMFE* and *EcJHEH* significantly inhibited the biosynthesis of cantharidin in male *E. chinensis* after mating, but the interference of *EcJHAMT did not* influence the biosynthesis of cantharidin.

Chemical synthesis of cantharidin

Earlier, cantharidin was being extracted from the bodies of Meloidae beetles, however, the provision of raw materials and the extraction process was

cumbersome and low yielding. The chemical synthesis program has become a good alternate solution for the production of cantharidin.

Bruchhausen has tried furan and dimethylmaleic anhydride as raw material, by Diels-Alder reaction for the synthesis of cantharidin [31]. However, the synthesis of cantharidin under natural conditions is prone to dehydrogenation and leads to a spontaneous retro Diels-Alder reaction.

In the 1950s, Gilbert Stork synthesized cantharidin in a multistep chemical reaction. In the final step, he purified liquid diene through chromatography and used ethyl acetate to ozonize it at -60°C [32]. The crude cantharidin with a melting point of 209-212°C was obtained by the decomposition of the ozonide with hydrogen peroxide. The crude cantharidin was recrystallized from acetone with a melting point of 212-213°C. The synthetic cantharidin was identified by comparing its X-ray powder diffraction pattern and infrared spectrum with those of natural cantharidin.

Subsequently, another chemist, Gnther Otto Schenck, improved this method. He used 1,4-butadiene and 3,4-dimethyl maleic anhydride as raw material, through the classic 7-step reaction to obtain the final product cantharidin [33]. Although some of the key steps of this method are still very demanding as regards reaction conditions, for a long time this method has been the main method of cantharidin synthesis.

In 1980, William G. Dauben proposed a two-step synthesis of cantharidin solution [34]. The reaction of furan and 2,5-dihydrothiophene-3,4-dicarboxylic anhydride in the presence of methylene chloride at 15 kbar at room temperature for 6 h gave a cycloadducts mixture of isomers. The Raney nickel desulfurization of one of the isomers gave cantharidin as identified by IR, NMR spectroscopy and melting point. Although this method is simple, the extremely high pressure required for the reaction limits its use for a large amount of synthetic cantharidin. So far, low-cost synthesis of cantharidin at a commercial scale was not successful.

CANTHARIDIN AS AN INSECTICIDE

Historical perspective of cantharidin use as an insecticide

Cantharidin has toxic effects on *Phyllopertha horticola*, *Malacosoma neustria,* and *Pyrrhocoris apterus* before the emergence of chemical insecticides [35]. The strong antifeedant activity of cantharidin was reported against insects [17].

Several canthariphilous insects were confirmed to have lower cantharidin contents, namely, ceratopogonids, *Atrichopogon oedemerarum* and *A. trifasciatus* trapped in the field [36]. Three canthariphilous insects were reported to have been attracted towards cantharidin bait in different parts of Africa. From the beetle family Anthicidae, two *Aulacoderus,* seven *Formicomus,* two *Mecynotarsus,* 11 *Notoxus,* three *Tomoderus,* and one *Cyclodinus, Omonadus, Pseudoleptaleus, Sapintus,* and *Tenuicomus* species were noted. The chrysomelid species *Barombiella vicina* and *Barombiella* sp. (Coleoptera: Chrysomelidae) were trapped at cantharidin besides *Pallenothriocera rufimembrls* (Coleoptera: Cleridae) [37].

The toxicity of cantharidin as an insecticide was determined in the laboratory, as well as by field tests for effective control of pests. Cantharidin was found highly effective in a laboratory bioassay against *Plutella xylostella* [38]. There are further reports of strong contact, stomach poisoning and antifeedant activity of cantharidin against larvae of *Plutella xylostella*.

In a previous research, 1.5% cantharidin aqueous solution had strong antifeedant activity, contact activity to armyworm, *Spodoptera frugiperda* [39]. The contact LD_{50} of the 4th instar larvae was 0.45 mg/ kg, the antifeedant EC_{50} value was 2.56 mg/ L. The effects of cantharidin on 6 different pests, *Mukaria pallipes*, *Bambusiphaga furca*, *Agrotis ipislon*, *Nilaparvata lugens, Sogatella furcifera* and *Plutella xylostella* were evaluated [40]. Cantharidin showed contact, stomach activity, but no systemic and fumigation activity was observed. Effects of 1.0 % cantharidin EC were tested using different bioassay methods on *Musca domestica*, *Stiophilus zeamais*, *Pryeria sinica*, *Lipaphis erysimi*, *Macrosiphoniella sanborni*, *Myzus persicae*, *Macrosiphum roswomm*, *Hyaloptera amygdali*, *Tetranychus cinnabarinus*, *Phethaleus major*, *Tetranychus viennensis* and *Myzus persicae*, and different degrees of toxicity against these pests were found [41]. Cantharidin also showed a significant synergistic effect. It has been reported that cantharidin mixed with different groups of insecticides such as abamectin, endosulfan, chlorfluazuron, bisultap, and methomyl showed different levels of synergism, and the best mix was found to be cantharidin with chlorfluazuron [42]. Recently, the researchers found that a sublethal dose of cantharidin can cause abnormalities in population parameters such as intrinsic rate of increase (r), finite rate of increase (rm), net reproductive rate (R_0) and mean generation time (T) index of *Helicoverpa armigera* [43]. The fertility and fecundity were also significantly affected. Besides, its effects on morphological abnormalities were also reported. The sublethal dose of cantharidin

caused similar effects in the armyworm, *Mythimna separata* under laboratory conditions [44].

Field efficacy tests showed that 0.1 % aqueous cantharidin solution was effective against certain sucking pests such as *Brevicoryne brassicae*, *Pieris rapae*, *Myzus persicae,* and *Schizaphis piricola* and chewing pests such as *Plutella xylostella* under field conditions [45]. The control of 0.01 % of cantharidin aqueous solution against the green peach aphid, *Myzus persicae,* and oriental aphid, *Schizaphis piricola* reached 90.2 % and 88 %, respectively, in the field control trials [46]. Toxicity and sublethal effects of cantharidin were documented against housefly, *Musca domestica*. Both low and high concentrations of cantharidin either caused negative effects on population parameters or caused mortality [47]. In more recent studies a microemulsion of norcantharidin was tested against *P. xylostella* in a laboratory bioassay and acute LC_{50} at 12.477 mg/L was determined [48].

Safety of cantharidin against non-target organisms

Although cantharidin has good toxicity to many insect pests, it is important to evaluate whether cantharidin has a negative impact on non-target organisms and the environment, which is an important prerequisite for the development of new pesticides. The toxicity of 1.0 % cantharidin EC to five different organisms such as bees, silkworms, tadpoles, earthworms, and soil microorganisms was determined [11]. The results showed that 1.0 % cantharidin EC showed low toxicity to earthworms and soil microbes, high toxicity to bees and silkworms, and moderate toxicity to tadpoles. Toxicity of pure cantharidin and 1.0 % cantharidin EC to some non-target organisms according to the "Experimental Guideline for Environmental Safety Evaluation of Chemical Pesticides" was also computed [49]. It was found that cantharidin and 1.0 % cantharidin EC showed low toxicity against quail, ladybugs and soil microorganisms, whereas moderate toxicity to fish.

Cantharidin and norcantharidin induced adverse effects on soil invertase and phosphatase activity and fungal gene structure, but the effect was transient in nature. The adverse effects of these biopesticides vanished within two weeks after application in soil. The degradation of cantharidin and norcantharidin in the soil can be completed within a few days in soil [50].

Insecticidal mode of action of cantharidin and its derivatives

Histological observation can directly detect the changes of cells and tissues after cantharidin poisoning, and provide a pathologic basis for clarification of insecticidal mechanism. For the first time in 1964, poisoning symptoms and other cytological changes after the treatment with cantharidin were observed in various tissues of late instar *Mythimna separata* [51]. The poisoning process of the armyworm is divided into three stages: paralysis, coma, and death. After the poisoning, the number of blood cells in the body was decreased; the mesenteric epithelium was separated from the basement membrane; the parietal cells of the Malpighian tubules were disintegrated and the lumen was filled with pus; the nerve cells were blurred and the nerve fibers were assembled and dissolved. Moreover, symptoms such as cell nucleus swelling, partial disintegration; male germ cell division held at the spermatocyte stage were also observed. It is speculated that cantharidin first acts on the nervous system, breaking the ring neurons, hindering nerve conduction, resulting in muscle movement, showing paralysis symptoms, and other tissue lesions as secondary symptoms. Mesenteric tissues of *M. separata* and *Plutella xylostella* were investigated by optical microscopy and transmission electron microscopy after the cantharidin poisoning [52]. There were obvious histopathological changes, such as cell microvilli shedding, mitochondrial dissolution, rupture, ribosome shedding, swelling of nuclei and so on, and it was speculated that there might be cantharidin-specific binding sites in the midgut cell membrane. Effects of cantharidin on cell proliferation were also reported [53]. Although cantharidin was found to inhibit the growth of both spex-VII and Sf9 in a dose-dependent manner, Sf9 showed more sensitivity towards cantharidin. Moreover, both cells showed apoptotic features such as chromatin condensation, nucleic fragmentation, intact cell membrane and formation of the apoptotic body. Cantharidin poisoning has also been reported to have an impact on the level of enzyme activity in insects. In a study, it was found that cantharidin had no significant effect on the activities of phosphatase and larval digestive enzymes of *Plutella xylostella* such as protease, lipase, and α-amylase but the activities of acetylcholinesterase and carboxylesterase significantly increased [54].

Changes of alkaline phosphatase, acid phosphatase, carboxylesterase, glutathione S-transferase and cytochrome P450 enzyme system in 5^{th} instar larvae of *Mythimna separata,* Walker at different times after feeding with cantharidin were investigated [55]. The activities of PPO and alkaline phosphatase decreased with time, the acid phosphatase activity increased at a later stage, the activity of glutathione S-transferase at first increased

and later on decreased; cytochrome P450 enzyme activity was inhibited at first, afterward activated. It was suggested that the toxicity of cantharidin to *Mythimna separata* may be related to its inhibitory activity on alkaline phosphatase and polyphenol oxidase. The activity of carboxylesterase increased with the increase in treatment time.

Although some scholars have done a lot of research on the insecticidal mechanism of cantharidin, at present, the specific mechanism of cantharidin is still unclear, and the main role of cantharidin in insects has not been reported. The main target of cantharidin in mammals is PP2A. In addition, it has also strong inhibitory activity on the protein serine/threonine phosphatase (PSP) family such as PP1, PP4, PP5, PP6, PP7, which catalyze the dephosphorylation of substrate proteins, participate in almost all physiological processes [56]. Once the enzymatic activity of PSPs is inhibited or lost, it can cause the disorder of normal cell activities and even lead to cell apoptosis. The current studies on the effects of cantharidin on PSPs are largely concentrated in mammals, plants, whereas, its role in the regulation of insects' PSPs has not been widely reported until 2014. The PSPs family is considered to be one of the most conserved proteins in eukaryotes. In a recent study, cantharidin, okadaic acid, and endothall were tested for their inhibitory effects on protein phosphatase 5 (PP5) in *Helicoverpa armigera*, *Mythmna separata,* and *Plutella xylostella*. Strong inhibitory effects of cantharidin were noticed on HaPP5, MsPP5 and PxPP5 compared to okadaic acid and endothall [57]. Apart from its inhibitory effects on PPs family cantharidin was found to have strong inhibitory effects on heat shock protein (HSP) at the transcriptional level. In the experiment, it was found that cantharidin in *P. xylostella* down-regulates sHSP19.23, sHSP 19.5, sHSP 20.06, sHSP 20.09, sHSP 20.1, sHSP 21.9, sHSP 23.4, sHSP 27.5 and sHSP 28.9 (Fig. 2).

Cantharidin and its analogue cantharidin-24 were used in combination with cry2ab on *Mythmna separata* and its effects on growth, hydrolytic and detoxifying enzymes were investigated. The mixture of cantharidin and its analogue with cry2ab had adverse effects on larval weight, In addition, alkaline phosphatase and acid phosphatase were inhibited, whereas glutathione S-transferase was unregulated in sublethal concentration. It was further suggested that the combination of cantharidin and its analogue has a potential in pest management [58].

The rationale behind the use of cantharidin and its analogues as an insecticide

Due to the high dependence on chemical insecticides in pest control, serious 3R (resistance, resurgence, residue) problems, especially the rapid development of insecticide resistance, have been caused.

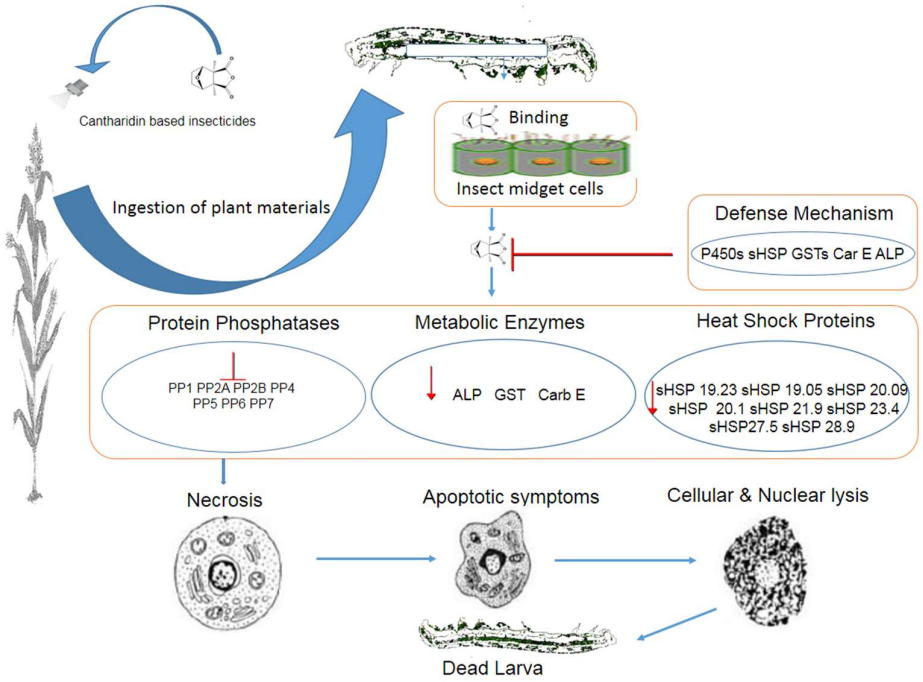


Figure 2. Putative model for the toxicity of cantharidin in insects

However, the development of insect resistance to biopesticides is relatively slow [59]. Biopesticides generally having better selectivity, less pressure on the environment, non-target biological safety, are becoming the focus of new pesticide research and development. Cantharidin is a kind of defensive toxin produced by the insects of Meloidae. A lot of studies have shown that it has good toxicity to many kinds of insects, and cantharidin can also be used in combination with other traditional chemical insecticides showing a significant synergistic effect. Cantharidin is of potential use in pest control and other agricultural applications.

Structural relationship of cantharidin's derivatives to insecticidal activity

Investigations into the structural relationship of cantharidin and its analogues with insecticidal and PPs inhibitory activity has been already documented. The methyl at either atomic site 4-C or 5-C does not significantly affect the activity of PP2B [60], however, it is considered beneficial for the PP1 and PP2A inhibition. Substitution at 3-C or 6-C causes decrease in inhibition capabilities to all PPs. Substitution at both positions will abolish the activity towards PPs. The oxygen bridge is essential for its activity. Anhydride oxygen at site 9 is considered good for its activity towards PP2A but S is considered better (Table 1) [61].

In a recent investigation complete loss of bioactivity was observed when anhydride oxygen of norcantharidin was replaced with nitrogen. The replacement of a cyclic anhydride oxygen atom with N–H and N–alkyl or aryl caused a total loss of larvicidal activity of the compound. Aliphatic amide moiety substituents containing $–CH_3$, $–CH(CH_3)_2$ and $–CH_2(CH_2)_2CH_3$ were used to see the effect of structure-activity relationship. The results showed that the compound containing $–CH_3$ showed significantly higher mortality on *Plutella xylostella* larvae compared to other moieties when used in a concentration of 500 µg mL^{-1}. Subsequently, electron-contributing $–OCH_3$ and electron-drawing $–CF_3$, $–OCF_3$, F and $–CO_2H$ substituents were substituted with aniline ring of the compound to see the effect of electron movement on activity. It was observed that the position of the substituents on the aniline ring has a direct impact on larval mortality. Moreover, substituents with electron-drawing ability demonstrated high larval kill against *Plutella xylostella* compared to the substituents with electron-contributing substituents (Table 2) [62].

Table 1. Structural parts of cantharidin molecule for bioactivity

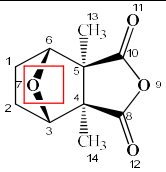
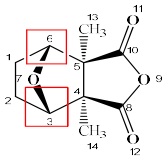
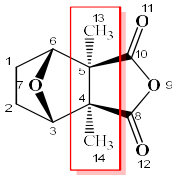
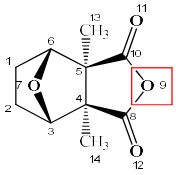
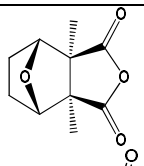
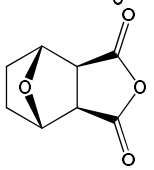
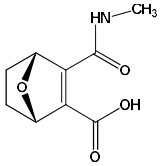
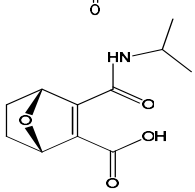
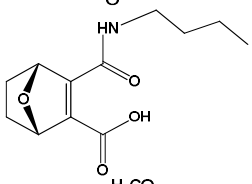
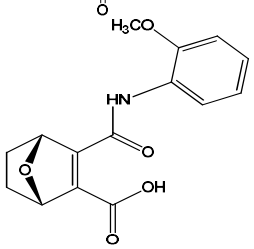
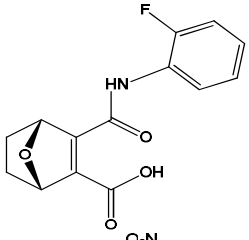
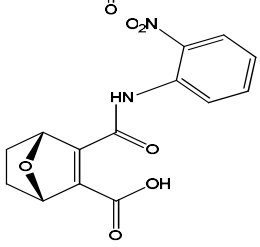
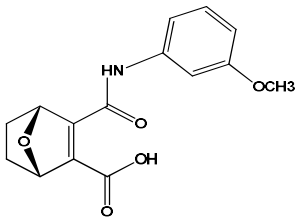
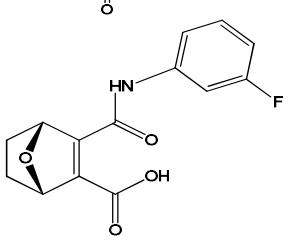
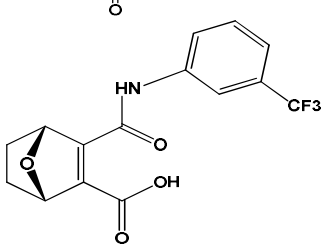
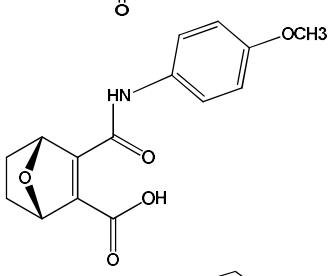
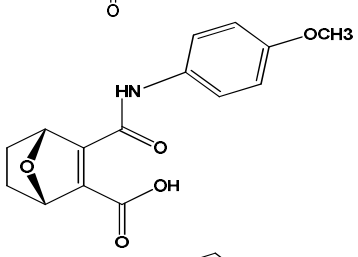
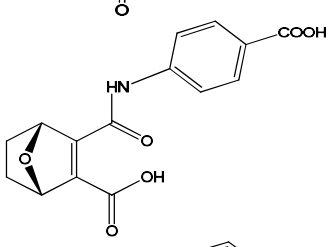
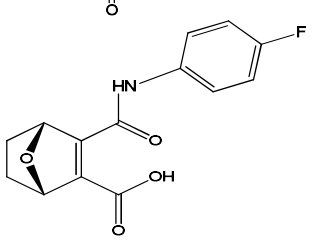
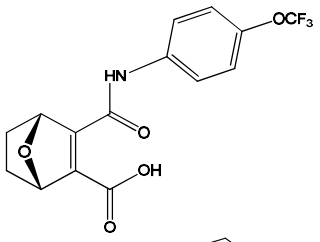
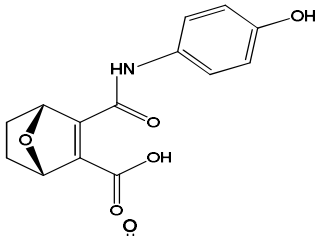
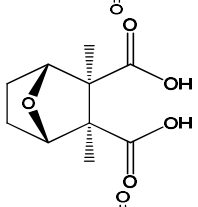
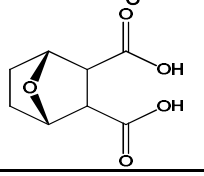
Atomic site	Structural part	Function
7		Bridging of O is indispensable for activity
3, 6		Substitution at either site may result in decreased inhibitory activity for all PPs, whereas substitution at both ends results in total loss of inhibitory activity.
13, 14		Sites 5 and 4: CH_3 at these sites are favorable for inhibitory activity to PP1 and PP2A.
9		Oxygen is considered good, whereas S is considered better.

Table 2. Cantharidin, its analogues and derivatives for insecticidal action.

S. No.	Structure	Insect	Mortality	References
1		*Helicoverpa armigera*; *Plutella xylostella*; *Mythimna separata*	+++++	Khan *et al*., 2014 [43] Fan *et al*., 2017 [58] Sun *et al*., 2013 [62]
2		*Plutella xylostella*; *Mythimna separata*	+++++	Shao *et al.,* 2018 [48] Fan *et al*., 2017 [58] Sun *et al*., 2013 [62]
3		*Plutella xylostella*	+++	Sun *et al*., 2013 [62]
4		*Plutella xylostella*	+	Sun *et al*., 2013 [62]
5		*Plutella xylostella*	+	Sun *et al*., 2013 [62]
6		*Plutella xylostella*	++	Sun *et al*., 2013 [62]
7		*Plutella xylostella*	+++	Sun *et al*., 2013 [62]
8		*Plutella xylostella*	+++++	Sun *et al*., 2013 [62]

9		*Plutella xylostella*	++	Sun *et al*., 2013 [62]
10		*Plutella xylostella*	+++	Sun *et al*., 2013 [62]
11		*Plutella xylostella*	++	Sun *et al*., 2013 [62]
12		*Plutella xylostella*	+	Sun *et al*., 2013 [62]
13		*Plutella xylostella*	+	Sun *et al*., 2013 [62]
14		*Mythimna separata*; *Plutella xylostella*	+++++	Fan *et al*., 2017 [58] Sun *et al*., 2013 [62]
15		*Plutella xylostella*	++	Sun *et al*., 2013 [62]

16		*Plutella xylostella*	+	Sun *et al*., 2013 [62]
17		*Mythimna separata*; *Plutella xylostella*	+++	Fan *et al*., 2017 [58] Sun *et al*., 2013 [62]
18		*Plutella xylostella*	+++++	Sun *et al*., 2013 [62]
19		*Plutella xylostella*	+++++	Sun *et al*., 2013 [62]

A plus sign (+) in the table above indicates level of mortality. (+) = 20% and (+++++) = 100%.

CONCLUSION

Pest control of either agriculturally or medically important pests generally relies on the application of insecticides. The indiscriminate and extensive use of insecticides is becoming ineffective owing to the resistance developed by insects against a broad range of insecticides. The introduction of cantharidin or its analogues with a novel mode of action for pest management will help to overcome the insecticide resistance problem.

The use of cantharidin as an insecticide or as a synergist for the control of Lepidoptera pests has been an established fact. However, the widespread use of cantharidin may raise environmental concerns. Though its safety has been established for some non-target organisms, still it is toxic for other organisms. This problem can be addressed by restricted application of cantharidin as a synergist.

As the extraction or chemical synthesis of cantharidin is a tedious process, it is, therefore, necessary to synthesize analogues and derivatives which may be effective on one hand and easy to produce chemically on the other hand. Norcantharidin may be a better candidate for the synthesis of effective analogues. The unique toxicological and insecticidal properties of these compounds will create an upsurge in research activities in the pesticide industry.

REFERENCES

1. G. S. Wang, *J. Ethnopharmacol.,* **26**, 147 (1989).
2. D. Mebs, W. Pogoda, M. Schneider, G. Kauert, *Toxicon.,* **53**, 466 (2009).
3. K. S. Ghoneim, *Int. Res. J. Biochem. Bioinforma.,* **3**, 75 (2013).
4. C. E. P. Galvis, L. Y. V. Mendez, V. V. Kouznetsov, *Chem. Biol. Drug Des.,* **82**, 477 (2013).
5. R. B. Woodward, R. N. Loftfield, *J. Am. Chem. Soc.,* **63**, 3167 (1941).
6. J. Piccard, *Ber. Dtsch. Chem. Ges.,* **10**, 1506 (1877).
7. V. J. Gadamer, *Arch. Pharm.,* **252**, 609 (1914).
8. Y. Y. Dang, C. Zhu, *J. Chinese Materia Medica,* **34**, 2088 (2009).
9. Y. J. Dang, C. H. Hu, L. N. An, C. Y. Zhu, *Methods Find. Exp. Clin. Pharmacol.,* **32**, 157 (2010).
10. T. S. Huang. Encyclopedia of chemicals & drugs, The University Book Publishing Company, Taipei, China, 1982, p. 117.
11. J. X. Feng, Y. L. Zhang, *Acta Agriculturae Boreali-Occidentalis Sin.,* **16**, 275 (2007).
12. J. Coloe, D. S. Morrell, *Pediatr. Dermatol.,* **26**, 405 (2009).
13. H. Jia, Y. Liu, W. Yan, J. Jia, *Development,* **136**, 307 (2009).
14. E. J. Davidowitz, I. Chatterjee, J. G. Moe, *Curr. Top. Biotechnol.,* **4**, 47 (2008).
15. K. Bonness, I. V. Aragon, B. Rutland, S. Ofori-Acquah, N. M. Dean, R. E. Honkanen, *Mol. Cancer Ther.,* **5**, 2727 (2006).

16. H. Brustel, G. Kakiopoulos, *Biocosme Mésogéen (Nice),* **26**, 65 (2009).
17. J. E. Carrel, T. Eisner, *Science,* **183**, 755 (1974).
18. J. R. Sierra, W. D. Woggon, H. Schmid, *Experientia,* **32**, 142 (1976).
19. J. P. McCormick, J. E. Carrel, Cantharidin biosynthesis and function in meloid beetles, Pheromone Biochemistry, Academic Press, Orlando, Florida, 1987, p. 307.
20. J. E. Carrel, M. H. McCairel, A. J. Slagle, J. P. Doom, J. Brill, J. P. McCormick, *Experientia,* **49**, 171 (1993).
21. J. E. Carrel, W. Thompson, M. McLaughlin, *Am. Zool.,* **13**, 1258 (1973).
22. H. R. Wang, Z. K. Wang, J. Chen, Y. W. Yang, Y. Q. Cao, Y. X. Xia, Y. P. Yin, *Acta. Entomol. Sin.,* **51**, 264 (2008).
23. L. Qiurong, Study on the artificial rearing technology of blister beetle and cantharidin biosynthesis in vivo [Master Thesis], Shaanxi, Northwest Agriculture and Forestry University, 2008.
24. C. Schlatter, E. E. Waldner, H. Schmid, *Experientia,* **24**, 994 (1968).
25. H. Guenther, E. Ramstad, H. G. Floss, *J. Pharm. Sci.,* **58**, 1274 (1969).
26. M. G. Peter, H. R. Waespe, W. D. Woggon, H. Schmid, *Helv. Chim. Acta,* **60**, 1262 (1977).
27. S. J. Kramer, J. H. Law, *Acc. Chem. Res.,* **13**, 297 (1980).
28. J. E. Carrel, J. P. Doom, J. P. McCormick, *J. Chem. Ecol.,* **12,** 741 (1986).
29. J. Ming, T. Huo, S. M. Lu, Y. L. Zhang, *Acta Entomol. Sin.,* **55**, 860 (2012).
30. J. Ming, S. Lü, Y. L. Zhang, *Sci. Rep.,* **7**, 2308 (2017). DOI:10.1038/s41598-017-02393-w
31. F. von Bruchhausen, *Ber. Dtsch. Pharm. Ges.,* **266**, 697 (1928).
32. G. Stork, E. E. van Temelen, L. J. Friedman, A. W. Burgstahler, *J. Am. Chem. Soc.,* **73**, 4501 (1951).
33. G. O. Schenck, R. Wirtz, *Naturwissenschaften*, **40**, 581 (1953).
34. W. G. Dauben, H. O. Krabbenhoft, *J. Am. Chem. Soc.,* **98**, 1992 (1976).
35. V. K. Gornitz, *Arb. Physiol. Angew. Entomol.*, **4**, 116 (1937).
36. M. Frenzel, K. Dettner, *J. Chem. Ecol.,* **20**,1795 (1994).
37. C. Hemp, K. Dettner, *Entomol. Gen.,* **22**, 97 (1997).
38. Z. Y. Zhang, F. Yuan, X. Zhang, *Acta Phytophyl. Sin.,* **25**, 166 (1998).
39. L. X. Wei, Q. L. Liang, H. M. Shen, S. Y. Yang, *Agrochemicals,* **46**, 272 (2007).
40. X. F. Li, X. S. Chen, X. H. Hou, *Guizhou Agric. Sci.,* **36**, 65 (2008).
41. R. R. Liu, Y. Ma, Z. Q. Ma, Y. L. Zhang, *J. Northwest Agric. For. Univ.,* **38**, 181 (2010).
42. S. Zhang, Y. L. Zhangp. A. Fenxia, *Acta Phytophyl., Sin.,* **34**, 83 (2007).
43. R. A. Khan, M. Rashid, D. Wang, Y. L. Zhang, *Pest Manage. Sci.,* **70**, 39 (2014).
44. M. Rashid, R. A. Khan, Y. Zhang, *J. Econ. Entomol.,* **106**, 2177 (2013).
45. S. Diao, J. Wang, M. Gu, H. Jiang, S. Zhou, *Agrochem.,* **42**, 36 (2003).
46. G. Mingjie, *China Fruits,* **5**, 35 (2007).
47. H. Yasoob, H. A. A. Khan, Y. Zhang, *J. Econ. Entomol.,* **110**, 2539 (2017).
48. H. Shao, N. Xi, Y. L. Zhang, *Sci. Rep.,* **8,** 10565 (2018). https://doi.org/10.1038/s41598-018-28626-0
49. F. L. Cui, X. Li, Z. Q. Ma, Y. L. Zhang, *J. Environ. Entomol.,* **31**, 143 (2009).
50. H. Shao, Y. Zhang, *Sci. Rep.,* **7**, 5521 (2017). https://doi.org/10.1038/s41598-017-05923-8.
51. B. L. Lu, *Acta Entomol. Sin.,* **13**, 824 (1964).
52. Y. L. Hang, Y. Zhou, Z. Y. Zhang, *Acta Entomol. Sin.,* **46**, 272 (2003).
53. L. P. Chen, B. D. Yang, Z. Y. Zhang, *J. Beijing Uni. Agric.,* **23**, 17 (2008).
54. Z. Y. Zhang, F. Yuan, X. Zhang, *Acta Phytophyl. Sin.,* **7**, 355 (2000).
55. Y. Ma, R. R. Liu, Z. Q. Ma, Y. L. Zhang, *Acta Entomol. Sin.*, **53**, 870 (2010).
56. Y. M. Li, J. E. Casida, *Proc. Natl. Acad. Sci.,* **89**, 11867 (1992).
57. X. Chen, S. Lu, Y. Zhang, *Plos One,* **9**, e97437, (2014). https://doi.org/10.1371/journal.pone.0097437.
58. J. B. Fan, S. Van de Velde, W. B. Sun, H. Yu, Y. L. Zhang, D. Wang, *J. Appl. Entomol.,* **141**, 363 (2017).
59. Y. Li, H. Sun, H. Yasoob, Z. Tian, Y. Li, R. Li, S. Zheng, J. Liu ,Y. Zhang, *Pestic. Biochem. Phys.,* 172 (2021).
60. M. Sodeoka, Y. Baba, S. Kobayashi, N. Hirukawa, *Bioorg. Med. Chem. Lett.,* **7**, 1833 (1997).
61. W. N. Zeng, Y. Lu., *Chinese J. Org. Chem.,* **26,** 579 (2006).
62. W. Sun, Z. Liu, Y. Zhang, *Int. J. Mol. Sci.,* **14**, 1 (2013).

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Green fuels: concepts, benefits, and studies in Nigeria

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A survey of the literature is presented on the concept of what green fuels are all about, their classes, showing benefits that could be derivable from different classes of daily waste generated in our communities. Several efforts of researchers, especially Nigerian ones, to ensure government and private investors' attention toward the establishment of functional green fuel refineries, otherwise known as biorefineries, were also reported. Areas lacking significant attention were highlighted, and emphasis was made on the need to give better attention to them in future studies. However, for green fuels to attract the attention of government and private investors, and government to be motivated to adopt the idea of investing in green fuels to promote the efficiency of waste management, it would be essential for the works to consider the exploration of the reaction kinetics and mechanism to facilitate process simulation and pilot plant development studies which would enable the possibility of unravelling the economic potentials of the fuels known for being environmentally friendly and renewable.

Keywords: Green fuels, Biomass wastes, Biofuels, Biorefinery, Renewable fuels.

INTRODUCTION

Fossil fuels are well-known for being a class of material that emerges from the decomposition of carbon-based organisms that have been buried millions of years ago. These buried materials get transformed into coal, gas, and oil deposits known to be non-renewable [1], [2]. Presently, they represent about eighty percent of the globe's energy supply, which finds applications in the production of plastics, pharmaceutical materials, detergents, and other products. Nigeria as a country has been reported to be blessed with coal, oil, and gas of proven deposits of about 639 million tonnes[3], 37-40 billion barrels [4], [5], and 182 trillion cubic feet [6] capacity, respectively. Oil and gas have formed a significant component of Nigeria's revenue generation and a monopoly sector determining the drive of the Nigerian economy ever since the post-independence era [7], [8]. The story of our great country has not been like this before the discovery of the oil deposit, even when coal is still discovered. Agriculture was the driving market before the discovery of crude oil; some of the major cash crops that Nigeria traded in the foreign exchange were cocoa, rubber, palm oil, groundnut, and a lot more. On discovering the oil in Nigeria, attention was shifted from sustaining agriculture as one of the primary drives in our economy to fossil fuels. Instead of seeing fossil fuels as a complementary drive to agriculture, it was taken as a substitute [9]. This movement has made our successful government give better attention to the exploration of crude oil and gas for export purposes even with lesser attention for the local processing of the extracted crude, while the agriculture sector degrades.

Also, the dependency of Nigeria's economy on fossil fuels has fetched the government much revenue, which could have been used to grow other sectors, but the case was different [9]. Not until now has our present government begun to look outside the box of fossil fuels to consider other alternatives like giving attention to taxation and agriculture[10], [11]. Many factors have contributed to this new resolution, some of which were the unpredictable future of fossil fuels, drop-in crude price, the global campaign to discourage the use of fossil fuels, the promotion of green fuels [12], green automobile companies, lesser countries to trade within fossil fuels in future and a lot more [13]–[16].

Fossil fuels have generally contributed to making our environment unsafe through their consistent release of greenhouse gases (GHG), non-biodegradable petroleum products, promotion of global warming, climatic changes like wildfires, and a lot more [17], [18], as shown in Fig 1 [19]. All these unfortunate events reportedly found to be associated with the processing and use of fossil fuels have been a significant driver for the ongoing campaign for the promotion of low-carbon and cleaner energy/fuels [12], [20]–[22]. Having our great nation be left out of the recent ongoing move in most developed nations would cause our economy much challenge to sustain our rising annual budget with the unpredictable fall/rise that fossil fuel would offer in the future. As a result, our nation must

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extend its economic drive and energy mix from petroleum to bio-based fuels, which could be produced from our agricultural materials to boost our agricultural sector and the opportunity of energy to extend our energy mix to the inclusion of green fuels.

Furthermore, our waste management approach is poor and has not gotten the required attention to make our environment friendly and promote good public health in our communities [23], [24]. Dumpsites are sited randomly within the residential places. Wastes are burnt as desired across different dumpsites resulting in the production of several toxic and carcinogenic gases, which would result in serious illnesses [25]–[28]. It is essential to consider the use of the waste-to-wealth approach to consider transforming our waste into green fuels, which has a vast potential of benefits to offer to our communities.

All these concerns have propelled our interest towards the need to preach the epistle of benefits from adopting green fuels through the investment of funds and significant attention into the sector that can improve waste management practice in Nigeria. To unfold these details, the report presents the concept of what the green fuels are all about, their common characteristics, their general classes, materials required for each class of the fuels, and report of studies carried out so far in Nigeria on the subject and future areas that works can pay attention to successfully gain the attention of government and private investors into investing into this noble idea.

CONCEPTS AND BACKGROUND OF GREEN FUELS

According to Finnish Energy Club [29], green fuel can be defined as a type of fuel distilled from plants and animal material, which some believe to be more environmentally friendly than the widely used fossil fuels that power most of the world. It is also known as biofuel, fuel obtained from bio-based sources like animal and plant materials. The desperate search for alternative energy sources gives rise to the green fuel evolving as a possible fuelling option as the world drains its fossil fuel resources. However, according to Finnish Energy Club [29], some detractors suggest that green fuel is a misnomer, as the processing of crops into biofuel creates a considerable amount of pollution that may be just as damaging to the environment current practices. Moreover, Othman *et al.* [30] define the fuel as green hydrocarbons, biofuels produced from biomass sources through various biological and thermochemical processes. It is also regarded as one of the most viable options for reducing CO_2 emissions in the transport sector [31] and as an energy source with the potential to solve a series of problems related to climate and sustainability [32]. Brito *et al.* [33] reported that the supply of these green fuels had claimed 3 % of the energy consumed worldwide in road transportation in 2011. For many years, the United States have been well known as the world's largest biofuel producer, claiming a large percent of the biofuels generated worldwide in 2011, and Brazil is known as the second-largest biofuel producer in the world. The economy of these countries has been promoted mainly through the diversification of the energy mix, which has also aided in extending the drives in their country's economy.

Fig. 1. Vehicles emit smoke on Lagos-Ibadan expressway and Apapa road [19].

COMMON CHARACTERISTICS OF THE GREEN FUELS

These green fuels have many advantageous characteristics that have been making them attractive and have been propelling to campaign for adopting these fuels. Some of the characteristics which have proven the fuel to be a largely suitable one to complement or possibly substitute the current use of fossil fuels are as follows:

Octane rating and greenhouse gas (GHG) emission of the fuels

The high value of octane rating [34] reported for the green fuels has been a significant force propelling the interest of the world towards these fuels over the fossil fuels whose lower octane rating has significantly contributed to some cases of incomplete burning leading to the emission of greenhouse gases (GHG) which have negatively affected the ozone layer of the earth [34], [35]. GHG emission has significantly affected our lives with recent reports on the temperature rise. The higher-octane rating of green fuels could go a long way to address this present challenge thoroughly.

Sustainability and renewability of the fuels

Due to the immense dependency of the green fuels on the biomass and bio-resources for their production, their production has been sustainable, unlike the fossil fuels with a projected timeline in which they are expected to be exhausted [36]. Green fuels have diverse sources from which feedstock could be obtained, including agricultural waste, municipal waste, lignocellulose-based materials, and many other bio-based materials that could go a long way in meeting the green refinery demands. According to Paliwal *et al.* [12], the consumption/ conversion of the environmental wastes in a potential green refinery would further make our environment much more friendly through the reduction of environmental waste deposits randomly littered in the communities, especially in the developing countries.

Heating value of the fuels

According to the literature survey, green fuels had shown a higher oxygen content compared to fossil fuels, and this high oxygen content [37] has resulted in a lower energy content often reported for green fuels like biodiesel [35], bioethanol, and others compared to the fossil fuels [38].

Cetane number of the fuels

This property qualifies the quality of the ignition of the fuel and goes by the European and US-based standards for fuel production. The values reported for the green fuels surpass the required minimum indicating its suitability of substituting and complementing the present use of fossil fuels, with a proven higher cetane value in the literature [34], [35].

CLASSES OF THE GREEN FUELS

In categorizing the green fuels into classes, they can be classified into three primary classes, which are as follows:

First-generation green fuels

This class of biofuels is said to be fuel derived from sources like starch, sugar, animal fats, and vegetable oil in which the oil is sourced *via* the use of conventional production techniques. Examples of some of the most popular types of first-generation biofuels include bioethanol, bio-ethers, biogas, biodiesel, other bio-alcohols, green diesel, biofuel gasoline, syngas, and solid biofuels. Much research works in Nigeria have looked into cassava-to-bioethanol [39], [40], waste food – to – biogas [41], and a lot more.

Second-generation green fuels

These are also known as advanced biofuels derived from different categories of biomass. The biomasses are significant sources from plant materials, as well as from animal materials. This report implies that this class of biofuel does not make use of food or edible materials. The precursors for their production are often referred to as non-food biomass, different from the precursors used in the production of the first-generation biofuels. Examples of this class of biofuels include bio-oil, butanol, cellulosic bioethanol, and mixed alcohols. Works in Nigeria have a look into the bagasse-to-bioethanol [42], [43], cob–to–bioethanol [44], molasses–to–bioethanol [45], [46], yam/cassava/potato peels [40], [42], [47]–[52], fruit peel–to–bioethanol [53], [54], and a lot more.

Third-generation green fuels

They are referred to in the case that the biofuel carbon is derived from aquatic autotrophic organisms like algae. Carbon dioxide, light (photons), and nutrients play essential roles in producing the feedstock or precursor used in the production of this class of biofuels. This report implies that a heterotrophic organism that uses cellulose, hemicellulose, or sugar to produce biofuels is not considered 3G. Biodiesel, bioethanol, and many more biofuels can be produced *via* this pathway. Some of the works done so far in Nigeria include investigating the transformation of algae-to-

biodiesel [55], sewage-to-biogas [56] and many others.

GREEN FUELS' BENEFITS TO WASTE MANAGEMENT

Some of the benefits that these classes of green fuels can offer in promoting waste management *via* the use of waste to wealth approach (that is, waste to fuel) are presented as follows:

First-generation green fuels

Our waste foods items from homes, markets, spoiled foods in stores, and the likes that are often thrown into a garbage can be considered for producing 1G-biofuels like bioethanol. Likewise, the used cooking oil can also be transformed into biodiesel [41], [57].

Fig 2. Waste food dumpsite in Port Harcourt, Nigeria [57].

The rising food wastage in Nigeria is significantly becoming a subject of concern irrespective of the rise in the hunger rate reported due to the COVID-19 pandemic and the resulting economic crisis. It was revealed that food worth about 750 billion dollars is wasted annually in Nigeria. Fig. 2 presents a site in Port Harcourt where food wastes are often dumped [58]. This rising food wastage in Nigeria is primarily due to the high cost of storage facilities and the lack of a steady power supply to power the device to preserve their food items at various homes and farm produces after harvest. However, the wasted agricultural produces and foods from various homes can be adequately collected and would be suitable for the production of green fuels like bioethanol, biodiesel, biogas (that is, bio-hydrogen, bio-methane, and many others), and other green chemicals like furan, furfural, hydroxyl methyl furfural, and a lot more.

Second-generation green fuels

Agricultural wastes like leaves, stalks, hulks, cobs, bagasse, and others of similar categories, which are often burnt or used for cooking in our homes, can be used in the production of cellulosic bioethanol, bio-oil, and mixed alcohols [53], [59]–[62]. Fig. 2 presents a case of agricultural wastes like bagasse, stem, leave, straw, and a lot more [62], which are commonly burnt in rural areas and could be converted into green fuels like bioethanol other green chemicals. This approach would help provide a complementary fuel to the present use of fossil fuels, which largely contribute to global warming.

Fig 3. Agricultural wastes like straw leaves and stems from the farm [63].

Fig 4. Open defecation in Bauchi [64].

As the rise in open defecation (as shown in Fig. 3) is becoming a significant subject matter not only in rural areas even in urban communities due to the absence of public toilets within the communities, but many people also refer to the option of defecating in open when they are pressed [64]. Building a public toilet around the community with a central sewage system would offer the best way to address this challenge. The sewage collection base can be used to source the feedstock for biogas like bio-methane, bio-hydrogen, and a lot more, which would go a long way to making our environment much more sustainable and friendly for the residents.

Third-generation green fuels

Cultivating algae for biofuels can be promoted as another aspect of agriculture promotion, which promotes the production of biofuels like biodiesel, which are obtainable from algae and bioethanol using the 3G technology [55], [65].

STUDIES CARRIED OUT SO FAR IN NIGERIA ON THE SUBJECT

Many works in the literature have engaged in the search for ways to advance and actualize green refinery, otherwise known as biorefinery, in Nigeria as a possible substitute or complementary means of relieving the considerable pressure and attention given to the exploration of fossil fuels in our country. Some of the areas that works have been making attempts to address looking into and a brief highlight of their attempts and extents are as follows:

Impact of different enzymes and other factors on the process

Several studies have explored the potential of different enzymes on the different transformation processes involved in producing these fuels, at different stages, some at hydrolysis, and others in digestion processes. At the same time, some considered studying different fermentation processes in search of enzymes that would yield a higher biofuels production. This subject is widely investigated on the production of bioethanol, biogas, and other bio-alcohols and the like fuels in the literature [56], [66]–[71] as shown in Table 1.

The report presented in Table 1 further indicates that some authors focus majorly on the production of green fuels [67], [72], [73] while some others try understanding the use of different feedstock, pretreatment approaches, the initial volume of water, reacting time length, temperature, etc. [69], [74]–[76]. Such studies have enabled to identify favorable conditions for producing various green fuels. Most importantly, ways of transforming our environmental wastes into valuable resources in the form of fuels have been established from their reports.

Table 1. Some experimental (laboratory) studies on green fuel production in Nigeria.

Ref.	Green Fuels	Feed	Study Focus	Findings Made
75	BG	Cassava peel waste & cow dung	Effect of different pretreatment methods, where NaOH, $Ca(OH)_2$ and NH_4Cl were studied	The use of NH_4Cl gives the highest yield of 104,961 cm^3
76	BG	Cow dung	Effect of the initial volume of water and time length	Biogas yield of 23 cm^3
71	BG	Fruit wastes (mango, watermelon, and pawpaw)	Effect of pH, temperature, and anaerobic counts variation over a 45 days retention time	Biogas yield of 4348 cm^3
69	BE	Rice husk	Effect of using different enzymes	*Aspergillus niger* (6.99% yield) and *Trichoderma harzianum* (6.25% yield)
74	BG	Poultry, cow, and kitchen wastes	Effect of using various feeds	Poultry droppings (0.0318 dm^3/day), cow dung (0.0230 dm^3/day), kitchen waste (0.0143 dm^3/day)
73	BD	Neem seed	Effect of variables change on the yield in the presence of CaO/MgO	The highest yield of 96.4% was obtained at 70oC, 60min, 500rpm, 6:1g-methanol-to-oil-ratio, and 1% w/w catalyst.

77	BD	Neem seed	Kinetic studies in the presence of CaO/MgO	The activation energy, Ea, of the reaction to be 406.53 J/mol, while the pre-exponential factor A was found to be 0.01618 1/min (or 0.9 1/h).
54	BE	Pineapple	Optimization studies	Max. bioethanol concentration of 5.82%
78	BD	Non-Edible Indigenous Feedstocks	Optimization studies in the presence of KOH	88.0 % (Rubber seeds), 92.0 % (Avocado Pear seeds) and 96.7% (Nipa Palm Kennel seeds)
79	BD	*Lophira lanceolata* Seed Oil	Optimization studies in the presence of sulphuric acid	Optimum biodiesel yield of 85.0%
80	BD	CaO from animal bone	Optimization studies in the presence of sulfuric acid & economic analysis	87.04% conversion with 3.62 wt% of catalysts
68	BE	Cassava starch hydrolysate	Production of green fuel	92% yield reported
67	BE	Corn stover	Production of green fuel	Bioethanol yield was 143.15mg/L
72	BD	Neem seed	Production studies in the presence of CaO	Biodiesel produced was within range of ASTM standard
77	BD	Neem seed	Production studies in the presence of CaO/MgO	The activation energy, Ea, of the reaction to be 406.53 J/mol, while the pre-exponential factor A was found to be 0.01618 1/min (or 0.9 1/h).
81	BD	Jatropha	Production studies in the presence of NaOH	87% was obtained at 333 Kelvin, oil-to-alcohol molar ratio of 1:6 and 1wt% NaOH catalyst concentration
66	BE	Cassava Starch	Suitability of new strain enzyme as an alternative to the conventional	Bioethanol yield of 5.3% which was found suitable

Note: BE - bioethanol, BG - biogas and BD – biodiesel

Table 2. Some non-experimental (theoretical) studies on green fuel production.

Ref.	Green Fuels	Feed	Study Focus	Findings Made
80	BD	Azadiricha Indica oil & CaO from animal bone	Techno-economic analysis & process modelling	The annual production cost, total capital investment, payback time and internal rate of returns are $ 3537105, $ 5243784, 2.67 and 43%, respectively.
82, 83	BE	Sugarcane	Process modelling and pilot plant	A functional pilot was fabricated. And the fuel produced was tested and confirmed to have worked effectively in a portable generating power set.
46	BE	Molasses	Techno-economic analysis & process modelling	The study identified that the project would be economically feasible if the molasses cost is lowered, 20-40% government subsidy or a significant decline in the Dollar/Naira exchange rate.
84–86	BE	Rice husk	Techno-economic analysis & process modelling	Findings from this study indicated that transforming rice husk into bioethanol would not be economically feasible without subsidy
43, 87	BE	Sorghum bagasse	Techno-economic analysis & process modelling	The best return on investment was found to be obtainable at 20 % subsidy (minimum), 0% tax rate (waiver), 150 NGN/$ (lowest), and 10 NGN/kg (maximum)
88, 89	BE	Sugarcane bagasse	Techno-economic analysis & process modelling	Based on the results obtained, the study shows that the plant would yield a benefit/cost ratio (1.46), net present worth ($ 4.29 million), payback period (10 yrs) and return on investment (8 %), which suggests that the proposed plant would be economically feasible.

Note: GF – green fuels, BE - bioethanol, BG - biogas and BD - biodiesel

Solid catalyst development

Other works give preferential attention to the study of the exploration of solid catalyst potential in the synthesis of green fuels, green chemicals, and renewable fuel additives like biodiesel, sorbitol, mannitol, 5-hydroxymethylfurfural, carboxylic acids, maleic acid, 2,5-furandicarboxylic acid, and a lot of green products from biomass. Researchers are looking into the possibility of improving yield *via* the identification of suitable solid catalysts with an economic benefit over the age-long use of homogeneous catalysts [72], [77], [81]. Report of works presented in Table 1 showcases some work attempted to synthesize solid catalysts to improve yield and catalyst recovery after being used. Some such works include the report of producing catalysts from animal bone [80]. Another is the blending of CaO with MgO to form a mixed catalyst [73] and many other reports to engineering biodiesel production.

Optimization of the process technology

Some other works explore production parameters involved in the transformation in search of improving the yield *via* advanced optimization approaches like factorial design, response surface methodologies, and many others. Some works (as in Table 1) have employed these methods the search for a way of improving production volume of bioethanol, biodiesel, and other fuels [48], [54], [78], [80], [90]–[93].

Reaction kinetics & molecular modelling

This aspect of study considered the search for insight into the kinetics involved in the reaction process and identification of the reaction mechanism *via* the use of molecular simulation tools to study what happens to the reaction at the molecular-scale level. Only a few works [94]–[97] have given attention to the study of kinetics involved in the process. The bulk of the studies has giving preferential attention to the optimization studies over reaction kinetics. In contrast, no attention is given to computational approaches to explore the kinetics and mechanisms involved in our country's production process, unlike the developed nations' works.

Process simulation and pilot plant development

The absence of vital kinetic data has also contributed to this aspect of study in our country to suffer a lot in terms of promoting the simulation of relevant green fuels production processes due to the significant attention that our experimentalists have been giving to the optimization studies instead of giving preference to reaction kinetics modelling and studies. As a result of these issues of concern, only a few processes have been so far investigated on this subject [43], [89], [98].

Economic and commercialization potentials

The development of technologies without establishing their economic significance remains unattractive to both private and government investors. Despite all the understanding of the facts, only a few works have reported on the economic feasibility of the developed processes in Nigeria, evident with the report presented in Table 2. The bulk of works [43], [46], [99], [100] for bioethanol must have been a contributing factor that began to attract the interest of government investment towards this green fuels as reported in the literature [36] for bioethanol. The literature survey indicated that only the absence of vital information (like reaction kinetics models, which can be used to model processes that could then be used in costing the process) on the other fuels has contributed to the absence of a report on their viabilities.

GOVERNMENT POLICIES FOR GREEN FUELS PROMOTION IN NIGERIA

In promoting the establishment of biorefineries in Nigeria, promoting green fuels affordability and commercialization across the nation, the government develops a structured plan for establishing biorefineries and policies that would attract private entrepreneurs to invest in green fuel productions.

Developmental structure to get green refineries established and green fuel utilization

A survey of the literature [101], [102] indicated that the government of Nigeria had put many measures to promote the adoption of green fuels in the Nigerian States, especially bioethanol fuel, which has recorded significant governmental effort unlike other green fuels like biodiesel, biogas, and many others.

One of the measures deployed by the Nigerian government includes the establishment of a policy to mandate the blending of 10% bioethanol with petrol, otherwise known as gasoline, whose resulting mixture is commonly referred to as E-10 or gasohol. The biofuel program was designed to be in two phases, including "seeding of the market" through the importation of bioethanol to initiate penetration of nation within 3-10 years.

In comparison, the second phase program would entail establishing agricultural plantations and building green refineries where the bioethanol would be produced locally to substitute the imported one

since phase one. The report indicated that 1.3 billion litres of bioethanol would be required to meet the demand of compliance with the E-10 policy within the country. Similarly, a policy of blending 20% biodiesel with the petro-diesel (B-20 policy) was made. This policy indicated that the biodiesel market demand would be about 900 million litres by 2020 in Nigeria. In summary, the phase is expected to domesticate the production of green fuels *via* the private investors' program. The Biofuels Energy Commission was charged with setting up policies and managing the green fuel's production and distribution across the nation. A Biofuel Research Agency was designed to be built to research the biofuels in Nigeria with the direction of the Biofuel Energy Commission [101], [103].

Attractive government policies for motivating private investors

Most importantly, the government has designed attractive policies to attract private investors to the sector. Some of the policies set include industry incentives, exemption of green fuel industries from taxation, waiver on imports and customs duties for green refineries, waiver on value-added tax on green production and its feedstocks, availability of long-term preferential loans. Insurance shall be provided for strengthening the production of green fuel's feedstock to adequately cover the inherent risks [101], [104]. Although, the bulk of government effort is majorly on the commercialization of bioethanol in Nigeria with less attention to other green fuels. State governments like Kogi [105], Osun [106], and many other states [107], [108] are already investing in the sector.

CONCLUSIONS

This report has successfully presented the concept of what green fuels are all about, their classes, showing benefits that could be derivable from different classes of daily waste generated in our communities. Several efforts of Nigerian researchers to ensure that government and private investors' attention is gained toward the establishment of functional green fuels refineries (otherwise known as biorefineries) were also reported. Areas lacking significant attention were highlighted, and emphasis was made on the need to give better attention to them in future studies.

For the green fuels to attract the attention of government and private investors, and government to be motivated to adopt the idea of investing in green fuel to promote the efficiency of waste management in Nigeria the further research works need to pay significant attention to the following in their respective drive to see green fuel sets established in Nigeria: (1) reaction kinetic studies and molecular simulation; (2) process simulation and pilot plant development; and (3) economic and commercialization potentials.

In addition, there is a need for government and private entrepreneurs to diversify their efforts into investing in other green fuels in Nigeria in terms of the practice of concentrating majorly on bioethanol, whose economic viabilities have mainly been reported in the literature with insignificant reports for the other green fuels. Hence, future research is encouraged to focus on the study of unfolding the economic significance of investing in other green fuels like biodiesel and biogas.

REFERENCES

1. National Geographic Society, Fossil Fuels, National Geographic Society, National Geographic Encyclopedia, 2019. https://www.nationalgeographic.org/encyclopedia/fossil-fuels/.
2. ClientEarth, Fossil fuels and climate change: the facts, ClientEarth, ClientEarth Communication, Nov. 11, 2020. https://www.clientearth.org/latest/latest-updates/stories/fossil-fuels-and-climate-change-the-facts/.
3. M. Chukwu, C. O. Folayan, G. Y. Pam, D. O. Obada, *Journal of Combustion*, 9728278 (2016), doi: 10.1155/2016/9728278.
4. U. B. Akuru, O. I. Okoro, *ISRN Renewable Energy*, (2011), doi: 10.5402/2011/285649.
5. US-EIA, Nigeria - International, *U.S. Energy Information Administration (EIA) Report*, 2020. https://www.eia.gov/international/analysis/country/NGA
6. C. A. Odumugbo, *Journal of Natural Gas Science and Engineering*, **2** (6), 310 (2010), doi: 10.1016/J.JNGSE.2010.08.004.
7. NNPC, History of the Nigerian Petroleum Industry, *NNPC* Publication, Sep. 25, 2021. https://nnpcgroup.com/NNPC-Business/Business-Information/Pages/Industry-History.aspx.
8. M. Watts, *Geopolitics*, **9** (1),. 50 (2004), doi: 10.1080/14650040412331307832.
9. H. Chris, Nigerian Oil Economy: Development or Dependence, *Arts and Science Publication*, 1998, https://artsandscience.usask.ca/economics/skjournal/sej-3rd/hajler3.htm
10. N. Levinus, *Vangard Newspaper*, Abuja, Dec. 11, 2020. Accessed: Sep. 25, 2021. https://www.vanguardngr.com/2020/12/recession-fg-not-increasing-introducing-new-taxes-in-2021-finance-minister/
11. N. Cheta, *The Guardian Newspaper*, Lagos, Sep. 17, 2021. https://guardian.ng/opinion/nigerias-states-
12. will-struggle-to-collect-vat/
13. M. K. Paliwal, V. Pandey, A. Kumar, V. Sharma, *International Journal for Research in Engineering Application & Management*, Special Issue-TMRI-

2019, 25 (2019), doi: 10.18231/2454-9150.2019.0536.
14. J. Speirs, C. McGlade, R. Slade, *Energy Policy*, **87**, 654 (2015), doi: 10.1016/J.ENPOL.2015.02.031.
15. W. Gary, *The Harvard Gazette*, Apr. 22, 2020. https://news.harvard.edu/gazette/story/2020/04/harvard-experts-discuss-climate-change-fears/ (accessed Sep. 25, 2021).
16. N. A. Sasongko, C. Thorns, I. Sankoff, S. T. Chew, S. Bista, *Renewable Energy and Environmental Sustainability*, **2**, 25 (2017), doi: 10.1051/REES/2017034.
17. Biofuels - Campaigning for cleaner transport in Europe, Transport & Environment, *Transport & Environment Report*, 2021. https://www.transportenvironment.org/challenges/energy/biofuels/ (accessed Sep. 25, 2021).
18. F. P. Perera, *Environmental Health Perspectives*, **125** (2), 141 (2017), doi: 10.1289/EHP299.
19. F. Perera, *International Journal of Environmental Research and Public Health*, **15** (1), 16 (2018), doi: 10.3390/IJERPH15010016.
20. A. Femi, *The Punch*, Lagos, Dec. 27, 2016. Accessed: Oct. 14, 2021. [Online]. Available: https://punchng.com/dirty-fuels-vehicles-put-millions-nigerians-danger/
21. M. Agyei-Sakyi, Y. Shao, O. Amos, A. Marymargaret, *Sustainability,* **13***, 6239*, (2021), doi: 10.3390/SU13116239.
22. L. Temper, S. Avila, D. D. Bene, J. Gobby, N. Kosoy, P. L. Billon, J. Martinez-Alier, P. Perkins, B. Roy, A. Scheidel, M. Walter, *Environmental Research Letters*, **15**(12), 123004, (2020), doi: 10.1088/1748-9326/ABC197.
23. J. Busch, T. J. Foxon, P. G. Taylor, *Environmental Innovation and Societal Transitions*, **29**, 114, (2018), doi: 10.1016/J.EIST.2018.07.005.
24. B. J. Ananya, O. M. Ogungbade, C. N. Emeribe, A. W. Butu, *Global Journal of Earth and Environmental Science*, **5** (2), 37 (2020), doi: 10.31248/GJEES2020.069.
25. A. I. Anestina, A. Adetola, I. B. Odafe, *Journal of Waste Management*, 1 (2014), doi: 10.1155/2014/868072.
26. D. A. Okpara, M. Kharlamova, V. Grachev, *Sustainable Environment Research,* **31** (1), 1 (2021), doi: 10.1186/S42834-020-00077-1.
27. A. Nwosu, S. Olofa, "*Ethiopian Journal of Environmental Studies and Management*, **8** (2), 976 (2016), doi: 10.4314/ejesm.v8i2.11S.
28. A. H. O. Abiola, F. C. Fakolade, B. A. Akodu, A. A. Adejimi, O. A. Oyeleye, G. A. Sodamade, A. T. Abdulkareem, *African Journal of Primary Health Care & Family Medicine*, **13** (1), 1 (2021), doi: 10.4102/PHCFM.V13I1.2677.
29. O. Toyese, O. Ademola, J. J. Olusanya, *The Journal of Engineering, Science and Computing (JESC)*, **3** (1), 1 (2021), Accessed: Aug. 19, 2021. [Online]. Available: https://jesc.iu.edu.sa/Main/Article/63
30. Finnish Energy Club, *Finnish Energy Club Report*, Feb. 2021. https://www.svek.fi/esimerkkisivu/what-is-green-fuel/ (accessed Sep. 25, 2021).
31. M. F. Othman, A. Adam, G. Najafi, R. Mamat, *Renewable and Sustainable Energy Reviews*, **80**, 694 (2017), doi: 10.1016/J.RSER.2017.05.140.
32. Md. S. Alam, Md. S. Tanveer, *Bioreactors*, 55, (2020), doi: 10.1016/B978-0-12-821264-6.00005-X.
33. A. Doerr, S. Cardenas, S. Jardine, H. Yoon, S. Bucaram, J. N. Sanchirico, *Encyclopedia of Energy, Natural Resource, and Environmental Economics*, 2013, p. 232, http://www.sciencedirect.com/science/article/pii/B9780123750679000474
34. C. H. Brito Cruz, G. Mendes Souza, and L. A. Barbosa Cortez, *Future Energy: Improved, Sustainable and Clean Options for our Planet*, 215 (2014), doi: 10.1016/B978-0-08-099424-6.00011-9.
35. BTG, Bioethanol, 2004. https://www.eubia.org/cms/wiki-biomass/biofuels/bioethanol/
36. S. K. Hoekman, A. Broch, C. Robbins, E. Ceniceros, and M. Natarajan, *Renewable and Sustainable Energy Reviews*,. **16** (1), 143 (2012), doi: 10.1016/J.RSER.2011.07.143.
37. T. Oyegoke, E. Obadiah, F. Adah, J. E. Oguche, G. T. Timothy, I. A. Mantu, A. D. Ado, *Journal of Renewable Energy and Environment*, **9** (1), 37 (2022), doi: 10.30501/JREE.2021.278037.1197.
38. S. De, *Hydrocarbon Biorefinery*, 327 (2022), doi: 10.1016/B978-0-12-823306-1.00001-7.
39. M. Mohammad, T. Kandaramath Hari, Z. Yaakob, Y. Chandra Sharma, K. Sopian, *Renewable and Sustainable Energy Reviews*, 121 (2013), doi: 10.1016/J.RSER.2013.01.026.
40. I. O. Ogundari, A. S. Momodu, A. J. Famurewa, J. B. Akarakiri, W. O. Siyanbola, *Energy & Environment*, **23** (4), 599 (2012), doi: 10.1260/0958-305X.23.4.599.
41. C. Obianwa, A. U. Edak, I. Godwin, *African Journal of Biotechnology*, **15** (30), 1608 (2016), doi: 10.5897/ajb2016.15391.
42. S. O. Dahunsi, U. S. Oranusi, *British Biotechnology Journal*, **3** (4),. 485 (2013).
43. Y. Isah, H. D. Kabiru, M. A. Danlami, S. F. Kolapo, *J. Chem Soc. Nigeria*, **44** (2), 233 (2019).
44. O. O. Ajayi, K. Rasheed, A. Onadeji, T. Oyegoke, *Journal of Engineering Studies and Research*, **26** (3), 154 (2020).
45. T. Oyegoke, E. Obadiah, S. Y. Mohammad, O. A. Bamigbala, O. A. Owolabi, A. Oyegoke, A. Onadeji, A. I. Mantu, *European Biomass Conference and Exhibition Proceedings*, 1270 (2021), doi: 10.5071/29THEUBCE2021-4BV.9.13.
46. K. T. Gaffa, *Nig. J. Biotechn.*, **8** (1), 35 (1997).
47. A. Abemi, T. Oyegoke, F. N. Dabai, B. Y. Jibril, in *National Engineering Conference*, 2018, p. 531.
48. S. B. Oyeleke, B. E. N. Dauda, Oyewole, I. N. Okoliegbe, T. Ojebode, *Advances in Environmental Biology*, **6** (1), 241 (2012).
49. A. Monday Osagie, *International Journal of Statistical Distributions and Applications*, **3** (3), 47 (2017), doi: 10.11648/j.ijsd.20170303.14.

50. A. M. Ebabhi, A. A. Adekunle, O. O. Adeogun, *Nigerian Journal of Basic and Applied Sciences*, **26** (2), 17 (2019), doi: 10.4314/njbas.v26i2.3.
51. E. A. Akponah, O. O. Akpomie, *International Research Journal of Microbiology (IRJM*, **2** (10), 393 (2011).
52. S. Olayemi, A. Ibikunle, J. Olayemi, *Technology, and Sciences (ASRJETS) American Scientific Research Journal for Engineering*, **52** (1), 67 (2019).
53. M. E. Ojewumi, A. I. Job, T. O. Samson, O. O. Mopelola, A. A. Ayoola, E. O. Ojewumi, E. A. Oyeniyi, *International Journal of Pharmaceutical and Phytopharmacological Research*, **8** (3), 46 (2018).
54. M. E. Ojewumi, M. E. Emetere, C. v. Amaefule, B. M. Durodola, O. D. Adeniyi, *International Journal of Pharmaceutical Sciences and Research*, **10** (3), 1246 (2019), doi: 10.13040/IJPSR.0975-8232.10(3).1246-52.
55. O. Oiwoh, B. V. Ayodele, N. A. Amenaghawon, C. O. Okieimen, *Journal of Applied Sciences and Environmental Management*, **22** (1), 54 (2018), doi: 10.4314/jasem.v22i1.10.
56. N. M. Aminu, Y. Aladire, *International Journal of Scientific & Engineering Research*, **4** (1), 1 (2013).
57. G. Pilarski, M. Kyncl, S. Stegenta, G. Piechota, *Waste and Biomass Valorization*, **11** (7), 3579 (2019), doi: 10.1007/S12649-019-00707-9.
58. A. Umo, K. Egemba, E. Bassey, B. Etuk, *Global Journal of Engineering Research*, **12** (1), 13 (2013), doi: 10.4314/gjer.v12i1.2.
59. O. Odimegwu, Port Harcourt, Jan. 2014. https://africaprimenews.com/2018/01/20/development/special-report-nigeria-wastes-40-of-food-but-millions-of-citizens-are-dying-of-hunger/
60. S. Ingale, S. J. Joshi, A. Gupte, *Brazilian Journal of Microbiology*, **45**, (3), 885 (2014).
61. W. Braide, I. Kanu, U. Oranusi, S. Adeleye, *Journal of Fundamental and Applied Sciences*, **8 (**2), 372 (2016), doi: 10.4314/jfas.v8i2.14.
62. C. J. Nyachaka, D. S. Yawas, G. Y. Pam, *American Journal of Engineering Research (AJER)*, **2**, 303 (2013).
63. H. O. Stanley, A. O. Erewa, C. N. Ariole, *Biotechnology Journal International*, **22** (3), 1 (2019), doi: 10.9734/bji/2018/v22i330057.
64. A. Wale, *Legit*, Jan. 06, 2017. https://www.legit.ng/1081294-how-agricultural-waste-conversion-fix-nigerias-power-supply.html (accessed Oct. 14, 2021).
65. B. Afe, *Vanguard*, Lagos, Dec. 25, 2019. https://www.vanguardngr.com/2019/12/the-scourge-of-open-defecation-in-nigeria-need-for-immediate-and-urgent-intervention/
66. F. Saïdane-Bchir, A. el Falleh, E. Ghabbarou, M. Hamdi, *Waste and Biomass Valorization*, **7** (5), 1041 (2016), doi: 10.1007/s12649-016-9492-6.
67. F. O. Ajibola, E. Oyewole, *Nigerian Food Journal Official Journal of Nigerian Institute of Food Science and Techonology*, **30** (2), 114 (2012).
68. O. Evuensiri Onoghwarite, N. Victor Ifeanyichukwu Obiora, E. Akachukwu Ben, N.-O. Ekpe Moses, *International Journal of Scientific & Engineering Research*, **7** (8), 290 (2016).
69. E. Betiku, O. S. Alade, *Energy Sources, Part A: Recovery, Utilization and Environmental Effects*, **36**, (18), 1990 (2014), doi: 10.1080/15567036. 2011.557690.
70. F. U. Ahmad, A. Bukar, B. Usman, *Bayero Journal of Pure and Applied Sciences*, **10** (1), 280 (2018), doi: 10.4314/bajopas.v10i1.56s.
71. E. O. U. Uzodinma, A. U. Ofoefule, J. I. Eze, Onwuka N.D., *Trends in Applied Sciences Research*, **2** (6), 554 (2007), doi: 10.3923/TASR.2007.554.558.
72. A. O. Chinwendu, A. O. B. Catherine, A. E. Bassey, E. U. Okon, A. O. Chinwendu, *WJARR,* **1,** 52 (2019), doi: 10.30574/WJARR.2019.1.3.0026.
73. H. Danjuma Banu, T. B. Shallangwa, T. O. Magu, L. Hitler, S. Ahmed, *J. Phys. Chem. Biophys.*, **8**, 1 (2018), doi: 10.4172/2161-0398.1000266.
74. I. K. Ajadi, T. Oyegoke, T. T. Geoffrey, O. Fasanya, A. O. Ojetunde, *Proceedings of the Materials Science & Technology Society of Nigeria*, **1**, (2020), doi: 10.3329/BJSIR.V45I2.5702.
75. S. J. Ojolo, S. A. Oke, K. Animasahun, B. K. Adesuyi, *J. Environ. Health Sci. Eng*, **4** (4), 223 (2007).
76. F. A. Aisien, E. T. Aisien, *Detritus Multidisciplinary Journal for Waste Resource & Residues*, **10** (10), 108, (2020), doi: 10.31025/2611-4135/2020.13910.
77. K. O. Adiotomre, E. F. Ukrakpor, *International Journal of Innovative Scientific & Engineering Technologies Research*, **3** (2), 52 (2015).
78. T. Oyegoke, K. A. Ibraheem, *European Journal of Chemistry*, **12** (3), 242 (2021), doi: 10.5155/eurjchem.12.3.242-247.2085.
79. C. I. A. Nwoko, A. U. Nkwoada, H. U. Ogu, C. I. A. Nwoko, A. U. Nkwoada, H. U. Ogu, *The International Journal of Biotechnology*, **8** (1), 84 (2019), doi: 10.18488/JOURNAL.57.2019.81.84.92.
80. M. Z. Kyari, S. M. Danggoggo, B. B. Usman, A. B. Muhammad, *Nigerian Journal of Basic and Applied Sciences*, **25 (**2), 75 (2018), doi: 10.4314/njbas.v25i2.9.
81. E. O. Oke, O. Adeyi, B. I. Okolo, C. J. Ude, J. A. Adeyi, K. K. Salam, U. Nwokie, I. Nzeribe, *Bioresource Technology*, **332**, 125141, (2021), doi: 10.1016/J.BIORTECH. 2021.125141.
82. E. Funmilayo Aransiola, M. O. Daramola, T. V. Ojumu, O. Aremu, S. Kolawole Layokun, B. O. Solomon, *International Journal Of Renewable Energy Research*, vol. 2, no. 2, pp. 318–325, 2012.
83. I. M. Misau, I. M. Bugaje, J. Mohammed, I. A. Mohammed, B. H.Diyau'deen., *International Journal of Engineering Research and Applications (IJERA)*, **2** (4), 1142 (2012).
84. I. M. Misau, B. I. Muhammad, M. I. Ali, M. Jibril, *Journal of Applied Phytotechnology in Environmental Sanitation*, **3** (1), 11 (2014).
85. T. Oyegoke, M. Y. Sardauna, H. A. Abubakar, E. Obadiah, *Renewable Energy Research and*

Application, **2** (1), 51 (2021), doi: 10.22044/RERA.2020.10287.1042.
86. T. Oyegoke, E. Obadiah, Y. S. Mohammed, O. A. Bamigbala, A. Owolabi, in: *Sustainability Challenges & Transforming Opportunities: "Amidst Covid19"*, **1**, S. Anuradha, B. Jyoti, K. S. Ravi, S. Subodhika (eds.) New Delhi, Authorspress Global Network, 2021, p. 54.
87. T. Oyegoke, E. Obadiah, Y. S. Mohammed, O. A. Bamigbala, O. A. Owolabi, T. T. Geoffrey, A. Oyegoke, A. Onadeji, *Renewable Energy Research and Application,* **3** (1), 1, 2022.
88. T. Oyegoke, O. O. Ajayi, *Journal of Energy Technology and Environment*, **3** (4), 1 (2021), doi: 10.37933/nipes.e/3.4.2021.1.
89. T. Oyegoke, F. Dabai, *Nigerian Journal of Technology*, **37 (**4), 913 (2018), doi: 10.4314/njt.v37i4.9.
90. T. Oyegoke, F. Dabai, J. Abubakar Muhammed, B. El-Yakubu Jibiril, in: *1st National Conference On Chemical Technology (NCCT 2017)*, 2017, p. 125.
91. J. O. Madu, B. O. Agboola, *Biotech*, **8** (1), 1 (2018), doi: 10.1007/s13205-017-1033-x.
92. B. Suleiman, S. A. Abdulkareem, E. A. Afolabi, U. Musa, I. A. Mohammed, T. A. Eyikanmi, *Advances in Energy Research*, **4** (1), 69 (2016), doi: 10.12989/eri.2016.4.1.069.
93. D. Adegunloye, D. Udenze, *Journal of Advances in Microbiology*, **4** (2), 1 (2017), doi: 10.9734/jamb/2017/34032.
94. O. O. Christopher, A. A. Felix, *Microbiology Research Journal International*, 1 (2019), doi: 10.9734/mrji/2019/v28i330134.
95. I. C. Nnaemeka, O. S. Egbuna, I. O. Maxwell, A. O. Christain, C. Onyekwulu, *Journal of Bioresources and Bioproducts*, **6** (1), 45 (2021), doi: 10.1016/J.JOBAB.2021.02.004.
96. N. Nosrati-Ghods, S. T. L. Harrison, A. J. Isafiade, and S. L. Tai, *ChemBioEng Reviews*, **7** (3), 68 (2020), doi: 10.1002/CBEN.201900024.
97. G. K. Latinwo, S. E. Agarry, *Journal of Natural Sciences Research* , **5** (14), 38 (2015).
98. E. T. Akhihiero, B. V. Ayodele, M. A. Alsaffar, T. O. K. Audu, E. O. Aluyor, *Journal of Engineering*, **27** (4), 33 (2021), doi: 10.31026/J.ENG.2021.04.03.
99. A. G. Adeniyi, J. O. Ighalo, in: *Handbook of Environmental Materials Management*, Springer International Publishing, 2020, p. 1. doi: 10.1007/978-3-319-58538-3_185-1.
100. T. Oyegoke, O. O. Ajayi, R. O. Kolawole, Techno-economic Assessment Of Transforming Sorghum Bagasse Into Bioethanol Fuel In Nigeria: 2 - Economic Analysis, 2020.
101. T. Oyegoke, F. Dabai, *Nigerian Journal of Technology*, **37 (**4), 921 (2018), doi: 10.4314/njt.v37i4.9.
102. Federal Republic of Nigeria, Nigerian Biofuel Policy Incentives, *Federal Republic of Nigeria Official Gazette of the Nigerian Bio-fuel Policy and Incentives,* 2007, p. 1.
103. Biofuels Digest, https://www.biofuelsdigest.com/bdigest/2020/07/26/nnpc-to-cultivate-cassava-plantations-for-biofuels/
104. R. Olurounbi, *The Africa Report*, 2020. https://www.theafricareport.com/42590/nigerias-biofuel-success-must-include-more-private-investment/
105. A. Galadima, Z. N. Garba, B. M. Ibrahim, M. N. Almustapha, L. Leke, I. K. Adam, *Journal of Sustainable Development*, **4** (4), 22 (2011), doi: 10.5539/jsd.v4n4p22.
106. Biofuels Digest, https://www.biofuelsdigest.com/bdigest/2020/02/17/site-work-begins-at-planned-nnpc-ethanol-project-in-kogi-state/
107. Biofuels Digest, https://www.biofuelsdigest.com/bdigest/2020/09/22/officials-break-ground-at-osun-ethanol-biorefinery/
108. Biofuels Digest, https://www.biofuelsdigest.com/bdigest/2020/12/28/mou-signed-to-develop-cassava-based-biorefinery-in-nigerias-plateau-state/
109. Biofuels Digest, https://www.biofuelsdigest.com/bdigest/2020/06/09/ethanol-push-in-kebbi-state-could-result-in-1b-in-annual-revenues/

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Chemical characterization of *Artemisia annua* L. subcritical extract

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The aerial parts of *Artemisia annua* L. from Bulgaria were extracted with 1,1,1,2-tetrafluoroethane. Static two-stage extraction at a pressure of 8 and 12 bar was used at a relatively low temperature. The yield was 2%. The chemical profile of the product was reported for the first time. GC-MS analysis showed that the extract contained the essential oil constituents, but in lower concentrations. In total, 28 components were identified, the main ones being artemisia ketone (26.2%), camphor (10.7%), and eucalyptol (9.1%). Artheanin B (3.7%) and arteannuic acid (3.7%) were found to be co-metabolites and precursors of artemisinin. The content of the sesquiterpene lactone artemisinin, determined spectrophotometrically, was found to be 1.28 ± 0.10 %. The results revealed that the extract is of interest with the presence of biologically active substances used as a modern anti-malaria and potential anti-coronavirus agent.

Keywords: *Artemisia annua* L.; extraction; chemicals; artemisinin.

INTRODUCTION

Artemisia annua L. (Asteraceae), known as wormwood, a highly aromatic annual herb of Asiatic and East European origin, is widely distributed in the temperate and tropical regions throughout the world. The plant is spread wild in Bulgaria and is used in traditional medicine for treatment of gastric insufficiency, in infusion as poison antidote and for activating blood circulation [1]. It is an important medicinal plant, whose biological activity is due to volatile and non-volatile secondary metabolites [2]. The essential oil which is rich in mono- and sesquiterpenes represents another source of potential commercial value. Besides significant variations in its percentage and composition have been reported (major constituents can be camphor, germacrene, artemisia ketone, and 1,8-cineole (eucalyptol)), the oil has been subjected to numerous studies supporting promising antibacterial and antifungal activities [3].

The wormwood is among the ten top pharmaceutical crops and a source of the life-saving drug artemisinin, an active ingredient of most antimalarial medicines, used every year by over 300 million people. It is mainly located in the flowers and leaves of the plant and stored in the glandular trichomes on the surface of leaves, because of its toxicity for the plants cells [2]. The isolation of artemisinin was firstly achieved by the Chinese scientist Tu Youyou and awarded with the 2015 Nobel Prize in Medicine together with William C. Campbell and Satoshi Ō [4]. Recent docking studies indicated that artemisinin and its derivatives artesunate and arteminol could bind the SARS-CoV-2 spike protein in a way that would interfere with its docking onto the human ACE2 receptor protein, which is the required first step in the host infection process of the coronavirus disease (COVID-19) [5].

However, other bioactive compounds in *A. annua* contribute to the overall activity of the extracts: flavonoids, arteannuin B and artemisitene, but also scopoletin and 1,8-cineole act synergically with artemisinin and exhibit beneficial activity [2, 6, 7].

For this reason, in recent years, efforts have been focused on the application of different approaches to obtain a complex end product.

The studies assessed the economic and environmental potential of the extraction by ethanol, supercritical CO_2, ionic liquids, freons, monoether-based solvents as compared to hexane and ethyl acetate [8-14]. Extraction by tetrafluoroethane was found to be the most promising approach among the considered new alternative processes [15]. The solvent (also known as refrigerant R134a) is nontoxic, nonreactive, nonflammable, and nonozone depleting. It has high volatility and boiling point at atmospheric pressure of -25.9°C, which means that it leaves negligible solvent residue in the products. It is in a gas form at room temperature, stable to aqueous acids and bases, immiscible with water and sparingly soluble in water (1500 ppm at 20°C). It is normally handled as a compressed gas under pressure in liquid form

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and has a liquid density of about 1.3 kg/L [16]. The biological products made by this process have extremely low residual solvent - less than 20 parts per billion or frequently below the levels of detection. It is neither acidic nor alkaline and has only minimal potential reaction effects on the botanical materials [11]. It can perfectly extract the essential oil with a particularly cleaner process comparing with other solvents, with contamination-free product and facile downstream processing. Despite the excellent indications for the efficacy of R134a as an extractant, there are no data in the literature for a complete chemical analysis of the product. The authors concentrate and comment on the content of artemisinin and its derivates, but other components are not mentioned [11, 13, 14]. Only two references comment on the camphor content in a supercritical extract with CO_2 [9, 12]. Adding the importance of the origin of *A. annua* for the quantitative and qualitative characteristics of the final product [1, 2, 17, 18], the question of the chemical profile of the subcritical extract of the native plant becomes important.

The theme is very actual and the aim of the present work was to evaluate the chemical composition of the freon extract from *A. annua* L., grown in Bulgaria and its potential to obtain artemisinin.

EXPERIMENTAL

Plant material

A. annua L. herbs (full blooming) were collected in the area of Kazanlak (Bulgaria) in July 2016. The plant material was air dried at room temperature in a shadow place. According to Ferrera *et al.* [19], that kind of drying provided the highest artemisinin yield. Later it was stored in a cool, dark and dry place prior to extraction and analysis. The moisture of the material (12.5%) was determined by drying up to constant weight at 105°C. The plants were grounded immediately prior to extraction.

Extraction

Extraction of wormwood material using tetrafluoroethane was performed in a pilot apparatus. The unit consists of a 1 L extraction vessel, a 5.5 L of collector vessel, equipped with a 200 W electric heater, a compressor, a heat exchanger unit, and a filtration set. The system is equipped with temperature and pressure sensors. It is controlled by a fully automatic Programmable Logic Controller (PLC) screen interface with first level safety functionality and user programmable extraction pressure, number of extractions, separation end pressure and extraction time. A heating mantle was constructed around the collector vessel to maximize the freon transfer rate from liquid to gas state.

The solvent was food grade 1,1,1,2-tetrafluoroethane (CAS number 811-97-2), purchased from Frigo Chem Ltd. (Bulgaria).

The experiments were conducted at conditions, shown in Table 1.

GC/MS analysis

GC/MS analysis was carried out on a 7890A gas chromatograph (Agilent Technologies) interfaced with a 5975C mass selective detector (Agilent Technologies). Separations were performed using a 30 m×0.25 mm (i.d.) DB-5 ms silica-fused capillary column coated with a 0.25 μm film of poly (dimethylsiloxane) as the stationary phase. The flow rate of carrier gas (helium) was maintained at 1.0 mL/min. The injector and the transfer line temperature were kept at 250°C. The oven temperature program used was 40°C for 2 min, then 5°C/min to 300°C for 10 min. The injection volume was 1 μL. The injections were carried out in a split mode 20:1. The mass spectrometer was scanned from 50 to 550 m/z. All mass spectra were acquired in an electron impact (EI) mode with 70 eV.

A mixture of aliphatic hydrocarbons (C_8-C_{40}) (Sigma) was injected into the system under the above temperature program in order to calculate the retention index RI (as Kovàts index) of each compound. Identification of compounds was obtained by comparing the RI and the spectral data from the NIST'08 (National Institute of Standards and Technology, USA).

Table 1. Extraction parameters of subcritical processing of *Artemisia annua* L.

*Variants	Charge weight, g	Pressure, bar	Duration, min	Temperature, °C	Number of extractions
Variant 1	95	6 - 7	60	30	2
Variant 2	95	10 - 12	60	45	2

Spectrophotometric determination of artemisinin

The quantitative determination of artemisinin in the extract was performed according to the procedure developed in our laboratory (unpublished data) based on that described by Zhao and Zeng [20]. Briefly, 50 mg of the extract was dissolved in $CHCl_3$ and applied on a TLC plate (Silica gel 60 $F_{254,}$ glass plate 20 × 20 cm, Merck) on both ends of which the standard (artemisinin) was also added. The plate was developed using n-hexane-diethyl ether (1:1/v:v). After developing, the plate was dried and a small part of it was sprayed with H_2SO_4 in order to locate the artemisinin on the plate. Further, the TLC zone containing artemisinin was scrapped, quantitatively transferred to a small glass column and eluted with diethyl ether (50 mL). Further, the solvent was evaporated under reduced pressure. The artemisinin-enriched sample was dissolved in 10 mL of ethanol, transferred to a 50 mL volumetric flask and made up to the mark with 0.2% aqueous NaOH. The resulting solution was kept in a water bath (50ºC) for 30 min and then the absorbance was measured at 291 nm on a spectrophotometer. Different concentrations of artemisinin (0.2-1.0 mg/50 mL) at the same conditions were used for preparation of the calibration curve ($y = 1.2037x + 0.0454$, $R^2 = 0.9975$). The amount of artemisinin in the extract was calculated using the following equation:

$$\text{Artemisinin (\%)} = (C/P) \times 100,$$

where C was the amount of artemisinin (mg/50 mL), calculated from the calibration curve and P was the amount of the extract (mg) used for the analysis. The analysis was performed in triplicate. The value is presented as a mean ±SD.

RESULTS AND DISCUSSION

After extraction the exhausted raw material seems like before - with the same color and type. This is due to the gentle technology and the specificity of the solvent. The extracts themselves are a green mass with a brownish hue.

Despite the different pressure, the product yield is 2%. This indicates that extraction at lower pressure reaches the extraction limit of the extractable substances. Literature data for the extraction of essential oil vary from 0.30% to 0.66% [3, 18]. A non-polar solvent extracts the essential oil and an additional large number of substances. As regards the yield it is always more efficient than distillation.

The same solvent achieved a yield of 1.66% at a pressure of 10 bar and a duration of 24 h [15]. Supercritical extraction with CO_2 at a pressure of 30 MPa and 2 h realized a total product within 3.90 - 4.46% [6, 12]. According to these data our result was over the average values and indicated the effectiveness of the experiment.

Our results on the chemical composition can be considered as the first ones. The profile of the chromatogram (shown in Fig. 1) revealed its complex picture. The composition of the *A. annua* essential oil with the same origin is the best basis for a parallel with our product. The chemical structure of the subcritical product is shown in Table 2.

Twenty-eight components with concentrations higher than 0.1% were identified, which represents 85.48% of the total detected compounds. Among them, the most abundant class was that of oxygenated monoterpenes, followed by oxygenated sesquiterpenes, monoterpene hydrocarbons, phenylpropanoids and hydrocarbons. The oxygenated terpenoids were the main chemicals with 74% of the composition. Compared with data on Bulgarian wormwood essential oil from the same ecological region, it can be seen that the number of its compounds represents 60% of the whole number in the extract. The main groups in the structure of the essential oil were sesqiterpene hydrocarbones, followed by oxygenated monoterpenes and monoterpene hydrocarbons. Phenylpropanoids were noted by about 1%. The results can be explained by the nature of the solvent and the extraction method.

Artemisia ketone and artemisia alcohol are specific for the genotype and the data showed that their amounts were at average three times over in the extract. The quantity of the camphor in a group with eucalyptol and camphene was also triple in our product.

The main compound in the subcritical extract was artemisia ketone, while in the essential oil it was β-caryophyllene. According to Martinez-Correa *et al*. [8], the volatile fraction of the CO_2 extract consists mainly of camphor, so our product should be classified as different. This result can also be attributed to the different origins of wormwood. The advantage is emphasized in the non-polar low-molecular weight constituents. With increasing molecular weight and reducing volatility, sesquiterpenes exhibit greater amounts in the distillation product (after α-copaene). It is rather due to the relative proportion of ingredients in the two products. The extract contains the valuable germacrene D, arteannuin B, and arteannuic acid in sufficient quantity. This result is a consequence of the specificity of the solvent and emphasizes the advantage of the method.

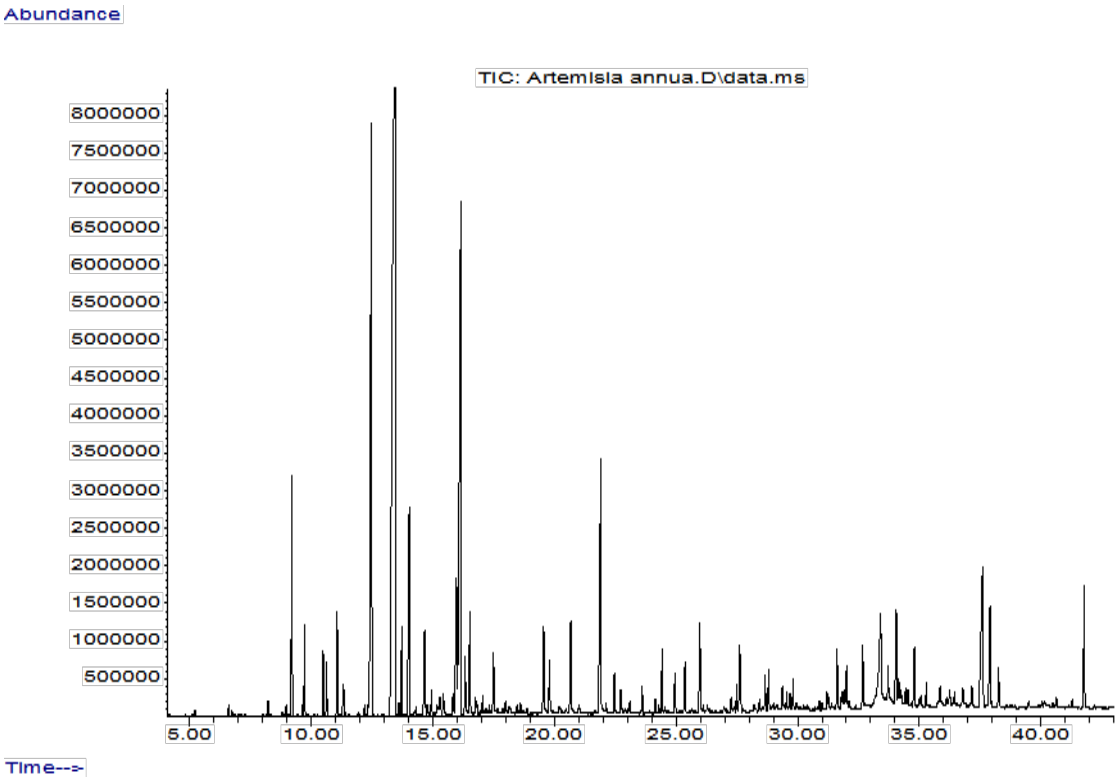

Figure 1. CG-MS profile of the subcritical extract from *A. annua*

Table 2. Chemical composition of the subcritical extract and essential oil from Bulgarian *Artemisia annua* L.

№	Component	Rel. %, as determined by GC/MS		
		KI	Subcritical Extract	Essential oil [1]
1	α-Pinene	939	2.52	1.26
2	Camphene	953	0.91	0.40
3	Sabinene	969	0.67	*
4	β-Pinene	974	0.52	0.32
5	β-Myrcene	991	1.06	*
6	(3E)-2,5,5-Trimethyl-3,6-heptandien-2-ol	1018	0.44	*
7	Eucalyptol	1027	9.06	2.55
8	Artemisia ketone	1051	26.15	8.45
9	Artemisia alcohol	1068	3.02	*
10	trans-Pinocarveol	1136	2.21	0.43
11	trans-Verbenol	1141	1.63	*
12	Camphor	1149	10.68	3.61
13	Pinocarvone	1154	1.29	0.73
14	Terpinen-4-ol	1177	0.86	<0.10
15	Eugenol	1358	4.33	<0.10
16	β-Elemene	1390	0.62	0.26
17	α-Copaene	1376	0.39	7.42

Table 2 (Continued)

№	Component	Rel. %, as determined by GC/MS		
		KI	Subcritical Extract	Essential oil [1]
18	β-Caryophyllene	1419	0.73	24.73
19	γ-Elemene	1433	0.21	0.26
20	α-Humulene	1454	0.37	3.86
21	Eugenyl acetate	1485	1.21	*
22	Germacrene D	1580	0.28	*
23	Caryophyllene oxide	1582	0.92	*
24	Arteannuin B	1692	3.69	*
25	n-Heptacosane	1700	1.41	*
26	n-Octacosane	1800	3.55	*
27	Arteannuic acid	1818	3.70	*
28	Lupenone	2856	3.08	*
Monoterpene hydrocarbons, %			6.67	3.50
Oxygenated monoterpenes, %			64.35	29.10
Sesqiterpene hydrocarbones, %			3.05	67.22
Oxygenated sesquiterpenes, %			9.75	*
Phenylpropanoids, %			6.54	<0.18
Hydrocarbons, %			5.84	*
Other, %			3.80	*
Total			85.48	54.48

Legend: KI - Kovats Index; * - not identified

The amount of artemisinin in the studied extract was determined spectrophotometrically using the known reaction of conversion of artemisinin into a UV-absorbing compound, Q292 (Fig. 2) by treating with 0.2% aqueous NaOH [20].

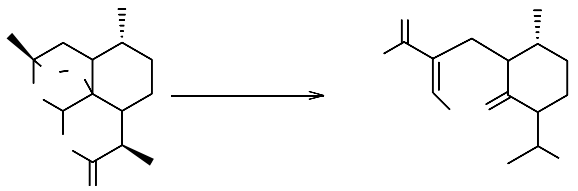

Figure 2. Conversion of artemisinin in Q292 derivative

Then it was subjected to alkaline hydrolysis and the absorbance of the hydrolyzed mixture was measured at 292 nm using a spectrophotometer. Pure artemisinin that underwent the same procedure was used to make a calibration curve. The content of artemisinin in the extract was found to be 1.28 ± 0.10%. With a method efficiency of 60% [17], we can assume with sufficient probability that the initial concentration in the plant is around 0.77%. The literature data for *A. annua* L. of different geographical origin indicated an artemisinin content of 0.33 to 0.97% [17]. The optimized analytical method of Kochler *et al*. [21] showed a maximum content of artemisinin of 0.96% in superctitical CO_2 extract. The high yield and selectivity of the solvent used by us in the intramolecular synthesis of the lactone is also proved in another study [22]. Our results correlate with these data and show a comparatively high potential for the availability and extraction of artemisinin in Bulgarian wormwood plants.

CONCLUSION

Subcritical extraction with 1,1,1,2-tetrafluoroethane of *Artemisia annua* L. from Bulgaria gave a yield of 2%. The chemical composition of the product consists mainly of oxygenated monoterpenes with the major compound artemisia ketone. The potential antimalarial constituents - artemisinin and its derivatives are proved to be in sustainable quantities.

REFERENCES

1. R. Tzenkova, Z. Kamenarska, A. Draganov, A. Atanassov, *Biotechnol. Biotechnol. Equip*., **24**(2), 1833 (2010).

2. R. Bhakuni, D. Jain, R. Sharma, S. Kumar*, Curr. Sci.*, **80**(1), 35 (2001).
3. A. Bilia, F. Santomauro, C. Sacco, M. Bergonzi, R. Donato, Evid. Based Complement. *Alternat. Med*., **2014,** 7 **(**2014).
4. Nobel Foundation. The Nobel Prize in Physiology or Medicine (2015). https://www.nobelprize.org / prizes/medicine/2015/.
5. M. Sehailia, S. Chemat, *J. Biomol. Struct. Dyn.,* **39**(16), 6184 (2021).
6. T. Tzeng, L. Lin, T. Jong, C. Chang, *Sep. Purif. Technol*., **56,** 18 (2007).
7. B. Ivanescu, A. Miron, A. Corciova, *J. Anal.* Methods *Chem*., **2015**, 21 (2015).
8. S. Laboukhi-Khorsi, K. Daud, S. Shemat, *ACS Sustainable Chem. Eng.*, **5**, 4332 (2017).
9. H. A. Martinez-Correa, R. G. Bitencourt, A. C. A. V. Kayano, P. M. Magalhães, F. T. M. Costa, F. A. *Cabral, Ind. Crops Prod*., **95**, 535 (2017).
10. P. Christen, J. Veuthey, *Curr. Med. Chem*., **8**(15), 1827 (2001).
11. A. Lapkin, P. Plucinski, M. Cutler, *J. Nat. Prod*., **69**(11), 1653 (2006).
12. S. Quispe-Condoria, D. Sánchez, M. Foglio, T. Paulo, R, Carsten, G. Brunnerb, A. Meireles, *J. Supercrit. Fluids*, **36**(1), 40 (2005).
13. Y. Zhang, P. Prawang, Ch. Li, X. Meng, Y. Zhao, H. Wang, S. Zhang, *Green Chem*., **20**, 713 (2018).
14. A. Zarrelli, A. Pollio, S. Aceto, V. Romanucci, F Carella, P. Stefani, A. De Natale, G. De Vico, *Phytochem. Anal.,* **30**, 564 (2019).
15. A. Lelono, S. Simanungkalit, I. Umarudin, H. Herdiawan, *J. Trop. Pharm. Chem*., **4**(3), 114 (2018).
16. S. Corr, *J. Fluor. Chem*., **118**, 55 (2002).
17. A. Lapkin, E. Adou, B. Mlambo, S. Chemat, J. Suberu, A. Collis, A. Clark, G. Barke, *C. R. Chim*., **17**, 232 (2014).
18. S. Zhigzhitzhapova, E. Dylenova, S. Gulyaev, T. Randalova, V. Taraskin, Zh. Tykheev, L. Radnaeva, *Nat. Prod. Res*., **34**(18), 2668 (2019).
19. J. S. Ferreira, J. E. Simon, J. Janick*, HortScience*, **30**(4), 888 (1995).
20. S. S. Zhao, M. Y. Zeng, *Planta Med.,* **51**, 233 (1985).
21. M. Kohler, W. Haerdi, Ph. Christen, J. L. Veuthey, *J. Chromatogr*., **785**, 353 (1997).
22. S. K. Karmee, B. Niemeijer, L. Casiraghi, B. Mlambo, A. Lapkin, L. Greiner, *Biocatal. Biotransformation*, **32**(2), 125 (2014).

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Lactic acid beverage based on wort and mint (*Menta piperita* L.)

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A new type of wort-based beverages with addition of mint in concentrations of 0.5 – 1.5 g/dm^3, fermented by probiotic lactic acid bacteria strains *Lactobacillus casei* ssp. *paracasei* PX3 and *Lactobacillus casei* ssp. *rhamnosus* LBRC11 was developed. The influence of the mint dose on the phenolic content, the antioxidant capacity and the fermentation dynamics was studied. Both lactobacilli strains grew well in wort with mint at each experimental mint dose and accumulated a sufficient number of viable cells necessary to achieve the desired probiotic effect. Mint addition led to an increase in the biological value of the beverages because the total phenolic content rose up to 22 % and the antioxidant capacity rose up to 53 %. The increase in the mint dose by 0.5 g/dm^3 was not sufficient to achieve a significant increase in the phenolics content, but it was enough to cause an increase in the antioxidant capacity. It can be hypothesized that the antioxidant capacity of the developed beverages was not determined only by the phenolic compounds. The maximum phenolic content and antioxidant capacity were observed at the maximum mint amount (1.5 g/dm^3).

Keywords: wort, mint, lactic acid fermentation, antioxidant capacity, phenolic content

INTRODUCTION

Functional foods contain not only the necessary nutrients for the body but also ingredients that contribute to improving the individual's health. One of the approaches to the development of functional foods involves the addition of components that are not present in most foods and which are not nutrients, but have proven a positive effect on the body, such as plant extracts, probiotic microorganisms, etc. [1].

In the last decade, articles related to the effect of fermentation on phenolic compounds and antioxidant activity in plant-based foods, the interaction between food phenolics and lactic acid bacteria, the antioxidant activity of lactic acid bacteria, cereal-based functional fermented food and drinks have appeared [2-5].

Fermentation of cereals such as oats, corn and wheat under the action of probiotic microorganisms is useful because it provides better digestibility of food. [6]. There is evidence in the literature that the survival of lactic acid bacteria is significantly improved by the use of barley malt as a basis for beverage production [7, 8].

Peppermint (*Menta piperita* L.) is a very widespread plant, which has a pleasant aroma and contains pharmacologically active ingredients. There are many articles related to the antioxidant and antimicrobial activities, the phenolic content and the essential oil of peppermint [9-11].

Wort from barley malt has a high nutritional value and is a suitable medium not only for alcoholic fermentation. However, scarce information is still available on the lactic acid fermentation of wort in the presence of herbs [2].

The two strains used in the present research have proven probiotic properties - antimicrobial activity against pathogenic and saprophytic microorganisms, antibiotic resistance and resistance to different concentrations of bile salts and different pH values, possibility for conduction of industrial processes (fermentation, freeze-drying) with accumulation and maintaining of a high concentration of viable cells, thus being very suitable for the development and production of functional beverages (unpublished data). Some members of the current scientific team behind the present research have worked on the examination of some probiotic properties of other representatives of the species *Lactobacillus casei*, proving their

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probiotic potential like antibiotic resistance and resistance to different concentrations of bile salts and different pH values [12], possibility for conduction of industrial processes (fermentation, freeze-drying) with accumulation and maintaining of a high concentration of viable cells [13].

The aim of the present study was to investigate the influence of mint (*Menta piperita* L.) addition on the fermentation dynamics, the antioxidant activity and the phenolic content of lactic acid beverages based on wort. The aim of the study will be achieved by studying the fermentation process of wort-based beverages with mint fermented by two *Lactobacillus casei* strains (*Lactobacillus casei* ssp. *rhamnosus* LBRC11 and *Lactobacillus casei* ssp. *paracasei* PX3).

EXPERIMENTAL

Materials

Pilsner malt from WEYERMANN, Germany was used. The malt was ground on a Corona hand mill. Dried milled peppermint (*Menta piperita* L.), bought from a local market was used. The used probiotic lactic acid bacteria (LAB) strains were *Lactobacillus casei* ssp. *rhamnosus* LBRC11 and *Lactobacillus casei* ssp. *paracasei* PX3. They are part of the culture collection at the department of Microbiology at the University of Food Technologies, Plovdiv.

Folin-Ciocalteu's phenol reagent and gallic acid were supplied by Merck - Germany. Trolox, DPPH radical and TPTZ were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals used were of analytical grade and were obtained from local producers.

Experimental design

Wort was obtained from barley malt by an infusion method using a brewery equipment Braumaister 20L of Speidel Germany. The wort was boiled for 10 min after mashing and lautering. The wort extract was 11.4°P. Immediately after boiling, peppermint was added to the wort at three different concentrations - 0.5 g/dm^3; 1.0 g/dm^3 and 1.5 g/dm^3. The mint was left to macerate for 30 min. After that the wort was filtered through cloth filter, cooled and inoculated with LAB. The wort without mint was inoculated too and served as a control. The quantity of the LAB suspension was 5 % of the wort volume. The initial concentration of viable LAB cells in all beverage variants was 10^7 CFU/cm^3. The fermentation was carried out at 15°C for 7 days.

Analyses

Sample preparation. Before determination of the total phenolic content and the antioxidant capacity, the samples were frozen and stored in a freezer until the day of analysis. After the samples were defrosted, they were treated with methanol, filtered to remove formed precipitates and diluted properly with methanol.

Wort extract and pH. Wort extract was determined using a pycnometer according to Analytica – EBC, Method 8.3 (2005). The pH value was determined using Sartorius PB-11 pH meter according to Analytica – EBC, Method 8.17 (2005) [14].

DPPH radical scavenging activity. The ability of the samples to scavenge DPPH radicals was determined by the modified method of Brand-Williams *et al.* (1995) [15]. Freshly prepared 6×10^{-5} M solution of DPPH was mixed with the diluted sample at a ratio of 9:1 (v/v). After 30 min incubation at room temperature, the absorption was measured at 517 nm. The DPPH radical scavenging activity was defined as a function of the Trolox concentration having equivalent antioxidant activity and was expressed as μmol TE per dm³ sample.

Ferric-reducing antioxidant power assay (FRAP). The FRAP assay was carried out according to the procedure of Benzie and Strain (1999) [16]. The suitably diluted sample (150 μl) was mixed with 2850 μl of FRAP reagent, allowed to react for 4 min at room temperature and the absorbance was measured at 593 nm. The results were expressed as μmol TE per dm³sample.

*Total phenolic compounds (TPC). T*he FC reagent according to ISO 14502-1 [17] and a version of the Glories method modified by Mazza *et al.* (1999) were used [18]. The diluted sample was mixed with 0.1% HCl solution in 96% ethanol and 2% solution of HCl at a ratio of 1:1:18.2 (v/v/v). After incubation for 15 min at room temperature the absorbance at 280 nm was measured. The calibration curves were prepared using gallic acid and the total phenolic content was expressed as g gallic acid equivalent (GAE) per dm³.

Determination of the concentration of viable lactobacilli cells. Appropriate ten-fold dilutions of the samples taken from the relevant fermentation stage were prepared. Amounts of 0.1 cm^3 from the last three dilutions were used for spread-plating on LAPTg10-agar. The inoculated Petri dishes were incubated at 37±1°C for 48-72 hours until the appearance of countable single colonies. The number of single colonies was used to estimate the

concentration of viable lactobacilli cells in the test sample.

The concentration of viable cells and the pH value were determined during the fermentation process – immediately after inoculation, on the first, fourth and seventh day of fermentation.

Statistical analysis. The obtained data were analyzed statistically using MS-Excel 2010 software. The analyses were performed in triplicate. The results were expressed as mean value ± standard deviation. One-way ANOVA and Scheffe's multiple range test at $p<0.05$ as described by Donchev *et al.* [19] were used.

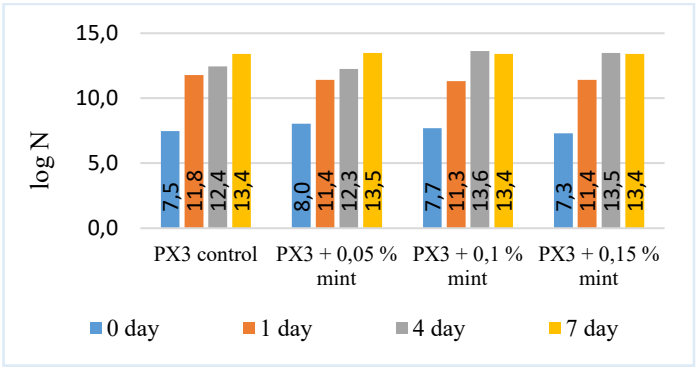
RESULTS AND DISCUSSION

In an initial series of experiments, both strains of lactic acid bacteria grew well on wort. Beverages with very good sweet/sour balance, with pronounced freshness and harmony were obtained as a result of fermentation. They were evaluated by an organoleptic panel as the most preferred among all beverages obtained by fermentation with different strains of different species of the genus *Lactobacillus* (unpublished data).

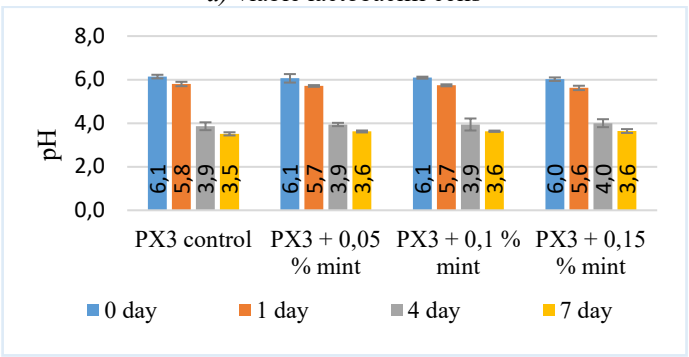
Development of a new type of wort-based beverages with mint addition fermented by L. paracasei PX3 or L. rhamnosus LBRC11

During the first day, an exponential phase in the growth of *L. paracasei* PX3 in all variants was observed, as the concentration of added mint did not significantly affect the reproduction and growth of the strain (Fig. 1). After the first day, the concentration of viable cells increased significantly and reached 10^{11}CFU/cm^3, at an initial concentration after inoculation of 10^7 CFU/cm^3 (the change was 4logN). Then their concentration continued to increase, and at the end of the process it reached 10^{13} CFU/cm^3. At higher mint doses (1.0 g/dm^3 and 1.5 g/dm^3), the entry of the cells into the stationary phase was after the fourth day of fermentation.

The pH of the medium in all beverage variants with *L. paracasei* PX3 gradually decreased from the beginning of the fermentation process (Fig. 1).



a) viable lactobacilli cells

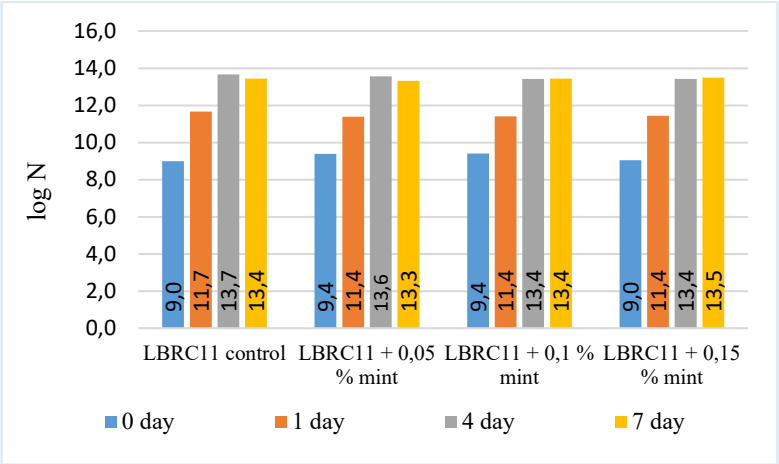


	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
0 day	a	a	a	a
1 day	b	b,c	b,c	c
4 day	d	d	d	d
7 day	e	e	e	e

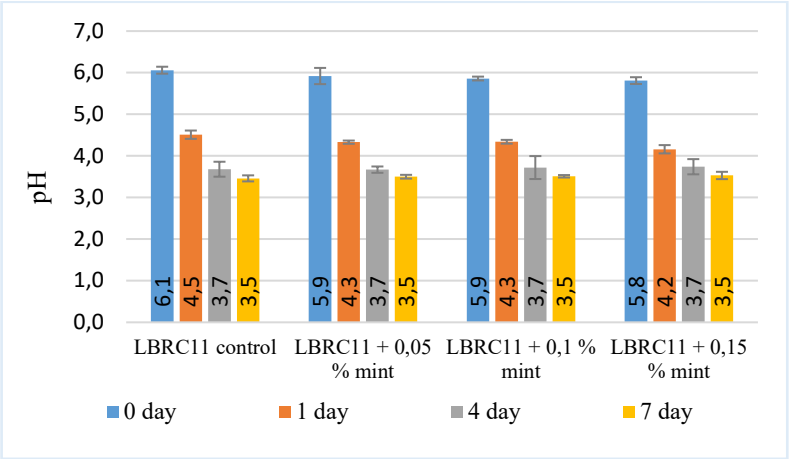
The same letter for a given sample means no significant differences (95% confidence level)

b) pH value

Fig. 1. Changes in the concentration of viable lactobacilli cells and pH value during lactic acid fermentation with *Lactobacillus casei* ssp. *paracasei* PX3



a) viable lactobacilli cells



	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
0 day	a	a	a	a
1 day	b	b, c	b, c	c
4 day	d	d	d	d
7 day	d	d	d	d

The same letter for a given sample means no significant differences (95% confidence level)

b) pH value

Fig. 2. Changes in the concentration of viable lactobacilli cells and pH value during lactic acid fermentation with *Lactobacillus casei* ssp. *rhamnosus* LBRC11

Due to the buffering nature of wort on the first day, the change in pH was within 0.3-0.4 units, then by the end of the fermentation process the total decrease in pH was 1.6-1.9 units. As a result of the fermentation process, the pH decreased from 6.0-6.1 to 3.5-3.6 for the different wort/mint combinations.

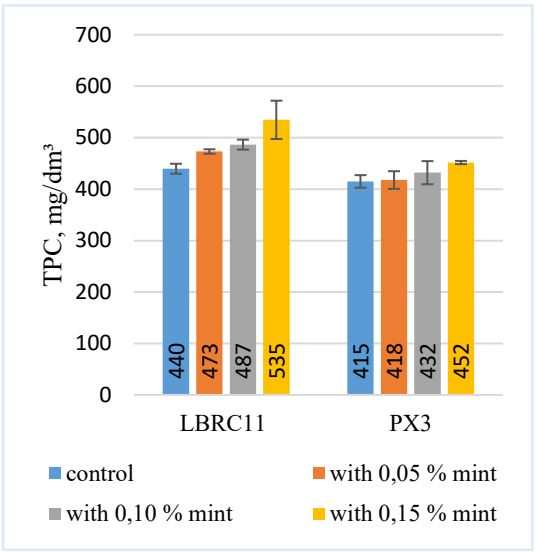
Within 24 hours of the start of the fermentation by *L. rhamnosus* LBRC11 the concentration of viable cells increased by two logarithmic units, and in the next 96 hours by two more logarithmic units (Fig. 2). After the fourth day, there was an entry into the stationary phase in all experimental variants. *L. rhamnosus* LBRC11 accumulated a high concentration of viable cells, regardless of the experimental combination of wort and mint.

Regardless of the specific percentage of added mint, the variants fermented by *L. rhamnosus* LBRC11 retained lower pH values (Fig. 2) compared to the beverages obtained with *L. paracasei* PX3. The pH value of the beverages with *L. rhamnosus* LBRC11 on the day of inoculation ranged from 5.8 to 6.1. Within 24 hours, the pH dropped sharply in all experimental variants. The pH values on the fourth and the seventh day of fermentation were comparable. In all variants of beverages fermented by *L. rhamnosus* LBRC11 a pH = 3.5 was reached at the end of the fermentation process. Both strains grew well in a medium of wort with mint added in amounts between 0.5 g/dm^3 and 1.5 g/dm^3 accumulating a sufficient amount of viable cells necessary to achieve the

desired probiotic effect upon consumption of the beverages. In the first 24 hours, both strains accumulated a significant amount of viable cells. In the fermentation with the strain *L. rhamnosus* Oly a slightly lower concentration of biomass was reported during the process [2], but in the end the biomass amount was equalized with that in the fermentation with the strains *L. rhamnosus* LBRC11 and *L. paracasei* PX3. In *L. rhamnosus* LBRC11 and *L. paracasei* PX3 the stationary phase was reached after the fourth day, while in the strain *L. rhamnosus* Oly biomass accumulation continued until the end of the process [2]. The fermentation process proceeded normally, organic acids, mainly lactic acid, accumulated and the pH of the medium decreased. *L. rhamnosus* Oly accumulated the least amount of acids and lowered the acidity of the medium slightly [2], while the greatest amount of acids accumulated in the fermentation with *L. rhamnosus* LBRC11.

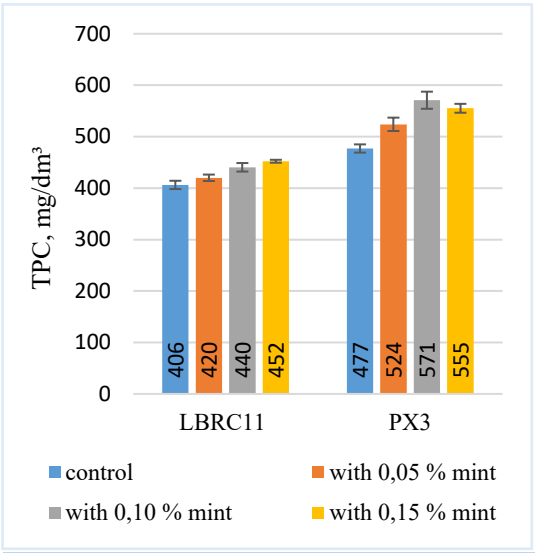
Phenolic content and total antioxidant capacity of beverages based on wort and mint, fermented by L. paracasei PX3 and L. rhamnosus LBRC11.

The total phenolic content determined by the direct spectral method (Glories method) in the beverages with mint and fermented by *L. paracasei* PX3 (Fig. 3) was between 10% and 20% higher than that of the beverage without mint. The increasing of the mint dose between 0.5 g/dm^3 and 1.0 g/dm^3 by a step of 0.5 g/dm^3 led to a significant increase in the phenolic content. The beverages with mint added in amounts of 1.0 g/dm^3 and 1.5 g/dm^3 had almost equal phenolic content. No significant change in the results obtained with the FC reagent (Fig. 3) when mint was added at doses up to 1.0 g/dm^3 was observed. Only the beverage with 1.5 g/dm^3 mint had 9% higher phenolic content compared to the control.



	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
LBRC 11	a	a, b	b	
PX3	c	c	c, d	d

a) colorimetric method with Folin-Ciocalteu reagent



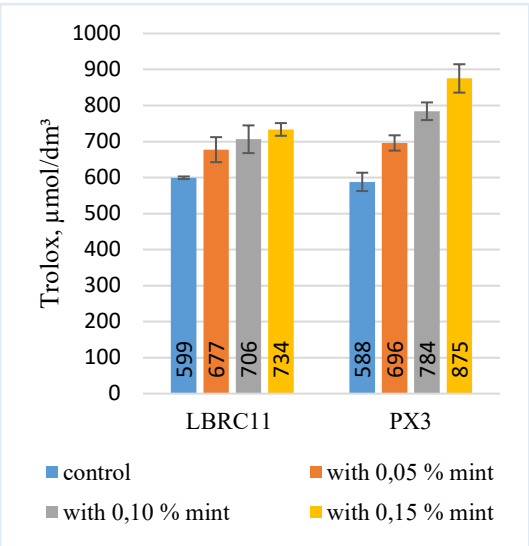
	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
LBRC 11		a	a, b	b
PX3			c	c

b) Glories method

The same letter for a given sample means no significant differences (95% confidence level)

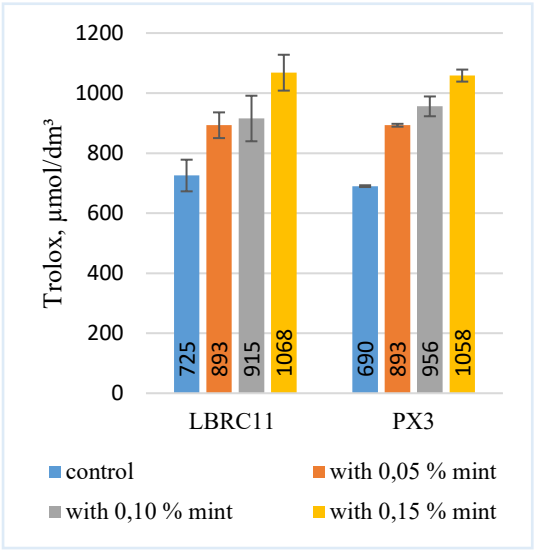
Fig. 3. Changes in the total phenolic content (TPC) during fermentation by *Lactobacillus casei* ssp. *rhamnosus* LBRC11 and *Lactobacillus casei* ssp. *paracasei* PX3

The mint addition and the increase in the mint dose in the beverages fermented by *L. paracasei* PX3, resulted in an increase of the beverage ferric-reducing antioxidant power (FRAP) between 29% and 53% compared to the control sample (Fig. 4). A similar trend was observed in the DPPH radical scavenging activity of the beverages (Fig. 4). It was by 18 – 49 % higher compared to the control and it increased with the increase in the amount of the mint.



	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
LBRC 11		a	a,b	b
PX3				

a) DPPH assay



	control	with 0,05% mint	with 0,10% mint	with 0,15% mint
LBRC 11		a	a	
PX3				

b) FRAP assay

The same letter for a given sample means no significant differences (95% confidence level)

Fig. 4. Changes in the total antioxidant capacity during fermentation by *Lactobacillus casei* ssp. *rhamnosus* LBRC11 and *Lactobacillus casei* ssp. *paracasei* PX3

The mint addition to the beverages fermented with *L. rhamnosus* LBRC11 was accompanied by an increase in the total phenolic content determined by the Glories method (Fig. 3). Compared to the control, it was by 8 - 11% higher. It turned out that the increase in the mint amount by 0.5 g/dm^3 did not lead to significant changes in the total phenolic content, but the increase in the mint dose by 1.0 g/dm^3 was sufficient for an increase in the total phenolics. A similar trend was observed in the results for the total phenolic content determined by the FC reagent (Fig. 3). The phenolic concentration was by 8 - 22% higher compared to the control sample and it was necessary to increase the mint dose by 1 g/dm^3 to have a significant increase in the total phenolic content.

The DPPH radical scavenging activity of the beverages with mint (Fig. 4) was by 13 – 22% greater than that of the control. More significant increase in the radical scavenging activity was observed when the mint dose rose from 0.5 g/dm^3 to 1.5 g/dm^3. A similar trend was established in the results from the FRAP assay (Fig. 4). The ferric-reducing antioxidant power of the beverages with mint was by 23 – 47 % higher compared to the control. The beverages with 0.5 g/dm^3 and 1.0 g/dm^3 mint did not show any significant difference in the FRAP value.

The obtained results for the total phenolic content determined by both methods showed that the addition of mint to wort led to an improvement in the biological value of the lactic acid beverages due to the higher content of phenolic compounds.

The increase in the total phenolic content was not proportional to the increasing values of the mint dose. In most cases, the increase in the mint amount by 0.5 g/dm^3 did not lead to a significant difference in the total phenolic content. A significant increase was achieved by increasing the mint dose by 1.0 g/dm^3. The observed trend was probably due to the different processes that occur when adding mint to wort and because of the influence of the lactic acid fermentation. On one side, various substances were extracted from mint, including phenolic compounds. On the other side, extracted compounds fall into a protein-rich environment, which is a prerequisite for the formation of insoluble protein-phenolic complexes [20]. In addition, it was possible that the cell wall of *L. rhamnosus* LBRC11 and *L. paracasei* PX3 adsorbed some of the phenolic compounds [21]. Therefore, in some cases, the increase in the mint dose by 0.5 g/dm^3 was not sufficient to cause an increase in the total phenolic content of the beverages despite the higher mint amount for extraction.

The differences in the total phenolic content determined by FC and Glories methods can be explained by the features of both methods. On one side, the Glories method determines the amount of oxidized and non-oxidized phenolics, unlike the FC method which determines the amount of only non-oxidized phenolics. On the other side, the FC reagent is not specific only to the phenolic compounds. It can react with many other substances such as ascorbic acid, monosaccharides, aromatic amines, etc. [22].

It is well known that phenolic compounds possess DPPH radical scavenging activity and ferric-reducing antioxidant power. However, they are not the only compounds in wort and mint that have such abilities. Wort is rich in peptides, which also have antioxidant properties [23]. Peppermint essential oil has iron-reducing and DPPH scavenging activities as well [10, 11]. The addition of mint to the boiling wort was a prerequisite for extracting some of the mint essential oil into the resulting beverages. The presence of compounds with antioxidant activity other than phenolics can be explained by the observed differences between the changes in the total phenolic content on one side and the values of the FRAP and the DPPH assays on the other side. The use of different strains to carry out lactic acid fermentation had certain impact as well. The increase in the mint dose by 0.5 g/dm^3 led to a significant growth in the antioxidant capacity of the beverages fermented by *L. paracasei* PX3. The same trend was observed in the fermentation by *L. rhamnosus* Oly [2], but the antioxidant capacity of the beverages fermented by *L. rhamnosus* LBRC11, especially the value determined by the DPPH assay, was not affected significantly when the mint dose rose by 0.5 g/dm^3.

CONCLUSION

Wort-based beverages with mint added in concentrations between 0.05 g/dm^3 and 0.15 g/dm^3, fermented under the action of two probiotic strains of the species *Lactobacilus casei* - *Lactobacillus casei* ssp. *rhamnosus* LBRC11 and *Lactobacillus casei* ssp. *paracasei* PX3 were prepared. It was found that both strains of lactic acid bacteria grew well in wort medium with added mint in all experimental concentrations, accumulating a sufficient number of viable probiotic lactobacilli cells necessary for the manifestation of the probiotic effect upon consumption. Their growth was strain-specific. Fermentation with both strains proceeded normally with accumulation of organic acids and lowering of the pH of the medium from 6.1 to 3.5.

The addition of mint (*Menta piperita* L.) and the increase in its dosage between 0.5 g/dm^3 and 1.5 g/dm^3 led to higher antioxidant capacity and total phenolic content in the obtained lactic acid beverages based on wort. Thus, the biological value of the finished beverages rose. Probably, the degree of the antioxidant capacity and the phenolic content changes were affected by different factors like extraction processes, formation of water-insoluble complexes, variety of the lactobacilli strains and adsorption of some phenolics onto the lactobacilli cell walls. Additional comprehensive investigations are needed to clarify the role of multiple mechanisms and the interactive influence of different factors on the observed changes in the phenolic content and the antioxidant capacity.

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REFERENCES

1. P. Sharma, S. K. Tomar, P. Goswami, V. Sangwan, R. Singh, *Food Research International*, **57**, 176 (2014).
2. G. Achmedova, M. Kaneva, R. Denkova, D. Teneva, Z. Denkova, I. Ignatov, P. Nedyalkov, *Scientific Works of the University of Food Technology*, **64** (1), 7 (2017).
3. S. Kumari, P.Guleria, N. Dangi, *International Journal of Enhanced Research in Science, Technology & Engineering* ISSN: 2319-7463, **4** (10), 134 (2015).
4. O. Livinska, O. Ivaschenko, I. Garamasheva, N. Kovalenko, *AIMS Microbiology*, **2(4)**, 447 (2016). https://doi.org/10.3934/microbiol.2016.4.447
5. H. Rodriguez, J.A. Curiel, J.M. Landete, B. de las Rivas, F.L. de Felipe, C. Gomez-Cordoves, J. M. Mancheno, R. Munoz, *International Journal of Food Microbiology,* **132**, 79 (2009).
6. C. R. Soccol, L. P. S. Vandenberghe, M. R. Spier, A. B. P Medeiros, C. T. Yamaguishi, J. De Dea Lindner, et al., *Food Technology and Biotechnology*, **48**, 413 (2010).
7. D. Charalampopoulos, S. S. Pandiella, C. Webb, *International Journal of Food Microbiology*, **82**, 133 (2003).
8. H. M. Patel, S. S. Pandiella, R. H. Wang, C. Webb, *Food Microbiology*, **21**, 83 (2004).
9. L. Georgieva, D. Mihaylova, *International Food Research Journal,* **22**(1), 240 (2015).
10. E. Schmidt, S. Bail, G. Buchbauer, I. Stoilova, T. Atanasova, A. Stoyanova, A. Krastanov, L. Jirovetz, *Nat. Prod. Commun.,* **4,** 1107 (2009). https://doi.org/10.1177/1934578X0900400819 ;
11. Zh. Wu, B. Tan, Y. Liu, J. Dunn, P. Martorell Guerola, M. Tortajada, Zh. Cao, P. Ji, *Molecules,* **24(15),** 2825(2019). .https://doi.org/10.3390/molecules24152825.

12. R. Denkova, L. Georgieva, Z. Denkova, S. Ilieva, V. Yanakieva, *Food and Environmental Safety*, XI, II, 5 (2012). ISSN 2068 – 6609
13. R. Denkova**,** B. Goranov, Z. Denkova, G. Kostov, I. Georgieva, *Scientific Works of the University of Ruse "Angel Kanchev*" - 2014, **53**, 10.2, 9 (2015). ISSN: 1311-3321.
14. Analytica-EBC, European Brewery Convention, Analysis Committee, Fachverlag Hans Care, Nürnberg, 2005.
15. W. Brand-Williams, M. E. Cuvelier, C. Berset, *LWT Food Science and Technology* **28**, 25 (1995).
16. F. F. Benzie, J. J. Strain, *Methods in Enzymology,* **299**, 15 (1999).
17. ISO14502-1 Part 1: Content of total polyphenols in tea — Colorimetric method using Folin Ciocalteu reagent.
18. G. Mazza, L. Fukumoto, P. Delaquis, B. Girard, B. Ewert, *J. Agric. Food Chem*., **47**, 4009 (1999).
19. D. Donchev, M. Dilcheva, V. Kinova, *Encyclopedia of Food Chemistry,* 2007, *p*. 550. https://doi.org/10.1016/B978-0-08-100596-5.21487-X.
20. M. P. Chapot-Chartier, S. Kulakauskas, *Microb. Cell Fact*., **13**, 9 (2014). https://doi.org/10.1186/1475-2859-13-S1-S9.
21. J. Dai, R. J. Mumper, *Molecules*, **15,** 7313 (2010). http://dx.doi.org/10.3390/molecules15107313.
22. T.-B. Zou, T.-P. He, H.-B. Li, H.-W. Tang, E.-Q. Xia, *Molecules,* **21**(1), 72 (2016). doi: 10.3390/molecules21010072.

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Essential oil composition and mineral element content *of Salvia Aethiopis* L. from Turkey

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Mediterranean sage (*Salvia aethiopis* L.) is a common species in Turkey belonging to the family Lamiaceae. It could be used for various medicinal purposes as an ointment, for treating multiple diseases, *etc*. Mediterranean sage was collected from the campus area of Yozgat Bozok University Erdoğan Akdağ in June 2017. The essential oil was isolated from leaves by hydrodistillation and analyzed utilizing GC and GC/MS. Among the 32 components identified in this essential oil (yield 0.15%), caryophyllene oxide (30.11%), aromadendrene (18.03%), α-humulene epoxide (5.78%), (E)-*α*-bisabolene (5.72%), and isoaromadendrene epoxide (4.30%) were found to be the major constituents. Concentrations of potentially harmful heavy metals (Al, Cd, Co, Cr, Ni) were below the respective toxic levels.

Keywords: *Salvia aethiopis*, essential oil, heavy metal

INTRODUCTION

Turkey showes a large distribution of various species (9753 species), 3035 of which are endemic. It is stated that there are 3649 (31.82%) endemic and 11707 taxon. Turkey is an important center of the Lamiaceae family [1]. Since ancient times, this family has had many medicinal plants, mainly in the Mediterranean basin [2]. It is a vast family with 200 genera and 3200 species. In Turkey, 45 genera, 546 species, and 731 taxa are spreading [3]. *Thymbra, Thymus, Origanum, Satureja, Mentha, Teucrium, Ballota, Stachys, Salvia, Ajuga, Prunella, Melissa, Lamium, Sideritis,* and *Marrubium* are among the known important genera of this family. Most of the members of the Lamiaceae family are rich in essential oils and other secondary metabolites. The family species are of great importance in areas such as medicine, pharmacy, food, cosmetics, and perfumery.

The leaves, flowers, and stems of *Salvia* species, which have been critical medicinal plants since ancient times, are used differently. Sage leaves are evaluated in various ways for the treatment of many ailments (soothing, pain relieving, antiperspirant, expectorant, cold and anti-cough, relieving muscle pain, lowering high blood pressure and disinfectant, *etc*.) in folk medicine [4, 5]. The consumption of some *Salvia* species as herbal tea and spices is widespread. *Salvia* species appeal to various consumer groups (food industry, pharmaceutical, chemical industry, *etc.*). Some of them are rich in macro- and microminerals. Herbal products can make an important contribution in meeting the daily mineral needs of the human body. Therefore, knowing the nutritional content and pharmacological functions of herbal teas it is crucial in determining the dosage of use [6].

Mediterranean sage (*S. aethiopis* L.) is a biennial or perennial herb that is 25-60 cm high. This species grows on steppes, igneous and limestone slopes, fallow fields, and roadside banks. Its leaves are simple, mostly basal, and ovate-elliptic to oblong [7].

The essential oil yield and composition have been studied in several countries such as Turkey [8-10], Serbia [11-13], Romania [14], Bulgaria [15], Spain [16], and Iran [17-20]. The eessential oil yield was determined between 0.2 and 0.5%, and the main components were sesquiterpene hydrocarbons β-caryophyllene (7.3-27.5%), α-copaene (9.15-22.4%), germacrene D (5.0-29.4%), bicyclogermacrene (9.3-41.5%), γ-muurolene (10.3%), β-cubebene (7.0-9.7%), δ-cadinene (5.0-8.7%), β-elemene (9.9%), and oxygen-containing sesquiterpene - spathulenole (8.3%). The variation in the yield and composition of the oil was probably due to the soil and geographical features of the plant's regions, even within a same country. The essential oil has been shown to have antimicrobial activity [14].

Mining, urban or industrial solid, gas and liquid wastes, pesticide and artificial fertilizer use, paint

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industry, and car exhaust gases cause excessive amounts of heavy metals to be released into nature. This heavy metal stress caused by environmental pollutants limits plant growth and reduces product yield and quality [21]. Some micronutrients such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and cobalt (Co) are necessary for plant growth and development. However, some heavy metals such as arsenic (As), mercury (Hg), cadmium (Cd), lead (Pb), and chromium (Cr) are elements that are not necessary for plant development [22]. The presence of heavy metals, with or without micronutrients, in the atmosphere, water, and soil, in a concentration above a certain level, causes severe problems for all living organisms [23]. This situation causes the accumulation of toxic elements in various vegetative organs of plants. The vast majority of medicinal, aromatic plants are collected from the natural area and exposed to multiple pollution factors.

The present study aimed to determine the yield and chemical composition of *Salvia aethiopis* L. essential oil obtained from Eastern Turkey characterized with continental climate, as well as the content of mineral elements, including heavy metals, to find opportunities for plant application as an additive in various food or cosmetic products.

MATERIAL AND METHODS

Yozgat Bozok University Erdogan Akdag Campus is located within the B5 square considering the grid system. In previous studies, it has been reported that there are 22 taxa, including 14 genera of Lamiaceae family, in the campus area of Yozgat Bozok University [27]. The aerial parts of the plant were collected in the complete flowering stage on 12 June 2017 from Yozgat Bozok University Campus (39º46′41.72′′; N 34º47′56.01′′; E, altitude 1346 m). Then dried and stored in a cool (20 °C), dark cabinet until further processed and analyzed. Species identification of the collected samples was made in the Department of Botany in Yozgat Bozok University. The flowers of the plants have white color (Picture 1), unlike species growing in other countries, registered that were colored pink and lilac.

Determination of essential oil content

After harvesting, the leaves were separated by hand, then dried to constant humidity in the dark, without direct sunlight, at a temperature of about 20 °C. The dried leaves were stored in double paper bags in a dark and dry place. Before analysis, they were crushed to a size of 1 cm, after which the moisture was determined [28].

Essential oil contents of dried leaves samples were determined by water distillation with a Clevenger type apparatus. An average of 50 g of dried plant sample was distilled in 700 mL of water for 3 h. Essential oil contents (%, v/w) of the samples were calculated on dry matter. The essential oils obtained were placed in dark colored bottles and stored in a refrigerator at +4 ºC until analysis.

Picture 1. *S. aethiopis* L. plant (The image is copyrighted).

Gas chromatography-mass spectroscopy analysis (GC / MS). The chemical composition of the essential oil was determined using standard methods [28].

Determination of mineral mater and heavy metal content. The mineral composition was determined using the iCAP-Qc ICP-MS spectrometer (Thermo Scientific) [28].

Each analysis in the study was carried out in triplicate and the results were presented as the mean value (± SD) from the three measurements. Significant differences ($p < 0.05$) were assessed by applying statistical tools such as ANOVA and Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Essential oil rate (%) and essential oil components (%)

The essential oil rate obtained from the samples collected from the natural environment during the full flowering period was recorded as 0.15%. The essential oil yield was lower than that reported in the literature for plants' aerial parts, for example 0.27% [8], 0.5% [12], 1.6% [18], 0.23% [20],

which could be explained by the growing conditions of the raw material.

The essential oil is a light yellow liquid with a characteristic -odor.

GC-MS results of essential oil obtained from the aerial parts of *S. aethiopis* species are given in Table 1.

Thirty-two components representing 91.18% of the essential oil obtained from the floral aerial parts of the *S. aethiopis* species were identified and caryophyllene oxide (30.11%) and aromadendrene (18.03%) were found as the main components. The major components were determined as follows: *α*-humulene epoxide (5.78%), (E)-*α*-bisabolene (5.72%), and isoaromadendrene epoxide (4.30%). The results indicated that the essential oil obtained from the aerial plants of *S. aethiopis* from Turkey was also a caryophyllene oxide and aromadendrene chemotype.

The classification of the identified compounds, based on functional groups, is summarized in Fig. 1.

According to the data analysis, oxygenated sesquiterpenes predominated in the oil. The comparative analysis of the main components in the essential oils of species growing in other countries of the Balkan Peninsula showed that the essential oil with a high content of sesquiterpene hydrocarbons was close to the data for Bulgaria [15], Serbia [12, 13], Romania [14], as in previous studies for Turkey [8-10].

Table 1. Chemical composition of *S. aethiopis* essential oil obtained from aerial parts.

Component	RI_{cal}*	Content, %
Copaene	1375	1.17 ± 0.01
β-Bourbonene	1382	1.19 ± 0.01
Caryophyllene	1384	0.16 ± 0.0
trans-β-Caryophyllene	1419	0.38 ± 0.0
Aromadendrene	1439	18.03 ± 0.16
Dihydro-β-ionol	1475	0.21 ± 0.0
Germacrene D	1477	1.87 ± 0.01
β-Selinene	1483	0.64 ± 0.0
Cubedol	1484	0.47 ± 0.0
Bicyclogermacrene	1491	1.14 ± 0.01
α-Cadinene	1509	2.22 ± 0.02
endo-1-Bourbonanol	1514	0.84 ± 0.0
(E)-*α*-Bisabolene	1536	5.72 ± 0.04
Germacrene B	1542	1.32 ± 0.01
Endo-1,5-Epoxysalvial-4(14)ene	1561	0.36 ± 0.0
Germacrene D-4-ol	1571	0.57 ± 0.0
Caryophyllene oxide	1575	30.11 ± 0.28
Viridiflorol	1584	0.79 ± 0.0
α-Humulene epoxide	1600	5.78 ± 0.05
Hexadecane	1601	0.44 ± 0.0
delta-Cadinol	1644	0.28 ± 0.0
α-Cadinol	1651	2.20 ± 0.02
Valerenol	1668	0.19 ± 0.0
Shyobunol	1682	1.92 ± 0.01
Isoaromadendrene epoxide	1739	4.30 ± 0.04
2-Pentadecanone, 6,10,14-trimethyl ester	1838	2.84 ± 0.02
Sclareoloxide	1991	2.51 ± 0.02
Phytol	2074	1.13 ± 0.01
7-Hexyldocosane	2192	0.16 ± 0.0
Tetracosane	2381	0.41 ± 0.0
Pentacosane	2494	1.27 ±0.01
Nonacosane	3119	0.56 ± 0.0

* RI_{calc} - Kovats Retention Index, calculated by authors; TIC - Total Ion Current.

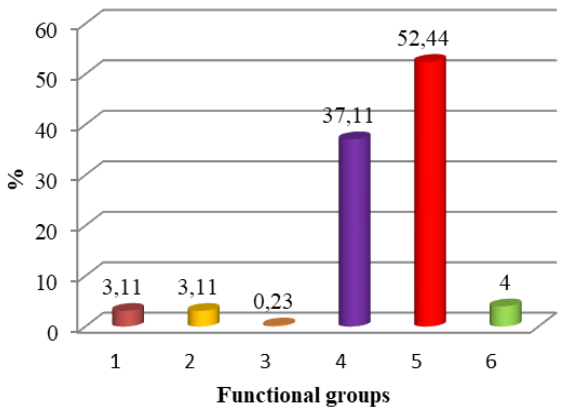

Fig. 1. Classification of the identified compounds, %. 1 - aliphatic hydrocarbons, 2 – oxygenated aliphatics, 3 - oxygenated monoterpenes, 4 - sesquiterpene hydrocarbons, 5 - oxygenated sesquiterpenes, 6 - diterpenes

According to [8] the main components of the *S. aethiopis* oil were germacrene D (29.0%), α-copaene (19.8%), β-cubebene+β-elemene (9.9%), bicyclogermacrene (9.3%), δ-cadinene (8.7%), and β-caryophyllene (7.3%). The variations observed in this study and those reported by other authors could be related to climatic alterations. Furthermore, differences in the amounts of some of the components obtained in this study could be explained by the variations of the soil and rainfed conditions between the countries.

Compared with the previous study results, the essential oil rates and composition of taxa were evaluated in the research scope; some results appeared to be similar or compatible but with very different results. These differences may be caused by genetic and environmental factors (temperature, precipitation, exposure time and intensity, altitude, orientation, drought, salinity, structure of soil and condition of plant nutrients, *etc*.) [9]. Plant parts used in the analysis, plant life cycle, and collecting time can also be effective [29]. Plants utilize secondary metabolites in order to counteract biotic and abiotic stressors. However, larger amounts of secondary metabolites are often synthesized when plants get stressed. For example, essential oil rates for medicinal and aromatic plants grown in hot and dry regions are higher than those for plants growing in cool and rainy areas [29]. In this context, it could be said that environmental factors significantly impact the differentiation of the findings obtained from our research from other studies.

Mineral matter and heavy metal content (ppm)

Some macro- (Ca, K, P and S) and microelement (Fe, Mn, Zn, Cu, B and Na) and heavy metal (Al, Cd, Co, Cr and Ni) contents of *S. aethiopis* species were determined and the results are presented in Figure 2.

In our study, Ca content was 270.15 ppm, K content was 468.80 ppm, and S content was 12.29 ppm. The proximity of agricultural areas to the cities, deterioration of urbanization, domestic and industrial wastes, heavy metals, or flue fumes emitted by motor vehicles cause undesirable results in all species. On the other hand, heavy metals from high doses of the food chain have a negative effect on human health. Medicine and aromatic plants collected from the natural environment or under cultural conditions may be exposed to various pollution factors. This situation causes the accumulation of toxic elements in the vegetative organs of plants, especially in their leaves. Toxic elements such as lead, cadmium, aluminum, and mercury can cause serious health problems. Therefore, the mineral content of medicinal and aromatic plants is an essential indicator of overall human health. Al, Cd, Co, Cr, and Ni contents of plants were evaluated as heavy metals. Among these, the amount of Al was determined as highest, which could be due to the level of precipitation, climatic factors and soil conditions (its pH) [21, 22]. The analytical determination of heavy metals in medicinal and aromatic plants is among the most important quality parameters in determining the plants' purity, safety, and effectiveness [30]. The limit values for Cd, Cr, and Ni, determined by the WHO/FDA (World Health Organization / American Food and Drug Administration), are 0.3, 0.02, and 1.63 ppm, respectively [31]. It was found that the detected quantities of the three metals were below the maximum allowed by the WHO/FDA.

CONCLUSION

Based on the results for the analysis of the chemical content of *S. aethiopis* essential oil, it was revealed that its main components were caryophyllene oxide, aromadendrene, *α*-humulene epoxide, *trans*-*α*-bisabolene, and isoaromadendrene epoxide. No heavy metals did occur in any significant concentrations in the plant samples. Therefore, *S. aethiopis* essential oil could be considered a prime potential additive or ingredient for application in the food and cosmetic industries, and will be a subject to further research.

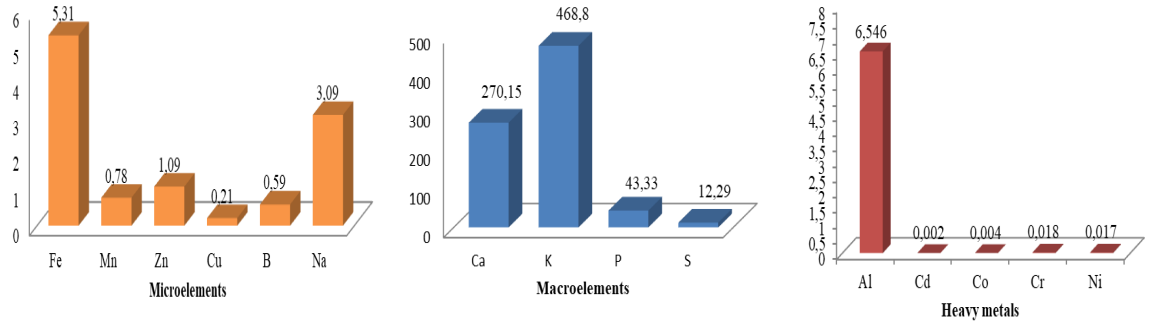

Fig. 2. Mineral matter and heavy metal content (ppm) of *S. aethiopis* L. taxa

REFERENCES

1. A. Güner, S. Aslan, T. Ekim, M. Vural, M. T. Babaç, List of Plants of Turkey (Vineary Plants), İstanbul: Nezahat Gokyigit Botanical Garden and Flora Research Association Publication, 2012 [in Turkish].
2. Z. Yaniv, A. Dafni, D. Palcvitch, Labiateae as medicinal plants in Israel, in: N. Maorgaris, A. Koedam, D. Vokou (eds.), Aromatic Plants: basic and applied aspects, Martinus Nijhoff Publishers, The Hague, 1982.
3. A. Kahraman, M. Dogan, *Acta Bot. Croat.*, **69**, 47 (2010).
4. A. Sarı, M. Kürşat, S. Civelek, *J. Drug Metabolism and Toxicology*, **3**, 1 (2012).
5. M. Bayram, M. Yılar, E. Özgöz, I. Kadıoğlu, *Nevşehir Science and Technology Journal TARGİD Special Issue,* 325 (2016).
6. L. Queralt, L., M. Ovejero, M. L. Carvalho, A. F. Marques, J. M. Llabres, *X-Ray Spectrometry*, **34**, 213 (2005).
7. P. H. Davis, Flora of Turkey and the East Aegean Islands, **5**, University Press: Edinburgh, 1982.
8. M. Güllüce, H. Ozer, O. Bariş, D. Daferera, F. Sahin, M. Polissiou, *Turkish J. Biology*, **30**, 231 (2006).
9. E. Bağcı, A. Koçak, *Fırat Univ J Engineering Science*, **19**, 435 (2007).
10. H. B. Karayel, PhD Thesis, Çanakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences, Çanakkale, Turkey, 2019.
11. J. C. Chalchat, M. S. Gorunovic, S. D. Petrovic, Z. A. Maksimovic, *JEOR,* **13**, 416 (1999).
12. A. Veličković, M. Ristić, D. Veličković, S. Ilić, N. Mitić, *J Serbian Chemical Society*, **68**, 435 (2001).
13. D. Velickovic, M. Ristic, A. Velickovic, *JEOR*, **15**, 346 (2003).
14. M. Coisin, I. Burzo, M. Stefan, E. Rosenhech, M. Zamfirache, *Analele Stiintifice ale Universitatii Äl. I. Cuza, Iasi s. II a. Biologie vegetala*, **58**, 51 (2012).
15. S. Damyanova, A. Stoyanova, P. Bozov, T. Atanasova, O. Gubenia, *Ukrainian Food Journal*, **5**,697 (2016).
16. E. Torres, A. Velasco-Negueruela, M. Perez-Alonso, M. Pinilla, *JEOR,* 9, 27 (1997).
17. A. Rustaiyan, S. Masoudi, A. Monfared, H. Komeilizadeh, *FFJ*, **14**, 276 (1999).
18. K. Morteza-Semnani, M. Saeedi, S. Changizi, M. Vosoughi, *JEOBP*, **8**, 330 (2005).
19. M. Tajbakhsh, A. Rineh, M. Khalilzadeh, B. Eslami, *JEOR*, **19**, 569 (2007).
20. F. Salimpour, A. Mazooji, A. Darzikolaei, *J Medicinal Plants Research*, **5**, 1795 (2011).
21. A. K. Shanker, C. Cervantes, H. Loza-Tavera, S. Avudainayagam, *Environment International,* **31**, 739 (2005).
22. D. Niess, *Appl. Microbiology and Biotechnology*, **51**, 730 (1999).
23. M. Benavides, S. Gallego, M. Tomaro, *Brazilian J. Plant Physiology*, **17**, 21 (2005).
24. O. Yılmaz, Bozok University Ersoğan Akdağ Campus Flora. Master Thesis, Bozok University, Institute of Science and Technology, Yozgat, 2016.
25. B. Cosge Senkal, T. Uskutoglu, C. Cesur, V. Özavci, H. Dogan, *Turk. J. Agric. For.* 43, 395 (2019).
26. H. Baydar, Science and Technology of Medicinal and Aromatic Plants (4th Edition). Isparta: Süleyman Demirel University Publications, 2013.
27. I. Baranowska, K. Srogi, A. Włochowicz, K. Szczepanik, *Polish J. Environmental Studies*, **11**, 467 (2002).
28. M. I. Lone, S. Saleem, T. Mahmood, K. Saifullah, G. Hussain, *Int. J. Agr. Biology,* **5**, 533 (2003).

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Bulgarian contribution to the investigation of natural aromatic products: a brief retrospective review

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Bulgaria is one of the countries with established traditions in the production and processing of aromatic (essential oil-bearing) and medicinal plants, as well as in the investigation of the respective plant-derived products. These natural aromatic products (such as essential oils, concretes, absolutes, and other extraction concentrates) combine valuable olfactory profiles and beneficial biological and pharmacological activities, which substantiate their use in perfumery, cosmetics, aromatherapy, medicine, food, and many other areas. Therefore, this work presents a brief retrospective review (1900 - 2019) of the achievements of Bulgarian researchers in the study of the chemical composition, the antimicrobial, antioxidant and other activities of traditional and contemporary natural aromatic products, obtained from different - indigenous or uncommon to the country - medicinal and aromatic plants. The review does not claim to be exhaustive in terms of Bulgarian research achievements in the indicated timespan, neither has the objective to cover world research on aromatic and medicinal plants.

Keywords: medicinal and aromatic plants, natural aromatic products, biological activity, Bulgaria

INTRODUCTION

Bulgaria has the prerequisites – abundant flora, favorable climate, and socio-economical potential – to produce valuable, high quality aromatic products derived from aromatic, medicinal and edible plants. Indeed, such production has been a fact for more than 350 years. The country is recognized worldwide for the aromatic products obtained from plants like Damask rose (*Rosa damascena* Mill.), lavender (*Lavandula angustifolia* Mill.), peppermint (*Mentha piperita* L.), bigroot geranium (*Geranium macrorrhizum* L.), thyme (*Thymus vulgaris* L.), chamomile (*Matricaria chamomilla* L.), *etc*. [1].

It should be outlined that one and the same plant material can be processed by different methods, thus obtaining aromatic products, which have substantially different chemical composition, olfactory and other properties, and subsequently – different use potential. Processing of rose flowers by distillation, for example, yields rose oil and rose water, by traditional solvent extraction – rose concrete and rose absolute, and these four natural aromatic products are diametrically different in their composition and properties. Common (traditional, standardized) aromatic products are obtained from essential oil-bearing plants by well-established techniques, and they generally include: essential oils – obtained by hydro- or steam distillation or cold pressing of citrus rinds; concretes – by extraction with nonpolar organic solvents, which are subsequently removed; absolutes – by re-extraction of concrete with polar solvents, and some others (resinoids, oleoresins, tinctures). Thus, the qualitative and quantitative composition of the aromatic products, and respectively – their physical, chemical, and biological properties, depend greatly on the isolation procedure. These aromatic products are mixtures of many substances originating from the metabolism of terpenes, phenylpropanoids, amino and fatty acids, and other phytochemicals [1].

This work presents a brief review of the achievements of Bulgarian authors (1900 – 2019) in the investigation of the biological properties of natural aromatic products and their application in cosmetics. The review highlights the leading research trends of the respective time period, with a focus on the following aspects of the characteristics of traditional and new aromatic products:

i) chemical composition and identification of fragrance compounds;
ii) antimicrobial and antioxidant activity;
iii) potential application in different cosmetic products.

The authors of this brief review would like to emphasize that its objective has not been to cover the extensive international research on the products obtained from the plants referenced herein or to neglect the numerous publications of authors around the world, but rather to present the less popular research of Bulgarian scientists to the wider

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international audience. We are truly convinced that despite the relatively small market share of the aromatic products produced in Bulgaria on a worldwide basis, there has been an undoubted contribution of Bulgarian scientists to the development of aromatic plants research not only in the past, as is the case with the famous Bulgarian rose, but also in present days. This brief review, too, does not claim to be exhaustive or to provide historical coverage of all achievements of Bulgarian researchers on natural aromatic products investigation in the indicated timespan; due to the limited volume of the article, many authors have probably remained unmentioned, for which we sincerely apologize.

1900 – 1940. The period of launching the first studies on certain indices of Bulgarian essential oils

- The oleaginous (oil-yielding) rose (*R. damascena*) has been introduced in Bulgaria as early as the 14th century, while the industrial processing of rose sets its beginning in the middle of 17th century. The emblematic production and the reputation of the Bulgarian rose essential oil awarded the country the brand "Bulgaria – the land of the oleaginous rose".

- Lavender (*L. angustifolia*) was introduced in 1903 and its cultivation for industrial purposes began soon after that, with the first few tonnes of lavender oil being produced in 1925 year.

- The first investigations about the physical and chemical properties of rose oils [2-7] and of peppermint and hyssop oils [8] are conducted.

1940 – 1970. The beginning of systematic research of Bulgarian essential oils

The period marks the beginning of systematic research focused on different aspects of the process of obtaining the traditional essential oils and on understanding their biological activities. A series of studies have been published, which reveal:

- The impact of different technological factors on the yield and the quality of Bulgarian rose oil [9-13] and lavender oil [14];

- The chemical composition of the aromatic products from rose [15-31], lavender [32, 33], pine [34-37], tobacco [38-45].

- The first data from pharmacological and clinical studies about the therapeutical properties of rose oil [46, 47].

- The antimicrobial activities of rose oil [48-57], of thyme, pelargonium, ajowan, and coriander oils [56-58], and of pine oil [59].

- Based on their wide variety of bioactivities (antibacterial, antimycotic, anti-inflammatory, antioxidant, and other), infusions and essential oils from medicinal and aromatic plants have been used in different cosmetic products - unguents [55] and hair-care products [59, 60]. Rose products (water, oil and concrete) have been used in a number of formulations for oral hygiene and stomatology [53].

1970 – 1990. Continued studies on traditional and new aromatic plants; beginning of studies on waste recovery

During this period, continue the studies on the technology, the chemical composition and the biological activities of the traditional rose and lavender essential oils, as well as those of new aromatic plants. The investigations on the use potential of plant waste begin.

- The influence of different technological factors on the yield and quality of different essential oils has been followed by a number of researchers: rose essential oil [61-63], lavender oil [64].

- The chemical composition of the traditional aromatic products has been investigated by different authors – products from rose [65-75], from lavender [76].

- The antimicrobial activities of rose oil and its major compound geraniol [77-79], of lavender, savory, peppermint, basil, and dill oils [80-83] have been established.

- Investigations of the waste remaining after the distillation and extraction of rose oil [84-89] and from rose and lavender waste after extraction and distillation [90] have been conducted.

- The use of extracts and essential oils as functional ingredients in different cosmetic products has been investigated – skin-care creams [91-95] and products for oral hygiene [96-98].

1990 – 2019. A period of disturbance and transition (1990-2000) and a new period (2000-2019) of marked revival in the research of traditional and novel aromatic products

The period between 1990 and 2000 reflected the post-communist disturbance and transition processes in the political, social and economic life in Bulgaria. For more than a decade after the political changes in the country, there has been a period of relative stagnation in the clinical studies of the traditional for Bulgaria aromatic products. The new period of marked revival in the research of traditional and novel aromatic products and their biological activities paralleled the beginning of the 21st century.

- The chemical composition and antimicrobial activities of essential oils obtained from a wide range of aromatic and medicinal plants, either traditional or nontraditional for Bulgaria, have been identified in numerous studies [99-127].

- In the years after 2000, many Bulgarian authors and their collaborating foreign partners have revealed the antimicrobial activity of the individual main components of different aromatic products against various microorganisms. It is determined that Bulgarian essential oils (rose, lavender, peppermint, basil, sage, oregano, *etc.*), demonstrate high antimicrobial activity against various pathogenic and spoilage microorganisms, belonging to the groups of Gram-possitive and Gram-negative bacteria, dimorph yeasts and fungi. It is of great importance that Bulgarian essential oils from these essential oil-bearing plants achieve antimicrobial activity against fluconazole resistant strains of *Candida albicans* and non-albicans Candida strains and Methicillin-resistant *Staphylococcus* strains. The obtained results expand the possibilities for application of Bulgarian essential oils not only as flavouring or perfuming agents in food industry and cosmetics, but even as preparations with wide pharmacological importance [128-149].

- Along with these studies, the antioxidant activity of various traditional or non-traditional for Bulgaria aromatic products, as well as that of their main individual components, has been investigated [150-155].

- A distinct trend in the period has been the continuation of investigations on the chemical composition and the biological activities of different waste materials, with the aim of their valorization and prospective use; for example, those on rose waste [156-174].

- Essential oils and extracted aromatic products from traditional and new medicinal and aromatic plants have been incorporated in different skin-care products; creams [148] and lotions [126].

- A new step in the development of aromatic products in Bulgaria has been the introduction of low-temperature extraction with liquefied gases. The main achievements of Bulgarian researchers in the field are discussed in more detail below.

Low-temperature extraction with liquefied gases

In many countries nowadays, essential oil bearing plants are processed by extraction with liquefied gases (CO_2, air, freons, and others). The produced extracts are considered harmless, and therefore they can be widely used in food and flavour industry, cosmetics and medicine. The use of liquefied gases overcomes some of the major drawbacks of installations working with volatile polar and nonpolar solvents [175-177].

There are currently six extracting installations working with liquified gases in Bulgaria. Three of them are operating with supercritical CO_2, situated respectively in the town of Dimitrovgrad, in the village of Mirkovo, Sofia region, and in the Bulgarian Academy of Sciences, Sofia. The other three installations (two industrial - in the town of Pavel Banya, Kazanlik region and in the city of Plovdiv, and one laboratory - at the University of Food Technologies in Plovdiv) are operating with 1,1,1,2-tetrafluoroethane (known also as hydrofluorocarbon-134a, HFC-134a, and freon 134a). These installations have been used in the last decades for processing different essential oil bearing plants – rose, lavender, peppermint, chamomile, sage, hyssop, juniper, and many others.

Extraction with CO_2

- In the last 15 years the installation in the town of Dimitrovgrad, Bulgaria has been set to obtain extracts from different essential oil bearing and medicinal plants, representing both industrially processed plant materials (e.g. fennel, coriander) and non-traditional for the country, experimentally processed ones (e.g. linden, hop). The specified plant materials have been processed for the purpose of providing an alternative of the respective imported CO_2 extracts in different fields of application, e.g. as ingredients in cosmetic preparations, in foods and drinks, and others. A summary of the essential oil bearing and medicinal plants processed on the installation is presented in a chronological order in Table 1, as well as data about the yield and the chemical composition (main components, above 3%) of the extracts.

- The research carried out by Bulgarian scientists confirms that CO_2 is a suitable extractant for processing plant materials that are rich in thermolabile components and therefore are not processed through high-temperature distillation, like linden.

- All of the obtained CO_2 extracts revealed antimicrobial activity against different test-microrganisms [184], and according to the data, their antimicrobial activity was similar to that of the respective essential oils.

- To the best of our knowledge, no data have been published about the inclusion of the obtained CO_2 extracts in cosmetic or other products, since they were considered economically not competitive to the imported ones.

Table 1. Yield and composition of extracts obtained with CO_2

Plants	Main components (above 3%), %	Yield, %	Ref.
Coriander	linalool[1] (71.6), α-pinene (6.3)	0.8	[178]
Fennel	anetole (72.3), fenchone (11.3)	1.4	[182]
Hop	*α*-acids (36.1), *β*-acids (19.5)	9.7	[179]
Ginger	*α*-zingiberene (36.9), *β*-phellandrene (15.3), *β*-bisabolene (8.8), (*E,E*)-*α*-farnesene (7.0), *α*-curcumene (6.6), camphene (3.2)	3.7	[180]
Black pepper	*β*-caryophyllene (43.9), limonene (17.1), *δ*-3-carene (11.8), *α*-phellandrene (4.4), *allo*-aromadendrene (3.3), myrcene (3.1)	2.2	[180]
Linden	benzaldehyde (14.2), phenylethyl alcohol (9.8), 2-methylpropanal (7.1), n-hexanal (4.8), *trans*-pentenal (4.3), ethylalcohol (3.9), nonanal (3.2)	1.0	[181]

[1]Allergic fragrance in accordance with [183]

Extraction with tetrafluoroethane (TFE)

- To the best of our knowledge, the research group from Bulgaria has been among the pioneers, who started to use and systematically studied 1,1,1,2-tetrafluoroethane as a low-temperature extractant of aromatic and medicinal plants.

- For the last 15 years, more than 25 different essential oil bearing and medicinal plants have been processed in the laboratory installation situated at the University of Food Technologies in Plovdiv, Bulgaria. The set of processed plant materials includes species traditional for the country [185-187], as well as non-typical for the essential oil industry aromatic plants and spices [126, 180, 188-208]. The former group has been analysed mainly with the view of comparison and providing of alternatives to the established products, and the latter group – with the purpose of diversifying the range of available aromatic products.

- Data about the yield, chemical composition (components in concentration higher than 3%) and antioxidant (DDPH radical scavenging) activity of some of the obtained extracts are listed chronologically in Table 2.

Table 2. Yield, composition and antioxidant activity of extracts with 1,1,1,2-tetrafluoroethane

Plants	Main components (above 3%), %	Yield, %	IC_{50}, mg/mL[1]	Ref.
Black pepper	*β*-caryophyllene (56.8), limonene[2] (18.9), terpinolene (3.9), *p*-cymene (3.1)	1.6	-[3]	[180]
Ginger	*α*-zingiberene (42.1), *β*-phellandrene (14.2), *β*-bisabolene (11.3), *cis*-menth-2-en-1-ol (9.8), *α*-curcumene (8.5), (E,E)-*α*-farnesene (5.9)	1.1	-	[180]
Anise	anethole (92.9-93.4)	2.7	8.3	[188]
Linden	phenylethyl alcohol (24.7), phenylethyl benzoate (5.7), nonadecane (10.8), heneicosane (9.1), tricosane (8.0), phytol (6.0), citronellol[2] (3.2)	0.1	-	[189]
Hyacinth	benzyl benzoate[2] (23.0), cinnamyl alcohol[2] (12.8), benzyl acetate (6.4), *p*-cumin-aldehyde (5.4), *p*-cymene (5.0), *α*-terpinene (4.7)	0.2	-	[190]
Lilac	*from violet lilac*: lilac alcohol (13.6), 1,2,4-trimethoxy benzene (9.0), squalene (8.0), tricosane	1.2	-	[191]

	(7.3), methyl eugenol (6.6), geranil geraniol (5.7), elemicin (4.4), lilac alcohol D (4.2), palmitic acid (3.7), 8-hydroxy linalool (3.5)			
	from white lilac: lilac alcohol (8.6), 1,2,4-trimethoxy benzene (10.1), squalene (6.9), tricosane (6.1), geranil geraniol (4.0), lilac alcohol D (7.0), palmitic acid (4.8), *β*-pinene (3.8)	1.0	-	[191]
Coriander	linalool[2] (71.4), *γ*-terpinene (7.8), *α*-pinene (5.9), geranyl acetate (4.0)	60-80[4]	17.7	[192]
Cinnamon	cinnamal[2] (77.3), coumarin[2] (4.3)	50-60[4]	0.4	[193]
Fennel	anetole (68.3), fenchone (17.7)	25-30[4]	6.0	[194]
Cardamom	terpinyl acetate (36.8), 1,8-cineole (29.2), linalyl acetate (5.2), sabinene (3.9), linalool[2] (3.1)	30-35[4]	63.3	[195]
Cumin	*γ*-terpinene (23.5), *p*-cymene (22.2), cumin aldehyde (18.8), *β*-pinene (15.3), *p*-mentha-1,4-dien-7-al (5.9), linalool[2] (4.3), *α*-pinene (3.1)	3.8-4.0	6.4	[154]
Pimento	methyl eugenol (55.9), myrcene (14.7), eugenol[2] (9.6), *β*-caryophyllene (4.4)	100-130[4]	< 0.1	[197]
Clove	eugenol[2] (69.7), eugenyl acetate (13.4), *β*-caryophyllene (9.3)	8-12[4]	<0.1	[198]
Lilium	1-hexacosanol (21.1), 1-octacosanol (20.4), n-dotriacontane (6.6), n-triacontane (5.9), n-nonanal (5.5), n-nonadecane (5.0), n-pentacosane (4.7), n-tricosane (4.5), n-heptacosane (4.1)	0.1	-	[196]
Dill (herba)	D-carvon (53.1), limonene[2] (37.1)	0.7	1.2	[199]
Sage	1,8-cineole (25.2), *β*-caryophyllene (7.5), *cis*-thujone (6.7), *α*-humulene (6.1), *trans*-thujone (5.4), *β*-pinene (5.4), camphor (4.8), *allo*-aromadendrene (4.6), borneol (3.7), *α*-pinene (3.6), bornyl acetate (3.6)	100-150[4]	-	[200]
Sumac	limonene[2] (23.7), *α*-pinene (15.7), caryophyllene oxide (10.7), 2-hexenal (4.0), *p*-cymene-8-ol (3.6), farnesyl	0.2	-	[201]

	acetone (3.4), *β*-pinene (3.2)			
Marigold	*α*-bisabolon oxide (8.3), *β*-farnesene (8.1), *α*-bisabolon oxide A (7.3), *α*-pinene (6.8), *γ*-cadinene (5.5), *p*-cymene (5.4), *γ*-terpinene (5.1), *δ*-cadinene (5.1)	2.7-3.0	10.3	[202]
Tobacco	*from Burley tobacco*: nicotine (72.9), phytol acetate (7.8)	0.5	-	[126, 203]
	from Virginia flue-cured tobacco: nicotine (60.9), phytol acetate (20.8)	0.3	-	[126, 203]
	from Oriental tobacco: phytol acetate (9.0), acetic acid (8.7), nicotine (6.2), norambreino lide (5.7)	0.7	-	[126]
Pepper-mint	menthone (30.5), menthol (21.1), menthyl acetate (9.9), *iso*-menthone (4.4), *cis*-sabinene hydrate (3.8), 1,8-cineole (3.4), pulegone (3.0)	50-70[4]	0.4	[187]
Savory	thymol (73.9)	0.8-1.0	0.6	[204]
Basil	estragol (47.0), linalool[2] (10.6), methyl eugenol (8.9), 1,8-cineole (3.7), *β*-bisabolene (3.0)	350-400[4]	0.3	[205]
Thyme	geraniol[2]	100[4]	0.2	[206]
	(20.5), thymol (14.9), carvacrol (10.3), geranyl acetate (7.4), linalool[2] (6.6), germacrene D (5.3), *p*-cymene (5.3), *β*-caryophyllene (3.4)			
Juniper	*α*-pinene (32.0), myrcene (21.1), germacrene D (8.3), sabinene (5.2), limonene[2] (4.0).	60-75[4]	43.2	[207]
Oregano	carvacrol (70.1), *p*-cymene (11.8), p-cymene-2,5-dione (3.8)	60-80[4]	-	[208]
Rose	phenylethyl alcohol (59.1), citronellol[2] (12.3)	1.2-1.5	-	[185]
Lavender	linalool[2] (32.5), linalyl acetate (23.0), borneol (5.1), *cis*-linalol oxide (4.5), (E)-*β*-farnesene (4.1), lavandulol (4.2), *β*-caryophyllene (3.3)	1.2	-	[186]

[1]DPPH radical scavenging activity; [2]Allergic fragrance in accordance with [183]; [3]Not determined; [4]Yield is given as kg raw/kg extract.

In order to evaluate the quality of the new products, a comparative analysis regarding selected representatives of traditional and non-traditional for Bulgaria plants has been carried out against current ISO standards that set up the variation limits of main or characteristic components of the produced essential oils. As a result of this comparison, it is established that the extracts are in accordance with the standards, except for the following specific features:

i) The TFE extract from lavender (clonal lavender, Bulgaria) [188] is with higher content of

linalool (32.5%) and lavandulol (4.2%), and lower – of linalyl acetate (23.0%) than ISO 3515:2002 lavender oil (22.0-34.0% linalool, min. 0.3% lavandulol, and 30.0-42.0% linalyl acetate, respectively);

ii) The TFE extract obtained from peppermint [187] is with higher content of menthone (30.5%) and menthyl acetate (9.9%), and lower content of menthol (21.1%) than ISO 856:2006 peppermint oil (13.0-28.0% menthone; 2.0-8.0% menthyl acetate, and 32.0-49.0% menthol, respectively);

iii) The TFE extract from sage [200] is with lower content of *α*-thujone (5.7%) and camphene (0.8%), and higher – of 1,8-cineole (25.2%) and bornyl acetate (3.6%) than ISO 9909:1997 sage oil (18.4% *α*-thujone, 1.5-7.0% camphene, 5.5-13% 1,8-cineole, and max. 2.5% bornyl acetate, respectively);

iv) According to the qualitative and quantitative content of the major constituents the produced TFE rose extract [185] is significantly different from the essential oil according to ISO 9842:2003, but is almost equal to the rose absolute [1];

v) The TFE extracts obtained from some typical spice species, such as anise [188], cardamom [195], coriander [192], *etc.* match up the major constituents pointed out as characteristic of the respective standardized oils, ISO 3475:2000, ISO 4733:2004, and ISO 3516:1997.

Thus, it can be summarized that 1,1,1,2-tetrafluoroethane extraction has been found appropriate for a set of essential oil bearing and medicinal plant materials, which either:

i) Contain thermolabile components and are not processed by distillation, like lilac, hyacinth, and linden;

ii) Require low pH of distillation water, like tobacco, or

iii) Represent fruits containing both essential and glyceride oil, for example – black pepper, coriander, cumin, anise, fennel, and dill.

- Some of the new aromatic products – CO_2 and TFE extracts, comprise representatives of the list of potentially allergenic substances defined by the Cosmetics Regulation No 1223:2009 [183] (Table 1 and Table 2). Although being main ingredients of the extracts, their concentration does not exceed the range specified by the respective ISO standards.

- The comparative analysis of data justifies the conclusion that the extracts obtained with CO_2 and TFE from the same plant material (as in the case of coriander, fennel, ginger, black pepper, and linden) possess different, characteristic chemical composition, which could be attributed to the selectivity of the solvent applied [175-177].

- The analysis of the references cited above reveals that the antimicrobial activity of respective TFE extracts has been determined, against different pathogenic and spoilage bacteria and yeasts, from clinical and food isolates and reference strains. All studied extracts demonstrated antimicrobial activity against the tested microorganisms, although lower in comparison with the common preservatives and antioxidants used in cosmetics. Extracts characterized with the highest content of thymol (thyme and savory) and of eugenol (clove and pimento), identified as the major constituents responsible for the antimicrobial activity of the extracts, have demonstrated the highest activity. The antimicrobial activity of the extracts was similar to that of the respective essential oils, as established by the Bulgarian authors cited earlier, as well as by the authors referenced in [209].

- Approximately half of the obtained TFE extracts have been analysed with respect to their antioxidant properties (mainly by determining the DPPH radical-scavenging activity). The extracts from clove, pimento, thyme, basil, peppermint, cinnamon, and savory have demonstrated higher antioxidant activity than the rest of the extracts, which is attributed to the higher concentration of eugenol [109, 198], thymol [153, 204], cinnamal [110, 193], menthol [142, 187], and other active components [115, 197, 205, 206].

- The produced TFE extracts from black pepper, cardamom, cumin, and coriander have been successfully included as antioxidants in different meat products [210-212] and as preservatives in food emulsions [213].

- The produced TFE extracts from lavender have been successfully included as antimicrobial agents in cosmetic emulsions of the type oil/water [188].

Therefore, it can be summarized that – given the pronounced antimicrobial and antioxidant properties of the extracts – they can be incorporated in various cosmetic preparations (creams, gels, *etc.*) as successful substitutes of chemical preservatives and antioxidants, or as substitutes of perfume in natural and biocosmetic products. As these extracts do not yield to the respective essential oils by biological activity (antimicrobial, antioxidant, *etc.*), they can easily be considered as promising bioactive components for incorporation in cosmetic products with anti-aging, anti-inflammatory, and other beneficial effects.

CONCLUSIONS

The review of the scientific publications by Bulgarian authors in the period 1900-2019 demonstrates that there have been established

historic and sound traditions in the obtaining of aromatic products from a plethora of indigenous and uncommon to the country essential oil bearing plants. The same is true for the study of their chemical composition and biological activity with a view to their application in cosmetic and other products.

For the last 15 years, there has been a clear trend of expanding the range of available plant-derived ingredients by introducing innovative methods for obtaining aromatic products worldwide, and Bulgarian scientists have contributed to that trend.

The scientific achievements in the study of CO_2, tetrafluoroethane and other extracts today are far from being comprehensive, but undoubtedly open doors to their industrial obtaining and to the production of innovative bio and natural cosmetics in Bulgaria.

REFERENCES

1. E. Georgiev, A. Stoyanova, A Guide for the Specialist in the Aromatic Industry. UFT Acad. Publ. House, Plovdiv, 2006.
2. H. Kyulyumov, M. Stefanova, *Journal of the Agricultural Experimental Institutes in Bulgaria,* **1-2**, 70 (1921).
3. M. Stefanova, *Chem. Ind.,* **1**, 86 (1927).
4. M. Stefanova, *Chem. Ind.,* **7**, 1 (1936).
5. G. Karaivanov, *Chem. Ind.*, **1**, 74 (1933).
6. G. Karaivanov, *Annual Bulg. Eng. Arch. Soc.,* **6**, 94 (1934).
7. G. Karaivanov, *Annual Bulg. Eng. Arch. Soc.,* 155 (1936).
8. M. Stefanova, *Chem. Ind.,* **9**, 417 (1936).
9. D. Ivanoff, C. Ivanoff, I. Ognyanov, *Compt. Rend. Acad. Bulg. Sci.,* **6**, 25 (1953).
10. V. Staikov, G. Zolotovich, *Compt. Rend. Acad. Bulg. Sci.,* **8,** 41 (1955).
11. G. Zolotovich, PhD Thesis, Kazanluk, 1964.
12. I. Irinchev, P. Delev, *Bull. Dev. Essent. Oil Industry*, 16 (1965).
13. I. Irinchev, *Bull. Dev. Essent. Oil Industry*, 32 (1967).
14. I. Irinchev, K. Georgiev, Lavender and Lavender Oil Production in Bulgaria, CCU, Sofia, 1959.
15. D. Ivanoff, C. Ivanoff, B. Stoianova-Ivanova, *Compt. Rend. Acad. Bulg. Sci.*, **6**, 29 (1953).
16. D. Ivanoff, C. Ivanoff, I. Ognyanov, *Compt. Rend. Acad. Bulg. Sci,* **6**, 25 (1953).
17. D. Ivanoff, C. Ivanoff, B. Stoianova-Ivanova, *Compt. Rend. Acad. Bulg. Sci.*, **7**, 17 (1954).
18. D. Ivanoff, C. Ivanoff, N. Marecoff, I. Ognyanoff, *Compt. Rend. Acad. Bulg. Sci.*, **7**, 25 (1954).
19. D. Ivanoff, N. Marekoff, *Compt. Rend. Acad. Bulg. Sci.,* **7**, 13 (1954).
20. D. Ivanoff, B. Stoianova-Ivanova, V. Ivanov, *Compt. Rend. Acad. Bulg. Sci.*, **8**, 33 (1955).
21. D. Ivanoff, N. Marecoff, S. Pavlova, L. Ivanova, *Ind. Parfum*., **2**, 105 (1956).
22. B. Stoianova-Ivanova, D. Ivanoff, *Ind. Parfum.,* **12**, 402 (1957).
23. B. Stoianova-Ivanova, M. Kouzmanova, *Compt. Rend. Acad. Bulg. Sci.*, **17**, 941 (1964).
24. B. Stoianova-Ivanova, P. Hadjieva, *Compt. Rend. Acad. Bulg. Sci.,* **18**, 145 (1965).
25. D. Ivanoff, *Medico-biological Information*, 21 (1969).
26. B. Stoianova-Ivanova, E. Ignatova, *Rivista Italiana EPPOS*, **51**, 375 (1969).
27. B. Stoianova-Ivanova, K. Mladenova, *Rivista Italiana EPPOS*, **50**, 72 (1968).
28. B. Stoianova-Ivanova, D. Nicolova, V. Tzvetkova, *Compt. Rend. Acad. Bulg. Sci.*, **18**, 141 (1965).
29. B. Stoianova-Ivanova, P. Hadjieva, K. Mladenova, *Rivista Italiana EPPOS*, **49**, 726 (1967).
30. B. Stoianova-Ivanova, P. Hadjieva, S. Popov, *Phytochemistry*, **8**, 1549 (1969).
31. B. Stoianova-Ivanova, D. Dinkov, M. Hrivnac, *Rivista Italiana EPPOS*, **51**, 315 (1969).
32. R. Vlahov, I. Ognyanov, D. Ivanov, G. Ganev, *Compt. Rend. Acad. Bulg. Sci.*, **20**, 1303 (1967).
33. G. Zolotovitz, *Compt. Rend. Acad. Bulg. Sci.*, **8**, 9 (1955).
34. G. Bojkov, H. Sivriev, G. Ganchev, I. Lazarov, *Bull. Dev. Essent. Oil Industry*, 68 (1964).
35. G. Bojkov, H. Sivriev, *Bull. Dev. Essent. Oil Industry*, 66, (1964).
36. G. Bojkov, H. Sivriev, *Bull. Dev. Essent. Oil Industry*, 54 (1964).
37. I. Ognyanov, R. Vlahov, E. Tzankova, *Rivista Italiana EPPOS,* **47**, 243 (1965).
38. I. Tzonev, V. Chenikov, *Bulg. Tobacco*, **7-8**, 37 (1962).
39. N. Ivanov, I. Ognyanov, *Compt. Rend. Acad. Bulg. Sci.*, **16**, 293 (1963).
40. N. Ivanov, I. Ognyanov, *Compt. Rend. Acad. Bulg. Sci.*, **18**, 1123 (1965).
41. I. Ognyanov, N. Ivanov, *Compt. Rend. Acad. Bulg. Sci.*, **18**, 1015 (1965).
42. I. Ognyanov, N. Ivanov, *Perf. Essent. Oil Record.*, **58**, 161 (1967).
43. I. Ognyanov, N. Ivanov, *SPC* (London), **40**, 631 (1967).
44. I. Ognyanov, N. Ivanov, *Compt. Rend. Acad. Bulg. Sci.*, **21**, 1287 (1968).
45. N. Ivanov, I. Ognyanov, *Compt. Rend. Acad. Bulg. Sci.*, **22**, 743 (1969).
46. P. Nikolov, *Bulgarian Clinic*, 201 (1941).
47. G. Hristov, *Compt. Rend. Acad. Bulg. Sci.*, **15**, 321 (1962).
48. G. Hristov, S. Klimentova, *Compt. Rend. Acad. Bulg. Sci.*, **6**, 17 (1953).
49. G. Hristov, *Compt. Rend. Acad. Bulg. Sci.*, **15**, 431 (1962).

50. L. Babinov, M. Simov, E. Georgieva, *Internal Diseases*, **4**, 73 (1965).
51. G. Hristov, *Compt. Rend. Acad. Bulg. Sci.*, **19**, 969 (1966).
52. G. Hristov, *Bull. Dev. Essent. Oil Industry*, 34 (1969).
53. G. Hristov, S. Klimentova, Proc. 3rd Int. Congr. Essent. Oils, Plovdiv, May 26-27, 1964, p. 301.
54. G. Hristov, K. Silianovska, *Bull. Dev. Essent. Oil Industry*, 44 (1969).
55. G. Hristov, *Medico-biological Information*, 16 (1969).
56. P. Pejchev, G. Apostolov, B. Topalov, N. Nikiforov, *Archives de Únion Medicale Balkanique (Bukarest)*, **2**, 653 (1964).
57. P. Pejchev, G. Apostolov, V. Topalov, N. Nikiforov, *Folia Medica*, **9**, 11 (1967).
58. P. Pejchev, Y. Dimitrova, *Bull. Dev. Essent. Oil Industry*, 58 (1969).
59. P. Toleva, *Sci. Works HIFFI*, **12**, 137 (1965).
60. I. Tolev, P. Toleva, *Sci. Works Medical Academy - Plovdiv*, **20**, 1 (1968).
61. D. Dimitrov, Y. Kamilev, *Sci. Works HIFFI,* **19**, 95 (1972).
62. A. Balinova-Tzvetkova, Y. Michailova, R. Atanasova, *Compt. Rend. Chim. Acad.*, **7**, 45 (1974).
63. V. Staikov, A. Balinova-Tzvetkova, R. Decheva, I. Kalaidjiev, *Rivista Italiana EPPOS*, **57**, 176 (1975).
64. B. Bojadjieva, V. Staikov, *Bull. Dev. Essent. Oil Industry*, 11 (1972).
65. B. Stoianova-Ivanova, P. Hadjieva, S. Gerova, *Rivista Italiana EPPOS*, **52**, 637 (1970).
66. B. Stoianova-Ivanova, K. Mladenova, *Rivista Italiana EPPOS*, **52**, 573 (1970).
67. B. Stoianova-Ivanova, B. Danieli, P. Hadjieva, *Rivista Italiana EPPOS,* **53**, 138 (1971).
68. B. Stoianova-Ivanova, K. Mladenova, S. Popov, *Phytochemistry*, **10**, 1391 (1971).
69. B. Stoianova-Ivanova, P. Hadjieva, S. Popov, *Compt. Rend. Acad. Bulg. Sci.*, **24**, 745 (1971).
70. B. Stoianova-Ivanova, K. Mladenova, *Rivista Italiana EPPOS*, **56**, 253 (1974).
71. B. Stoianova-Ivanova, P. Hadjieva, K. Ubik, A. Tzutzulova, *Rivista Italiana EPPOS*, **56**, 665 (1974).
72. B. Stoianova-Ivanova B., Petsev N., Gigova M. *Compt. Rend. Acad. Bulg. Sci*, **30**, 1427 (1977).
73. B. Stoianova-Ivanova, *Hung. Acad. Sci.,* 213 (1979).
74. I. Ognyanov, *Medico-biological information*, 3 (1976).
75. J. Michailova, R. Atanasova, A. Balinova-Tzvetkova, *Compt. Rend. Acad. Bulg. Sci.*, **30**, 89 (1977).
76. I. Ognyanov, L. Panajotov, *Rivista Italiana EPPOS*, **55**, 560 (1973).
77. A. Maleev, S. Stoyanov, G. Neshev, *Medico-biological information,* 21 (1970).
78. A. Maleev, S. Petrovski, S. Stoyanov, *Farmacie*, **20**, 38 (1970).
79. S. Petrovski, P. Uzunov, *Dermatology and Venerology*, **10**, 243 (1971).
80. S. Petrovski, *Modern Medicine*, **22**, 51 (1971).
81. S. Petrovski, *Farmacie*, 41 (1974).
82. S. Petrovski, Proc. 2nd Nat. Conf. "Problems of Cosmetic and Perfumery Industry", Varna, Bulgaria, May 21-23, 1975, p. 343.
83. V. Grancharov, *Internal Medicine*, **14**, 91 (1975).
84. G. Ganchev, V. Nalbantian, S. Todorova, Z. Demireva, P. Boyadzhiev, E. Georgiev, L. Kupenov, Proc. 4th Nat. Conf. "Problems of the Cosmetics and Perfume Production", Varna, April 2-4, 1982, p. 293.
85. L. Kupenov, E. Georgiev, Proc. Nat. Sci. Session "Scientific and Technical Progress and Problems of Essential Oil and Medicinal Plants", Kazanlak, June 28-29, 1984, p. 60.
86. L. Kupenov, G. Ganchev, A. Konarev, L. Stefanov, Proc. Nat. Sci. Session "Scientific and Technical Progress and Problems of Essential Oil and Medicinal Plants", Kazanlak, June 28-29, 1984, p. 59.
87. L. Kupenov, I. Murgov, A. Konarev, M. Kostova, Proc. 6th Congr. Microbiol., Varna, Oct. 13-15, 1985, p. 337.
88. G. Ganchev, Collected Lectures for Postgraduate Qualification in New Natural and Synthetic Raw Materials for the Perfume and Cosmetics Industry, HIFFI, Plovdiv, 1988, p. 3.
89. G. Ganchev, Collected Lectures for Postgraduate Qualification in New Natural and Synthetic Raw Materials for the Perfume and Cosmetics Industry, HIFFI, Plovdiv, 1988, p. 12.
90. G. Papanov, P. Malakov, M. Minkov, K. Tomova, *Riechstoff Aromen Kosmetica*, **4**, 92 (1983).
91. P. Mihailov, N. Berova, *Medico-biological Information*, 17 (1971).
92. P. Mihailov, N. Berova, M. Angelakova, Proc. 1st Nat. Conf. "Problems of Cosmetic and Perfumery Industry", Varna, Bulgaria, 1972, p. 140.
93. R. Kumanova, PhD Thesis, Sofia, 1988.
94. Y. Savova, R. Yankova, S. Dobreva, D. Todorova, *Medico-biological Information*, 31 (1990).
95. Y. Savova, R. Yankova, S. Dobreva, D. Todorova, *Medico-biological information*, 34 (1990).
96. B. Markovska, O. Stoyanova, *Stomatology,* **57**, 188 (1975)
97. T. Burkova, V. Markovska, *Problems of the Dentistry*, **12**, 31 (1982).
98. M. Kirov, S. Vankov, Rose, Rose Oil, Girosital, Medicine and Sports, Sofia, 1986.
99. V. Gochev, P. Merdzhanov, L. Kozhuharova, A. Konakchiev, B. Zaharieva, A. Stoyanova, *Sci. Works University of Rousse*, **38**, 113 (2001).

100. L. Jirovetz, G. Buchbauer, A. Stoyanova, E. Georgiev, S. Damianova, *J. Agric. Food Chem.*, **51**, 3854 (2003).
101. L. Jirovetz, G. Buchbauer, A. Stoyanova, Z. Denkova, I. Murgov, *Euro Cosmetics*, 30 (2004).
102. L. Jirovetz, G. Buchbauer, A. Stoyanova, E. Georgiev, S. Damianova, *Ernährung,* **27**, 463 (2004).
103. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, E. Georgiev, R. Langer, *Österreichische Apotheker-Zeitung*, **59**, 585 (2005).
104. L. Jirovetz, G. Buchbauer, A. Stoyanova, E. Georgiev, S. Damianova, *Int. J. Food Sci. Technol.*, **4**, 305 (2005).
105. L. Jirovetz, S. Bail, G. Buchbauer, Z. Denkova, A. Slavchev, A. Stoyanova, E. Schmidt, M. Geissler, *Scientia Pharmaceutica*, **74**, 189 (2006).
106. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Slavchev, A. Stoyanova, E. Schmidt, *Ernährung*, **30**, 152 (2006).
107. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, V. Gearon, S. Birkbeck, E. Schmidt, M. Geissler, *Flavour Fragr. J.*, **21**, 465 (2006).
108. L. Jirovetz, G. Buchbauer, E. Schmidt, Z. Denkova, A. Stoyanova, R. Nicolova, M. Geissler, *Recent Research Devel. Agronomy Horticulture*, **2**, 1 (2006).
109. L. Jirovetz, G. Buchbauer, I. Stoilova, A. Krastanov, A. Stoyanova, E. Schmidt, *J. Agric. Food Chem.,* **54**, 6303 (2006).
110. E. Schmidt, L. Jirovetz, G. Buchbauer, G. Eller, I. Stoilova, A. Krastanov, A. Stoyanova, M. Geissler, *J. Essent. Oil Bear. Plants*, **9**, 170 (2006).
111. A. Stoyanova, A. Konakchiev, S. Damianova, I. Stoilova, S. Phan, *J. Essent. Oil Bear. Plants*, **9**, 93 (2006).
112. A. Dobreva, N. Kovacheva, T. Astatke, V. Zheljazkov, *Ind. Crops Prod.*, **34**, 1649 (2011).
113. A. Dobreva, *J. Essent. Oil Bear. Plants*, **16**, 404 (2013).
114. A. Edris, L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, A. Slavchev, *J. Essent. Oil Res.*, **19**, 186 (2007).
115. L. Jirovetz, G. Buchbauer, E. Schmidt, A. Stoyanova, Z. Denkova, R. Nicolova, M. Geissler, *J. Essent. Oil Res.*, **19**, 288 (2007).
116. L. Jirovetz, G. Buchbauer, K. Wlcek, V. Gochev, T. Girova, A. Stoyanova, E. Schmidt, M. Geissler, *J. Essent. Oil Bear. Plants*, **10**, 430 (2007).
117. L. Jirovetz, K. Wlcek, G. Buchbauer, V. Gochev, T. Girova, A. Dobreva, A. Stoyanova, E. Schmidt, *J. Essent. Oil Bear. Plants*, **10**, 412 (2007).
118. L. Jirovetz, K. Wlcek, G. Buchbauer, V. Gochev, T. Girova, A. Stoyanova, E. Schmidt, *Int. J. Essent. Oil Therapeutics*, **1**, 153 (2007).
119. L. Jirovetz, K. Wlcek, G. Buchbauer, V. Gochev, A. Stoyanova, E. Schmidt, M. Geissler, *Int. J. Essent. Oil Therapeutics*, **1**, 61 (2007).
120. V. Gochev, K. Wlcek, G. Buchbauer, A. Stoyanova, A. Dobreva, E. Schmidt, L. Jirovetz, *Nat. Prod. Comm.*, **3**, 1063 (2008).
121. V. Gochev, A. Stoyanova, T. Girova, T. Atanasova, *Sci. Works University of Plovdiv "Paisii Hilendarskii"*, **36**, 78 (2008).
122. V. Gochev, L. Jirovetz, K. Wlcek, G. Buchbauer, E. Schmidt, A. Stoyanova, K. Dobreva, *J. Essent. Oil Bear. Plants*, **12**, 1 (2009).
123. V. Gochev, A. Dobreva, T. Girova, A. Stoyanova, *Biotechnol. Biotechnol. Equip.*, **24***,* 512 (2010).
124. M. Mileva, E. Krumova, J. Miteva-Syaleva, N. Kostadinova, A. Dobreva, A. Galabov, *Compt. Rend. Acad. Bulg. Sci.*, **67**, 1363 (2014).
125. A. Marchev, I. Ivanov, P. Denev, M. Nikolova, V. Gochev, A. Stoyanova, A. Pavlov, V. Georgiev, *J. BioSci. Biotechnol.*, **4**, 219 (2015).
126. V. Popova, DSc Dissertation, UFT, Plovdiv, 2015.
127. D. Nedelcheva-Antonova, P. Stoicheva, L. Antonov, *Ind. Crops Prod.*, **108**, 36 (2017).
128. L. Jirovetz, G. Buchbauer, A. Stoyanova, Z. Denkova, I. Murgov, *Ernährung,* **28**, 257 (2004).
129. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, M. Geissler, *Scientia Pharmaceutica*, **73**, 27 (2005).
130. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, L. Ho, E. Schmidt, M. Geissler, *Euro Cosmetics*, 22 (2005).
131. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, E. Schmidt, M. Geissler, in: Processing, Analysis and Application of Essential Oils. HKB, Dehradun, India, 2005, p. 266.
132. E. Schmidt, L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, M. Geissler, *J. Essent. Oil Bear. Plants*, **8**, 99 (2005).
133. L. Jirovetz, G. Buchbauer, Z. Denkova, A. Stoyanova, I. Murgov, E. Schmidt, M. Geissler, in: Recent Progress in Medicinal Plants, J.N. Govil, V.K. Singh, C. Arunachalam (eds.), vol. 13, Studium-Press, India, 2006, p. 461.
134. M. Höferl, L. Jirovetz, G. Buchbauer, E. Schmidt, A. Stoyanova, Z. Denkova, A. Slavchev, M. Geissler, *Planta Medica*, **73**, 855 (2007).
135. L. Jirovetz, G. Buchbauer, V. Gochev, T. Girova, A. Stoyanova, E. Schmidt, Proc. 3rd Int. Conf. "Forest Biologically Active Resources", Habarovsk, Russia, September 25-27, 2007, p. 145.
136. E. Schmidt, L. Jirovetz, K. Wlcek, G. Buchbauer, V. Gochev, T. Girova, A. Stoyanova, M. Geissler, *J. Essent. Oil Bear. Plants*, **10**, 421 (2007).
137. L. Jirovetz, G. Buchbauer, E. Schmidt, Z. Denkova, A. Slavchev, A. Stoyanova, M. Geissler, *J. Essent. Oil Res.,* **20**, 82 (2008).

138. M. Höferl, G. Buchbauer, L. Jirovetz, E. Schmidt, A. Stoyanova, Z. Denkova, A. Slavchev, M. Geissler, *J. Essent. Oil Res.*, **21**, 459 (2009).
139. L. Jirovetz, G. Buchbauer, S. Bail, Z. Denkova, A. Slavchev, A. Stoyanova, E. Schmidt, M. Geissler, *J. Essent. Oil Res.*, **12**, 363 (2009).
140. E. Schmidt, S. Bail, S. Friedl, L. Jirovetz, G. Buchbauer, J. Wanner, Z. Denkova, A. Slavchev, A. Stoyanova, M. Geissler, *Nat. Prod. Comm.,* **5**, 1365 (2010).
141. E. Schmidt, S. Bail, G. Buchbauer, I. Stoilova, T. Atanasova, A. Stoyanova, L. Jirovetz, *Nat. Prod. Comm.*, **4**, 1107 (2009).
142. E. Schmidt, J. Wanner, S. Bail, L. Jirovetz, G. Buchbauer, V. Gochev, T. Girova, A. Stoyanova, T. Atanasova, *Perfum. Flavor.*, **34**, 46 (2009).
143. V. Gochev, A. Dobreva, T. Girova, A. Stoyanova, *Biotechnol. Biotechnol. Eq.*, **24**, 512 (2010).
144. T. Girova, V. Gochev, L. Jirovetz, G. Buchbauer, E. Schmidt, A. Stoyanova, *Biotechnol. Biotechnol. Equip.,* **24**, 547 (2010).
145. J. Wanner, E. Schmidt, S. Bail, L. Jirovetz, G. Buchbauer, V. Gochev, T. Girova, T. Atanasova, A. Stoyanova, *Nat. Prod. Comm.,* **5**, 1349 (2010).
146. J. Wanner, S. Bail, L. Jirovetz, G. Buchbauer, E. Schmidt, V. Gochev, T. Girova, T. Atanasova, A. Stoyanova, *Nat. Prod. Comm.*, **5**, 1355 (2010).
147. E. Schmidt, J. Wanner, M. Höferl, L. Jirovetz, G. Buchbauer, V. Gochev, T. Girova, A. Stoyanova, M. Geissler, *Nat. Prod. Comm.*, **7**, 1095 (2012).
148. Y. Hristova, PhD Thesis, University of Plovdiv "Paisii Hilendarski", Plovdiv, 2015.
149. M. Mileva, M. Nikolova, N. Nikolova, L. Mukova, A. Georgieva, A. Dobreva, A. Galabov, *Acta Microbiol. Bulg.*, **31**, 48 (2015).
150. I. Stoilova, A. Krastanov, A. Stoyanova, P. Denev, S. Gargova, *Food Chem.*, **102**, 764 (2007).
151. I. Stoilova, S. Bail, G. Buchbauer, A. Krastanov, A. Stoyanova, E. Schmidt, L. Jirovetz, *Nat. Prod. Comm.*, **3**, 1035 (2008).
152. I. Stoilova, S. Bail, G. Buchbauer, A. Krastanov, A. Stoyanova, E. Schmidt, L. Jirovetz, *Nat. Prod. Comm.*, **3**, 1043 (2008).
153. I. Stoilova, S. Bail, G. Buchbauer, A. Krastanov, A. Stoyanova, E. Schmidt, L. Jirovetz, *Nat. Prod. Comm.*, **3**, 1047 (2008).
154. I. Stoilova, N. Nenov, T. Atanasova, A. Stoyanova, V. Gochev, T. Girova, Proc. Int. Res. Pract. Conf., Almaty Technological University, Kazakhstan, October 12-13, 2012, p. 13.
155. I. Stoilova, J. Wanner, L. Jirovetz, D. Trifonova, L. Krastev, A. Stoyanova, A. Krastanov, *Bulg. J. Agric. Sci.*, **20**, 780 (2014).
156. P. Mollov, K. Mihalev, A. Schiber, N. Berardine, R. Carle, *Sci. Works UFT*, **52**, 99 (2005).
157. A. Schieber, K. Mihalev, N. Berardini, P. Mollov, R. Carle, *Zeit. Naturforsch.*, **60**, 379 (2005).
158. V. Shikov, K. Mihalev, N. Yoncheva, P. Mollov, V. Karagiozov, *Sci. Works UFT*, **53**, 83 (2006).
159. P. Mollov, K. Mihalev, V. Shikov, N. Yoncheva, V. Karagyozov, *Innov. Food Sci. Emerg. Technol.*, **8**, 318 (2007).
160. V. Shikov, D. Kammerrer, P. Mollov, K. Mihalev, N. Yoncheva, R. Carle, *Travaux scientifiques Universite de Plovdiv „Paisii Hilendarski"*, **36**, 59 (2008).
161. V. Shikov, D. Kammerer, K. Mihalev, P. Mollov, R. Carle, *J. Agric. Food Chem.*, **56**, 8521 (2008).
162. V. Shikov, PhD Thesis, UFT, Plovdiv, 2011.
163. V. Shikov, D. Kammerer, K. Mihalev, P. Mollov, R. Carle, *Food Res. Int.*, **46**, 552 (2012).
164. V. Shikov, K. Mihalev, N. Yoncheva, V. Karagyazov, P. Mollov, *J. EcoAgri Tourism*, **8**, 231 (2012).
165. V. Shikov, D. Kammerer, K. Mihalev, P. Mollov, R. Carle, *Sci. Works UFT*, **65**, 722 (2013).
166. A. Slavov, I. Vasileva, V. Shikov, M. Nikolova, D. Murdjeva, A. Dinchev, N. Yancheva, *Sci. Works UFT*, **61**, 366 (2014).
167. A. Slavov, H. Kiyohara, H. Yamada, *Int. J. Biol. Macromol.*, **59**, 192 (2013).
168. N. Yancheva, D. Markova, D. Murdzheva, I. Vasileva, A. Slavov, *Acta Scientifica Naturalis*, **3**, 7 (2016).
169. A. Slavov, I. Panchev, D. Kovacheva, I. Vasileva, *Food Hydrocoll.*, **61**, 469 (2016).
170. A. Slavov, I. Vasileva, L. Stefanov, A. Stoyanova, *Rev. Environ. Sci. Biotechnol.*, **16**, 309 (2017).
171. A. Slavov, P. Denev, I. Panchev, V. Shikov, N. Nenov, N. Yantcheva,
172. I. Vasileva, *Ind. Crops Prod.*, **100**, 85 (2017).
173. A. Slavov, I. Vasileva, N. Nenov, L. Stefanov, V. Shikov, K. Mihalev, A. Stoyanova, *Inf. Bull. BNAEOPC*, **76**, 39 (2018).
174. L. Stefanov, A. Stoyanova, *Inf. Bull. BNAEOPC,* **67**, 49 (2016).
175. A. Slavov, D. Karashanova, B. Georgieva, I. Vasileva, V. Bankova, R. Dinkova, N. Yantcheva, Proc. 2nd Conf. "Propolis in Human and Bee Health", Sofia, September 28-29, 2018, p. 88.
176. N. Nenov, *Sci. Works UFT*, **53**, 195 (2006).
177. N. Nenov, T. Atanasova, *Sci. Works UFT*, **55**, 255 (2008).
178. N. Nenov, S. Dichev, T. Atanasova, *Food Flav. Industry*, **2**, 40 (2008).
179. A. Stoyanova, S. Damianova, A. Konakchiev, I. Djurdjev, *Herba Pol.*, **49**, 235 (2003).
180. A. Stoyanova, I. Djurdjev, E. Georgiev, *Herba Pol.*, **51**, 24 (2005).
181. A. Stoyanova, Z. Denkova, N. Nenov, A. Slavchev, L. Jirovetz, G. Buchbauer, L. Ho, E. Schmidt, M. Geissler, *Elec. J. Env. Agric. Food Chem.*, **5**, 1615 (2006).
182. T. Atanasova, V. Gochev, A. Stoyanova, I. Djurdjev, *Sci. Works Univ. Plovdiv "Paisii Hilendarskii"*, **36**, 85 (2008).

183. S. Damianova, A. Stoyanova, A. Konakchiev, I. Djurdjev, *J. Essent. Oil Bear. Plants*, **7,** 247 (2004).
184. Regulation (EC) No 1223/2009 of the European Parliament and of the Council, of 30 November 2009, on Cosmetic Products, *Official Journal of the European Union*, **L342**, 59 (2009).
185. S. Damianova, A. Stoyanova, *J. Essent. Oil Bear. Plants*, **10**, 440 (2007).
186. N. Nenov, T. Atanasova, V. Gochev, P. Merdzhanov, T. Girova, T. Djurkov, A. Stoyanova, *World Sci.*, **7**, 17 (2016).
187. T. Atanasova, V. Gochev, N. Nenov, T. Djurkov, T. Girova, P. Merdzhanov, A. Stoyanova, *World Sci.*, **6**, 10 (2016).
188. N. Nenov, V. Gochev, I. Stoilova, T. Girova, T. Atanasova, A. Stoyanova, Proc. Int. Conf. "Agri-Food Sciences, Processes and Technologies", Sibiu, Romania, May 14-15, 2014, p. 70.
189. T. Atanasova, PhD Thesis, UFT, Plovdiv, 2007.
190. T. Atanasova, N. Nenov, T. Girova, A. Lazarov, T. Takov, A. Stoyanova, Proc. Int. Sci. Conf. "Economics and Society Development on the Base of Knowledge", Stara Zagora, June 4-5, 2009, p. 515.
191. T. Atanasova, N. Nenov, T. Girova, A. Lazarov, T. Takov, A. Stoyanova, *Sci. Works University of Rousse*, **48**, 131 (2009).
192. T. Atanasova, N. Nenov, T. Girova, A. Lazarov, T. Takov, A. Stoyanova, *Sci. Res. Union of Scientists in Bulgaria – Plovdiv*, **12**, 151 (2009).
193. T. Atanasova, T. Girova, V. Gochev, I. Stoilova, N. Nenov, M. Stoyanova, A. Stoyanova, *Sci. Works UFT,* **57**, 363 (2010).
194. N. Nenov, V. Gochev, T. Girova, I. Stoilova, T. Atanasova, V. Stanchev, A. Stoyanova, *J. Essent. Oil Bear. Plants*, **14**, 67 (2011).
195. T. Girova, V. Gochev, I. Stoilova, K. Dobreva, N. Nenov, V. Stanchev, A. Stoyanova, Proc. Int. Sci. Conf. eRA-6*,* Greece, September 19-24, 2011, p. 1.
196. V. Gochev, T. Girova, I. Stoilova, T. Atanasova, N. Nenov, V. Stanchev, A. Stoyanova, *J. BioSci. Biotechnol.*, **1**, 135 (2012).
197. P. Merdzhanov, R. Denkova, V. Yanakieva, N. Nenov, T. Atanasova, A. Stoyanova, *J. State University Voronezh*, **38**, 116 (2013).
198. T. Atanasova, N. Nenov, V. Gochev, T. Girova, P. Merdzhanov, I. Stoilova, A. Stoyanova, Proc. Int. Res. Pract. Conf. Almaty Technological University, Kazakhstan, Oct. 17-18, 2013, p. 40.
199. T. Atanasova, N. Nenov, V. Gochev, T. Girova, I. Stoilova, A. Stoyanova, Proc. Int. Res. Pract. Conf.*,* Voronezh, Russia, June 19-20, 2013, p. 20.
200. N. Nenov, T. Atanasova, I. Stoilova, V. Gochev, T. Girova, A. Stoyanova, *Sci. Works UFT*, **60**, 561 (2013).
201. T. Atanasova, N. Nenov, I. Dimitrova-Dyulgerova, V. Miladinova, V. Iliev, A. Stoyanova, *Sci. Works UFT*, **61**, 275 (2014).
202. T. Atanasova, N. Nenov, I. Dimitrova-Dyulgerova, V. Miladinova, V. Iliev, Stoyanova, *Sci. Works University of Rousse*, **53**, 107 (2014).
203. T. Atanasova, N. Nenov, V. Gochev, I. Stoilova, T. Girova, A. Stoyanova, *Technol. Commod. Sci. Agric. Prod.*, **2**, 51 (2014).
204. T. Ivanova, PhD Thesis, UFT, Plovdiv, 2014.
205. N. Nenov, V. Gochev, I. Stoilova, T. Girova, T. Atanasova, A. Stoyanova, *World J. Pharm. Pharm. Sci.*, **3**, 2128 (2014).
206. N. Nenov, V. Gochev, T. Girova, I. Stoilova, T. Atanasova, A. Stoyanova, Proc. Int. Res. Pract. Conf., Voronezh, Russia, November 19-21, 2014, p. 140.
207. N. Nenov, V. Gochev, I. Stoilova, T. Girova, T. Atanasova, A. Stoyanova, *Agro-Industrial Complex of Russia*, 162 (2015).
208. T. Atanasova, N. Nenov, T. Djurkov, I. Stoilova, V. Gochev, T. Girova, A. Stoyanova, *Sci. Works UFT*, **62**, 259 (2015).
209. M. Ivanova, N. Nenov, M. Dushkova, R. Vlaseva, A. Stoyanova, Proc. 3rd Int. Sci. Pract. Conf., Voronezh, Russia, February 11-13, 2015, p. 163.
210. K. H. C. Baser, G. Buchbauer (eds.), Handbook of Essential Oils: Science, Technology, and Applications, CRC Press Taylor & Francis Group LLC, Boca Raton, FL, 2010.
211. D. Balev, N. Nenov, S. Dragoev, K. Vassilev, D. Vlahova-Vangelova, F. Smolnikova, *Polish J. Food Nutr. Sci.*, **67**, 59 (2017).
212. S. Dragoev, D. Balev, K. Wassilev, N. Nenov N., Proc. 56 Int. Congr. Meat Sci. Technol., Jeju, South Korea, August 15-20, 2010, p. E034.
213. G. Kirisheva, PhD Thesis, UFT, Plovdiv, 2017.
214. D. Taneva, PhD Thesis, UFT, Plovdiv, 2017.

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Factors affecting the amount of biologically active substances in extracts of Bulgarian medical plants typical of Western Rhodopes

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To obtain extracts maximally enriched with biologically active compounds (BAC), it is necessary to study and select suitable conditions for carrying out the extraction process. Five Bulgarian plants that thrive in the Western Rhodopes were selected: *Cirsium ligulare* Boiss. and *Crataegus monogyna* (flowers), *Hypericum perforatum* L. and *Thymus callieri* Borbás ex Velen. (stems), *Crataegus monogyna* and *Juniperus communis* L. (fruits). The effect of processing of fresh herbs (drying and freezing) on the content of BAC was investigated. Higher amounts of total phenols and flavonoids were contained in the dried materials. The highest amount of BAC from the dried herbs was found in the *Thymus callieri* Borbás ex Velen. and from the frozen ones – in the *Hypericum perforatum* L. The method of extraction (conventional and ultrasonic) was found to influence the amount of extracted BAC. In the conventional method of extraction, the yield of BAC was almost twice as high as in ultrasonic extraction. The concentration of ethanol (0%, 30%, 50%, 70%, or 95%) had a significant effect on the amount of BAC, as 70% ethyl alcohol showed the best results. Of the studied 5 medicinal plants, dried *Thymus callieri* Borbás ex Velen. and frozen *Hypericum perforatum* L. might be successfully used to prepare 70% ethanolic extracts by the conventional method.

Keywords: antioxidant activity; flavonoids; phenols; DPPH; FRAP

INTRODUCTION

The use of plant products for treating various diseases started with the beginning of human civilization. The earliest document shedding light on the use of medicinal plants was written between 4500 and 1600 BC [1].

Medicinal plants are usually perennial and their shoot system contains BAC that are of great interest due to their antioxidant and antibacterial properties. Synthetic antioxidants are mostly used in the food industry and cosmetics to prolong the stability of foods and cosmetic products. However, the use of these antioxidants has been questioned due to their potential health risks and toxicity [2]. Therefore, the search for antioxidants from natural sources, such as medical plants, attracts researchers' attention. The presence of phenols and terpenes in their composition allows their use as stabilizers in food. In cosmetics, essential oils derived from medicinal plants are used for flavoring and due to their antiseptic action, in the composition of lotions, eaux de toilette, and soaps. Dried herbs are used in medicine as tinctures and teas for colds, coughs, stomach and intestinal diseases. According to Naczk and Shahidi [3], approximately 10 to 20% of plants are used in a positive way in health care to treat harmful diseases.

Varied medicinal plants are known as a source of antioxidants that can protect organisms from oxidative stress and various chronic diseases [4]. The group of antioxidants includes water-soluble antioxidant metabolites (ascorbate and glutathione) and secondary metabolites, such as polyphenols, flavonoids, and terpenoids [5] which are present mainly in plants [6], and are distributed in different parts, mainly in flowers, leaves, and fruits. However, environmental factors can affect the production of antioxidants and secondary metabolites. Furthermore, different extraction techniques are used for the isolation of BAC to achieve maximum process efficiency. Conventional (classical) extraction (CE) is the most widespread technique for the extraction of antioxidant compounds from plant materials but this method consumes a great amount of energy, due to the heating process and is characterized by long duration. Non-conventional extraction methods include ultrasound-assisted extraction (UAE) which uses less energy and has a shorter duration, allows

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full extractions to be completed in minutes with high reproducibility, reduces the consumption of used solvents, simplifies manipulation and work-up.

The extraction efficiency of all methods mainly depends on the choice of using selective solvents. The solvent polarity, its environmental safety, and toxicity are the most important factors while selecting a solvent for the extraction of BAC. Ethanol is a solvent that is safe for human consumption due to its low toxicity [7].

Indisputably, fresh herbs have the highest quality but they can be used only in one season. Different preservation techniques can be exploited to ensure the quality, safety, and shelf-life extension of plants. Among these, freezing is recognized as one of the main processes for long-term preservation which has a low impact on the nutritional quality of food products [8, 9]. Air-dried herbs are also a good alternative to fresh ones, as the process itself is easy to perform and inexpensive. Drying is by far the most widely used treatment [10].

The higher plants that grow in Bulgaria are very diverse. The vegetation in the municipality of Dospat, which is located in the sub-region of the Western Rhodopes, is characterized by rich biodiversity [11]. The relief is typical mountainous and significant variations in altitude (560 – 1653 m), and specific microclimatic conditions are the prerequisites for the rich floristic diversity [12]. Typical for the Western Rhodopes and the municipality of Dospat and with long-term traditional use (widely used in folk medicine, as medicines and less often in drinks and food) are the medicinal plants like thyme, St. John's wort, cirsium, hawthorn , juniper, etc.

Thymus callieri Borbás ex Velen. is a new species in the Balkans floristic regions [13] and *Thymus* extracts obtained with polar solvents are an attractive target for the screening of BAC for possible industrial applications in distinct fields, including food, cosmetics or pharmaceutical industries [14, 15]. St. John's Wort (*Hypericum perforatum* L.) is presently one of the most consumed medicinal plants in the world [16, 17]. So far, data on *Cirsium ligulare* Boiss. in literature are scarce. However, plants from the *Cirsium* genus are rich in phenolic compounds [18]. Hawthorn (*Crataegus monogyna*) is a medicinal plant widely used in phytotherapy for the treatment of many cardiovascular diseases [19], as from the plant are most often used its flowers, leaves and fruits. *Juniperus communis* L. is an evergreen aromatic shrub with high therapeutic potential for the treatment of diseases in human and animals [20]. These five medicinal plants are typical for the Bulgarian flora but little is known about their antioxidant activity.

This study aimed to evaluate the influence of the method of herbs processing (drying or freezing), extraction approach (conventional or ultrasound), and the concentration of the extracting agent (ethanol) on the antioxidant activity of herbal extracts prepared from different morphological parts of five different Bulgarian medical plants collected from Dospat (Western Rhodopes, Bulgaria).

MATERIALS AND METHODS

Plant materials and treatments (drying and freezing)

The different morphological parts of the five Bulgarian medicinal plants grown in the Western Rhodopes, Dospat municipality were selected: TC – thyme (*Thymus callieri* Borbás ex Velen.) stem, HP – St. John's wort (*Hypericum perforatym* L.) stem, CL – cirsium (*Cirsium ligulare* Boiss.) flower, CM-flower – Hawthorn (*Crataegus monogyna*) flower, CM-fruit – Hawthorn (*Crataegus monogyna*) fruit, JC – juniper (*Juniperus communis* L.) fruit.

A portion of fresh plant material was inspected, cut into small pieces, dried in a thin layer in the shade at 22 – 25°C and stored in tightly closed bags in a dry place until the time of analysis (dried herb). A second portion of fresh plant material was inspected, cut into small pieces, placed in plastic bags, and frozen in a refrigerator at –18 °C until the time of analysis (frozen herb).

Preparation of plant extracts

1. Conventional (classical) method of extraction. An aqueous extract (0%) and 30%, 50%, 70% or 95% ethanolic extracts from dried and frozen plant mass were obtained according to [21] with small modifications: 15 g (20 g) of the dried (frozen) plant were mixed with 300 mL of H_2O, 30%, 50%, 70% or 95% ethanol and kept for 1 h at 60ºC, then were left for 24 h at room temperature under constant stirring. The obtained mixtures were filtered through nylon cloth (250 mesh), and insoluble residues were extracted with an additional 200 mL of the same extractant at the same conditions. The two filtrates were combined and homogenized well.

2. Ultrasound-assisted extraction. The extraction process of BAC from the used experimental plants was carried out with the appropriate concentration of ethanol (solid to liquid ratio 1:20) in an ultrasonic bath (VWR, Malaysia;

45 kHz, 30 W) at 45 °C for 15 min, according to [22] with same modifications. The extracts were centrifuged at 1800×g for 15 min (MPW-251, Med. Instruments, Poland) and used for further analysis.

Chemical analyses

1. *Total flavonoid content* was evaluated using $Al(NO_3)_3$ reagent and measuring the absorbance at 415 nm as described by Kivrak *et al*. [23]. The calculation was made by using a standard curve prepared with quercetin.

2. *Total phenols* were determined according to [24]. The calculation was made by using a standard curve prepared with gallic acid.

Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated by two methods: FRAP (ferric reducing antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method.

1. *FRAP method* is based only on a single electron transfer mechanism and was measured according to [25] with some modification. Three ml of freshly prepared FRAP reagent (10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 1 part 20 mM $FeCl_3.6H_2O$ in d. H_2O) were mixed with 0.1 ml of investigated extract. The reaction time was 10 min at 37 °C in darkness and the absorbance was measured at 593 nm against blank prepared with the same solvent. A standard curve was built with $FeSO_4.7H_2O$. The results of FRAP analysis were expressed as μmol Fe^{2+} equivalents per gram plant.

2. *DPPH method* is based on mixed hydrogen atom transfer and single electron transfer mechanisms and was estimated according to [25] with some modification. Briefly, 0.15 ml of extract was mixed with 2.85 ml 0.06 mM DPPH fresh solution in 96% ethanol. The mixture was left for 30 min (kept in the dark at room temperature) so that a reaction could take place, and then the absorbance at 517 nm was read by a spectrophotometer against a blank containing the same solvent. The results of DPPH analysis were expressed as mmol Trolox equivalents (TE) per gram plant.

Statistical analyses

Analyses were performed in triplicate. Results are presented as means ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) using Statgraphics Centurion statistical program. Mean differences were established by Fisher's least significant difference test for paired comparison with a significance level $p \leq 0.05$.

RESULTS AND DISCUSSION

Influence of herbs processing (drying or freezing) on the amount of biologically active substances

According to our data (Table 1), the difference in plants processing had a significant effect on the amount of phenols and flavonoids. The results showed that the amount of BAC in the dried herbs was higher than in the frozen ones. Cell breakages during freezing can lead to the decompartmentalization of antioxidants such as anthocyanins and other phenolic compounds, and their degradation due to the interaction with oxidative enzymes [26]. The values of total flavonoids and phenols varied in a wide range (Table 1) - from 2.27 to 26.43 mg QE/g weight for flavonoids and from 7.55 to 86.19 mg GAE/g weight for phenols, respectively. The highest content of total flavonoids and phenols from dried herbs showed TC (26.43 mg QE/g weight and 86.19 mg GAE/g weight) and from frozen plants – HP (12.05 mg QE/g weight and 29.29 mg GAE/g weight). On the other hand, the 70% ethanolic extracts from CM – fruit and CL were found to be least rich in total phenols and flavonoids in both dried and frozen herbs. The content of BAC in different parts of the plant (flower and fruit) in CM showed variations, as significantly more noticeable was this difference in the dried samples. However, in both studied materials (dried and frozen), the amounts of both total phenols (81.36 mg QE/g weight in dried flowers and 26.88 mg QE/g weight in dried fruits) and flavonoids (14.96 mg QE/g weight in dried flowers and 2.89 mg QE/g weight in dried fruits) were higher in flowers. The established results were in agreement with Abdulkadir *et al.* [27] who investigated total phenolic and flavonoid contents of the ethanolic extracts from fruit, stem, and leaf of *Solanum torvum*. Their data showed the highest level of phenolic content in the stem (43.92 mg GAE/g), lower in the leaf (37.48 mg GAE/g) and the lowest in the fruit (16.15 mg GAE/g). Similar to phenols, the flavonoid content of 2.89 mg QE/g weight in dried CM – fruit was found to be significantly lower than that of the dried flower – 14.96 mg QE/g weight.

Table 1. Quantities of total flavonoids and phenols of 70% ethanolic extracts obtained by conventional extraction of dried and frozen plant material

70 % C_2H_5OH extracts	Total flavonoids, mg QE/g weight		Total phenols, mg GAE/g weight	
	dried	frozen	dried	frozen
TC	26.43±0.09^{a,A}	7.47±0.18^{b,B}	86.19±0.36^{a,A}	20.32±0.61^{b,B}
HP	8.32±0.22^{c,B}	12.05±0.42^{a,A}	36.83±0.42^{c,A}	29.29±0.44^{a,B}
CL	4.95±0.19^{e,A}	2.49±0.08^{e,B}	19.07±0.31^{f,A}	7.55±0.29^{f,B}
CM – flower	14.96±0.37^{b,A}	3.79±0.11^{d,B}	81.36±0.35^{b,A}	14.73±0.43^{d,B}
CM– fruit	2.89±0.07^{f,A}	2.27±0.05^{e,B}	26.88±0.41^{e,A}	13.19±0.42^{e,B}
JC	6.92±0.17^{d,A}	6.49±0.20^{c,B}	29.85±0.21^{d,A}	18.02±0.32^{c,B}

$^{a-f}$: Means in a column without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a row for a dried and a frozen plant (for a particular method) without a common letter differ significantly ($p \leq 0.05$).

Table 2. Antioxidant activity of 70% ethanol extracts obtained by conventional extraction of dried and frozen plant materials

70 % C_2H_5OH extracts	DPPH method, mM TE/g weight		FRAP method, µmol Fe^{2+}/g weight	
	dried	frozen	dried	frozen
TC	218.97±0.28^{a,A}	121.30±0.23^{b,B}	1110.77±0.85^{a,A}	218.96±0.94^{b,B}
HP	216.47±0.28^{b,A}	184.59±0.39^{a,B}	296.76±0.56^{c,B}	339.23±0.52^{a,A}
CL	90.57±0.48^{e,A}	26.74±0.68^{e,B}	155.85±0.28^{f,A}	66.74±0.81^{f,B}
CM- flower	217.06±0.27^{b,A}	97.02±0.38^{c,B}	966.01±0.31^{b,A}	171.95±0.87^{d,B}
CM- fruit	176.23±0.72^{c,A}	85.43±0.21^{d,B}	278.24±0.63^{d,A}	130.91±0.88^{e,B}
JM	126.13±0.75^{d,A}	120.95±0.56^{b,B}	223.76±0.86^{e,A}	188.28±0.42^{c,B}

$^{a-f}$: Means in a column without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a row for a dried and a frozen plant (for a particular method) without a common letter differ significantly ($p \leq 0.05$).

Table 3. Influence of the solvent concentration on the amount of total flavonoids and phenols

Bioactive compounds	Herb	Ethanolic extracts				
		0 %	30 %	50 %	70 %	95 %
Total flavonoids, mg QE/g weight	Dried TC	12.22±0.03^{d,A}	18.09±0.08^{c,A}	24.29±0.05^{b,A}	26.43±0.09^{a,A}	10.63±0.04^{e,B}
	Frozen HP	4.23±0.04^{d,B}	9.59±0.17^{c,B}	10.72±0.30^{b,B}	12.05±0.42^{a,B}	11.06±0.22^{b,A}
Total phenols, mg GAE/g weight	Dried TC	57.47±0.43^{d,A}	59.11±0.42^{c,A}	69.19±0.42^{b,A}	86.19±0.36^{a,A}	33.96±0.36^{e,A}
	Frozen HP	18.54±0.29^{e,B}	21.32±0.17^{d,B}	24.38±0.33^{c,B}	29.29±0.44^{a,B}	27.35±0.60^{b,B}

$^{a-e}$: Means in a row without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a column for dried and frozen plants (for a particular method) without a common letter differ significantly ($p \leq 0.05$).

Alam *et al.* [28] reported a decrease in phenols content in the order leaf>fruit>stem and in flavonoid content in the order leaf>stem>fruit in *Solanum nigrum*. Our study indicates that the different parts of a plant species might accumulate various levels of polyphenols and flavonoids.

Antioxidant properties of 70% ethanolic extracts obtained by conventional extraction of dried and frozen plant materials were determined by two different methods - DPPH and FRAP. Our study revealed that the extracts with higher phenolic and flavonoid contents presented higher antioxidant activities (Table 2). Concerning the antioxidant activity quantified by DPPH in each of the study species, the lowest value was obtained for CL (90.57 mM TE/g weight for dried one and 26.74 mM TE/g weight for frozen one). The species with the highest values were dried TC (218.97 mM TE/g weight) and frozen HP (184.59 mM TE/g weight). Regarding the antioxidant activity quantified by FRAP, the results for highest activity were the same but this method established a difference of around

3.3 times higher antioxidant activity for dried TC compared to frozen HP.

In all the methods used, except for the total flavonoids and FRAP method for HP, the dried plants gave higher results for BAC. For this reason, in the next series of experiments, one dried (TC) and one frozen (HP) herb, showing the best results, were used in the next phase of the study.

Influence of ethanol concentration of biologically active substances

The extractability of antioxidants from two herbs – dried TC and frozen HP was studied at 0%, 30%, 50%, 70%, and 95% ethanol (Tables 3 and 4). As different concentrations of ethanol affect the physical properties of the solvent [29], this is likely to change the extraction yield of the various BAC in both studied herbs. Also, antioxidant compounds in a plant have different polarity and solubility [30] and extraction solvent properties may affect the extraction yield. The ethanol with the highest concentration (95%) had a negative effect on the extractability of BAC in the same studied methods. Generally, the extractability increased with increasing alcohol concentration, reaching a maximum at 70% ethanol. The amount of total phenolics in the ethanolic extracts ranged from 33.96 to 86.19 mg GAE/g weight for dried TC and from 18.54 to 29.29 mg GAE/g weight for frozen HP, as shown in Table 3.

Flavonoids (including flavones, flavanones, isoflavones, flavonols, and anthocyanidins), which are most commonly found and widely distributed in plant polyphenol compounds, were in the range of 10.63 to 26.43 mg QE/g weight in ethanolic extracts from dried TC and from 4.23 to 12.05 mg QE/g weight for ethanolic extracts from frozen HP, respectively, in this study. The highest total flavonoids and phenols values were determined in 70% ethanolic extracts of the two herbs but in dried TC the lowest content was in 95% ethanolic extract, while in frozen HP the lowest content was in a water solvent. Our results were consistent with the previous studies. For example, Sun *et al.* [31] who evaluated the effect of different ethanol/water solvents on the total phenols and flavonoids, observed their highest contents in 75% ethanol. According to those authors, water and 25% ethanol seemed to be less effective in extracting phenolics than ethanol/water extraction solvents with high concentrations. Kim *et al.* [32] suggested that a natural antioxidant extracted by a diluted ethanol solution has higher extraction yield compared to that extracted by pure ethanol.

DPPH radical scavenging activity and ferric reducing antioxidant power of ethanolic extracts from the two plants are shown in Table 4.

Table 4. Influence of the solvent concentration on the amount of bioactive compounds, determined by DPPH and FRAP methods.

Method	Herb	Ethanolic extracts				
		0 %	30 %	50 %	70 %	95 %
DPPH, mM TE/g weight	Dried TC	198.84±0.82^{d,A}	206.20±0.53^{c,A}	212.92±0.79^{b,A}	218.97±0.28^{a,A}	191.36±0.30^{e,A}
	Frozen HP	100.10±0.72^{e,B}	163.73±0.73^{d,B}	173.35±0.48^{c,B}	184.59±0.39^{a,B}	177.90±0.72^{b,B}
FRAP, µmol Fe^{2+}/g weight	Dried TC	697.88±1.32^{d,A}	889.07±2.47^{c,A}	1083.20±1.11^{b,A}	1110.77±0.85^{a,A}	438.98±1.96^{e,A}
	Frozen HP	102.56±1.30^{e,B}	254.83±0.52^{d,B}	266.27±1.12^{c,B}	339.23±0.52^{a,B}	289.73±0.89^{b,B}

$^{a-e}$: Means in a row without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a column for dried and frozen plants (for a particular method) without a common letter differ significantly ($p \leq 0.05$).

Table 5. Influence of the type of extraction on the amount of total flavonoids and phenols.

70 % C_2H_5OH extracts	Total flavonoids, mg QE/g weight		Total phenols, mg GAE/g weight	
	Conventional extraction	Ultrasonic extraction	Conventional extraction	Ultrasonic extraction
Dried TC	26.43±0.09^{a,A}	15.01±0.33^{b,A}	86.19±0.36^{a,A}	42.20±0.38^{b,A}
Frozen HP	12.05±0.42^{a,B}	6.97±0.31^{b,B}	29.29±0.44^{a,B}	13.89±0.30^{b,B}

$^{a-b}$: Means in a row for a particular method for determination of bioactive compounds and a herb without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a column for a particular method of extraction without a common letter differ significantly ($p \leq 0.05$).

Table 6. Influence of the type of extraction on the amount of bioactive compounds determined by DPPH and FRAP methods.

70 % C_2H_5OH extracts	DPPH method, mM TE/g weight		FRAP method, µmol Fe^{2+}/g weight	
	Conventional extraction	Ultrasonic extraction	Conventional extraction	Ultrasonic extraction
Dried TC	218.97±0.28^{a,A}	216.40±0.59^{b,A}	1110.77±0.85^{a,A}	604.83±0.29^{b,A}
Frozen HP	184.59±0.39^{a,B}	152.56±0.40^{b,B}	339.23±0.52^{a,B}	138.48±0.39^{b,B}

$^{a-b}$: Means in a row for a particular method for determination of bioactive compounds and a herb without a common letter differ significantly ($p \leq 0.05$); $^{A-B}$: Means in a column for a particular method of extraction without a common letter differ significantly ($p \leq 0.05$).

In extracts from dried TC, as ethanol concentration increased, DPPH and FRAP increased too. The 70% ethanolic extracts showed the highest results but with the next tested concentration (95%) the values decreased. The results obtained by us agreed with those in the literature [33], where an ethanol/water solvent was more efficient for extracting antioxidant compounds compared to pure solvents. The extracts from frozen HP showed different extractability in water/ethanolic solutions. The best results were achieved with 70% ethanol followed by 95% concentration. Lowering the concentration of ethanol in the range from 0 to 50% led to a reduction in the number of participants in the reaction BAC, determined by both DPPH and FRAP methods. Thus, the addition of 70% ethanolic extracts from the two herbs as natural antioxidants might be the most effective.

Influence of the method of extraction - conventional or ultrasound-assisted (CE or UAE) on the amount of biologically active substances

The total phenols and flavonoids contents were affected significantly ($p<0.05$) by the type of extraction process. As shown in Table 5, the CE gave higher results, while dried TC revealed significantly higher activity (86.19 mg GAE/ g weight and 26.43 mg QE/g weight) than frozen HP (29.29 mg GAE/ g weight and 12.05 mg QE/g weight). In general, the yield of BAC was about two times higher during CE. These results were not in agreement with those of a study by Um *et al.* [34]. They found that the yield using UAE was significantly higher than that obtained using CE. Probably the higher results obtained by the CE method were due to the longer extraction time and the higher temperature used in this method. The UAE was done at a lower temperature for a shorter time. According to [34], at a temperature of 40 – 50 °C the yield of total phenols in the extract decreased with increasing reaction time because of the oxidative degradation of phenolic compounds. Albu *et al.* [35] investigated the difference in the application of CE and UAE on the concentration of BAC in sage and they concluded that the content of BAC was by approximately 60% higher under the influence of ultrasound. The higher values of polyphenols extracted with UAE were also reported by Dent *et al.* [36]. Under optimal conditions (output power of 400 W, 11 min) using 30% ethanol these authors achieved a 20% higher yield of BAC than with CE (60°C, 30 min). To achieve better results in ultrasonic extraction, the conditions of the process (frequency (kHz), amplitude (%), applied cycle (%), nominal output power (W), and geometrical parameters of the sonotrode (length and diameter – mm) itself must be optimized in details in our further research.

DPPH and FRAP were also affected by the method of extraction (Table 6). Regardless of statistical differences, the quantity of BAC in 70% ethanolic extract obtained by the CE method was only by 1.17% (for dried TC) and by 17.35% (for frozen HP) higher than that achieved by the UAE method. The lower ultrasonic extraction results may be a consequence of the shorter extraction time (15 min). According to [37], the best time of sonication was 40 min with an ethanol concentration of 35%. To achieve better results, the time of sonication in our research must be also optimized.

CONCLUSIONS

The herbs processing (drying or freezing) had a significant effect on the amount of BAC. The different concentrations of ethanol affected the antioxidant extractability as 70% ethanolic solution showed the highest results. The yield using ultrasound-assisted extraction was significantly lower than that obtained using a conventional method of extraction. Of the studied five Bulgarian medical plants, *Thymus callieri* Borbás ex Velen. and *Hypericum perforatum* L. were the richest sources of secondary metabolites. Hence, their 70%

ethanolic extracts (as antioxidants) might be of interest for application in the food industry.

REFERENCES

1. R. Rastogi, B. Mehrotra, *Compendium of Indian medicinal plants*, National Institute of science communication, New Delhi, India, 2002, p. 1.
2. S. P. Wong, L. P. Leong, J. H. W. Koh, *Food Chem*., **105**, 775 (2006).
3. M. Naczk, F. Shahidi, *J. Pharm. Biomed.*, **41(5)**, 1523 (2006).
4. K. Hasplova, A. Hudecova, E. Miadokova, Z. Magdolenova, E. Galova, L. Vaculcikova, F. Gregan, M. Dusinska, *Neoplasma*, **58** (5), 386 (2011).
5. A. M. Hashim, B. M. Alharbi, A. M. Abdulmajeed, A. Elkelish, W. N. Hozzein, H. M. Hassan, *Plants*, **9**, 869 (2020).
6. I. B. Suffredini, H. S. Sader, A. G. Gonçalves, et al., *Braz. J. Med. Biol. Res.*, **37**, 379 (2004).
1. 7.N. P. E. Hikmawanti, S. Fatmawati, A. W. Asri, *IOP Conf. Series*, **755**, 012060 (2021).
7. A.V. Cardello. *Appetite*, **40(3)**, 217 (2003).
8. G. Sacchetti, E. Chiodo, L. Neri, G. Dimitri, A. Fantini, *Acta Hort.*, **844**, 53 (2009).
9. M. B. Hossain, C. Barry-Ryan, A. B. Martin-Diana, N. P. Brunton, *Food Chem.*, **123,** 85 (2010).
10. D. Zahariev, L. Taneva, K. Racheva, *J. Biosci. Biotechnol.*, **6,** 59 (2017).
11. I. Brambarov, Rhodopi – recreation area. *Galik Publishing House*, Sofia, p. 213 (in Bulgarian) (2001).
12. V. Vladimirov, F. Dane, K. Tan *Phytologia Balcanica*, **19**, 131 (2013).
13. G. Ozkan, S. Kamiloglu, T. Ozdal, D. Boyacioglu, E. Capanoglu, *Molecules*, **21**, 257 (2016).
14. M.S. Brewer, *Compr. Rev. Food Sci. Food Saf.*, **10**, 221 (2011).
15. R. B. H. Wills, K. Bone, M. Morgan, *Nutritional Research Reviews*, **13**, 47 (2000).
16. V. Butterweck, *CNS Drugs*, **17**, 539 (2003).
17. M. Kozyra, K. Skalicka-Wozniak, *Acta Pol. Pharm.,* **71(5)**, 877 (2014).
18. H. Bahri, Ch. Benkirane, B. Tazi. *AJMP*, 7(2), 030 (2019).
19. R. Raina, P. K. Verma, R. Peshin, H. Kour, *Heliyon*, **5(8)**, e02376 (2019).
20. M. Kratchanova, M. Gocheva, E. Pavlova, I. Yanakieva, D. Nedelcheva, V. Kussovski, A. Slavov, *Bulg. Chem. Commun.*, **40**, 561 (2008).
21. I. Vasileva, R. Denkova, R. Chochkov, D. Teneva, Z. Denkova, Tz. Dessev, P. Denev, A. Slavov, *Food Chem*., **253**, 13 (2018).
22. Ş. Kivrak, T. Göktürk, I. Kivrak, E. Kaya, E. Karababa, *Food Sci. Technol., Campinas*, **39(2)**, 423 (2019).
23. E. A. Ainsworth, K. M. Gillespie, *Nat. Prot.*, **2**, 875 (2007).
24. I. Dimov, N. Petkova, G. Nakov, I. Taneva, I. Ivanov, V. Stamatovska, *Ukr. Food J.*, **7(4)**, 671 (2018).
25. R. Khattab, G. B. Celli, A. Ghanem, M. S. L. Brooks, *J. Berry Res.*, **5**, 231 (2015).
26. A. R. Abdulkadir, N. Mat, M. M. Hasan, M. S. Jahan, *Sci Asia*, **42(3)**, 184 (2016).
27. M. N. Alam, S. Roy, Sh. M. Anisuzzaman, M. Rafiquzzaman, *Pharmacogn. Commun.*, **2(3)**, 67 (2012).
28. J. E. Cacace, G. Mazza, *Food Eng. Phys. Properties*, **68**, 240 (2003).
29. T. Sun, C. Ho, *Food Chem.*, **90(4)**, 743 (2005).
30. C. Sun, Zh. Wu, Z. Wang, H. Zhang, *eCAM*, **2015**, 9 (2015).
31. N. M. Kim, H. S. Sung, W. J. Kim, *Korean J. Food Sci. Technol.* **25**, 204 (1993).
32. H. I. Jun, Y. A. Kim, Y. S. Kim, *J. Korean Soc. Food Sci. Nutr*., **43**, 381 (2014).
33. Min Um, Tae-Ho Han, Jae-Won Lee *Food Sci Biotechnol*., **27(2)**, 375 (2018).
34. S. Albu, E. Joyce, L. Paniwnyk, J. P. Lorimer, T. J. Mason, *Ultrason. Sonochem.,* **11**, 261 (2004).
35. M. Dent, V. Dragović-Uzelac, I. Elez Garofulić, T. Bosiljkov, D. Ježek, and M. Brnčić, *Chem. Biochem. Eng. Q.*, **29(3)**, 475 (2015).
36. D. B. Muñiz-Márquez, G. C. Martínez-Ávila, J. E. Wong-Paz, R. Belmares-Cerda, R. Rodríguez-Herrera, C. N. Aguilar, *Ultrason. Sonochem.*, **20(5)**, 1149 (2013).

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Physico-chemical characteristics of polysaccharides isolated from lavender by-products

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The essential oil industry, in addition to the main aroma products, such as essential oil, water, concrete, etc., results every year large quantities of lignocellulosic under-valorized biomass. Such by-products are obtained from processing of common lavender (*Lavandula angustifolia* Mill.) used industrially in Bulgaria, France, etc. chiefly for essential oil production. The solid biomass is discarded in locations neighboring distilleries. By this way potentially useful biomass is lost, hence the idea behind the present work was utilization of industrial lavender by-products as a source of polysaccharides. Two industrial solid residues were investigated: the first one obtained after supercritical CO_2 extraction (CO2-L), and the second: obtained after steam distillation of lavender (SD-L). The by-products were pretreated with 70% ethanol and alcohol insoluble residue (AIR) was obtained. The AIRs were subjected to a dilute acid extraction whereat the CO2-L and SD-L yielded 6.97±0.14% and 5.95±0.23% acid-soluble polysaccharide, respectively. The monosaccharide profile determined by HPLC revealed presence predominantly of galacturonic acid: 672.44±4.89 µg/mg and 619.17±5.23 µg/mg polysaccharide for CO2-L and SD-L, respectively. The degree of methoxylation: 53.7±1.5% and 48.9±2.5% and degree of acetylation (DAc) 2.7±0.4% and 2.3±0.1% for CO2-L and SD-L, respectively, suggested that the isolated polysaccharides were middle esterified with low DAc. The surface tension of SD-L 58.5±1.2 mN m^{-1} was lower compared to CO2-L (63.2±1.4 mN m^{-1}) which suggests that SD-L polysaccharide has better emulsification properties. The results of the present study suggested that the lavender by-products from essential oil industry could be successfully valorized and serve as a source of pectic type polysaccharides.

Keywords: lavender; by-products; valorization; polysaccharides; CO_2 extraction

INTRODUCTION

The lavender is among the most processed crops by the essential oil manufacturers. The main industrially exploited species are the true lavender (*Lavandula angustifolia* Mill.), lavandin (*Lavandula x intermedia* Emeric ex Loiseleur) and spike lavender (*Lavandula spica* D.C.). Bulgaria, France, UK, China, Ukraine, Spain, and Morocco are the biggest worldwide producers of essential lavender oil. In the last years Bulgaria has overtaken on lavender plantations and lavender oil yield (around 100 tons produced yearly) the long-standing leader in this field France [1]. The main species grown in Bulgaria is the true lavender (*Lavandula angustifolia* Mill.). Due to the fact that the concentration of essential oil in the plant materials (0.8-1.3% / fresh plant) is relatively low, after extraction or distillation of the important biologically active substances large quantities of by-products remain. Throwing simply away or composting are among the very often used procedures to eliminate these by-products. But they could also serve as initial materials for recovery of valuable substances which could be used in the food, cosmetic and perfumery industries. Alternative methods of valorization have also been applied in recent years - fermentation of distilled lavender and biotransformation of terpene compounds into valuable and difficult to chemically synthesize substances [1-5], isolation of substances with strong antioxidant activity – apigenin, rosmarinic acid, luteolin, etc. [1, 6], as well as the use of ethanol extracts from lavandin by-products for potential antifungal activity against *Penicillium verrucosum* (Dierckx), a common microorganism causing loss in cheese production [1]. Pectic polysaccharides recovered from lavender essential oil industry by-products were not reported in the literature. Pectic polysaccharides have two major applications: as texturizing agent in food industry [7] and for medical purposes [8, 9]. When used as gelling agent and viscosity modifier several physicochemical parameters are important, such as surface tension, viscosity, molecular weight, composition, etc. and hence, the hypothesis behind the present work was to explore two lavender essential oil industry by-products as a source of pectic polysaccharides and to compare their properties.

Although lavender was already explored as a source of water-soluble pectic polysaccharides and they have expressed potent immunomodulating activities [8, 9], very few studies are currently

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available on isolation and determination of physico-chemical properties of polysaccharides of true lavender (*Lavandula angustifolia* Mill.) by-products. Additionally, in the literature are missing data on attempts for valorization of by-products after supercritical CO_2 extraction of lavender. These observations determined the aim of the present work – to explore the possibility of utilization of spent lavender biomass (obtained by traditional steam distillation and supercritical CO_2 extraction), with an emphasis on obtaining polysaccharides and to investigate their physico-chemical properties.

MATERIALS AND METHODS

Materials

The lavender by-products were provided by ECOMAAT distillery (Mirkovo, Bulgaria, 2018). The first one was residue from supercritical CO_2-extracted lavender (CO2-L), and the second one – after steam distillation of fresh lavender (SD-L). After treatment the SD-L waste was cooled down, inspected for elimination of impurities and dried at 50°C. The CO2-L waste was removed from the extraction cylinder and checked for impurities. Both wastes were stored at -18 °C until further treatment. All the solvents used were of analytical grade and purchased from local distributors.

Methods

Alcohol-insoluble residue (AIR) was prepared according to Slavov *et al.* [10]. Lavender AIR (70 g) was treated with 1.4 L of 0.1M HCl (pH=1.2) at 85ºC for 1 h while stirring (120 min^{-1}). The mixture was filtered through a nylon cloth (250 mesh), the solid residue was returned in the extraction vessel, 1 L of 0.1M HCl was added and extraction was allowed to proceed at the same conditions. The mass was filtered again and both filtrates were collected. The combined filtrate was precipitated with 3 volumes of 96% C_2H_5OH overnight at 4ºC, and the resulting precipitate was centrifuged (9660 RCF, 4ºC, 25 min). The supernatant was eliminated, the precipitate was dissolved in 150 mL of deionized water and extensively dialyzed (Spectra/Por 1, Breda, the Netherlands, Mr. cut-off 6000-8000 Da) for 72 h against deionized water. The mass remaining in the dialysis tube was lyophilized and represented acid-soluble polysaccharides from lavender by-products. The anhydrogalacturonic acid content (AUAC) was investigated using the *m*-hydroxydiphenyl method with D-galacturonic acid as standard, the degree of methylesterification (DM): according to Slavov *et al.* [7] and the degree of acetylation (DAc) was investigated spectrophotometrically using hydroxamic acid and *β*-D-glucose pentaacetate (Sigma-Aldrich Chemie Gmbh, Germany) as a standard [11]. The monosaccharide composition of the isolated polysaccharides was ddetermined as follows: 10 mg of polysaccharide was hydrolyzed with 15 mL of 2M trifluoroacetic acid (Sigma-Aldrich, Germany) for 3 h at 120 ºC. The trifluoroacetic acid was removed by evaporation to dryness under vacuum and to the dry mixture 10 mL deionized water was added and evaporated again (repeated three times). The residue from the last evaporation was dissolved in 1 mL of deionized water. The quantities of galactose, rhamnose, arabinose and fucose, galacturonic acid and glucuronic acid were determined on a chromatographic system ELITE LaChrome (Hitachi) HPLC with a VWR Hitachi Chromaster 5450 refractive index detector using Aminex HPX-85H column. The samples and standards were eluted with 5 mM H_2SO_4 (Sigma-Aldrich, Germany) at an elution rate of 0.5 mL min^{-1}, column temperature 50ºC, and detector temperature 35ºC. The amounts of xylose and mannose were determined separately with the same chromatographic system using Sugar SP0810 (Shodex®) column. The samples and standards were eluted with ultrapure water at an elution rate of 1.0 mL min^{-1}, column temperature 85ºC, and detector temperature 35ºC.

The protein content and molecular weight of polysaccharides were determined as described by Slavov *et al.* [12]. The viscosimetric measurements were performed according to Slavov *et al.* [13]. The polysaccharides foam-forming capabilities, emulsifying activity and emulsion stability of model systems were determined as described by Yancheva *et al.* [14].

Statistical analysis

The analyses were run three times, and the data were given as mean values. Statistical significance was determined by analysis of variance (ANOVA, Tukey's test; value of $p<0.05$ indicated statistical difference).

RESULTS AND DISCUSSION

The dried biomass obtained after fresh lavender processing: extraction with supercritical CO_2 and steam distillation, was subjected to 70% ethanol treatment and alcohol insoluble residues (AIR), CO2-L-AIR and SD-AIR, respectively, were obtained. From the AIRs polysaccharides were extracted by dilute hydrochloric acid and the yield and characteristics are presented in Table 1. The yield of polysaccharides from CO2-L AIR was higher (6.97±0.14%) and significantly different from the yield of polysaccharides isolated from SD-L AIR

(5.95±0.23%). The influence of the type of industrial treatment of the raw materials on the yield of polysaccharides was observed also in previous works where similar data were obtained for essential rose oil biomass [10], chamomile by-products [15] and pot marigold [12] used as a source of polysaccharides. The neutral sugar content of CO2-L polysaccharide was also slightly higher: 882.93±24.87 μg/mg, but statistically not different compared to SD-L acid-soluble polysaccharide (854.14±19.17 μg/mg). Both polysaccharides had a molecular mass in the 10^4 Da range. The DAc: of 2.7±0.4% and 2.3±0.1% for CO2-L and SD-L acid-soluble polysaccharides, respectively, was substantially lower compared to pectic polysaccharides obtained from chicory roots: 11%, endive: 19%, and sugar beet: 44% [16] and comparable with pectic polysaccharides obtained from pot marigold: 3.3-3.5% [12]. The AUAC content and DM values for both polysaccharides were similar (not statistically different).

Table 1. Extraction of AIRs with dilute HCl – yield and characteristics of the polysaccharides

AIR	Yield, %	AUAC, μg/mg	DM, %	DAc, %	Neutral sugars, μg/mg	Molecular weight × 10^4, Da	Proteins, μg/mg
CO2-L	6.97±0.14[a]	653.35±5.28[a]	53.7±1.5[a]	2.7±0.4[a]	882.93±24.87[a]	26.6±0.2[a]	8.6±0.8[a]
SD-L	5.95±0.23[b]	642.94±5.21[a]	48.9±2.5[b]	2.3±0.1[a]	854.14±19.17[a]	20.7±0.1[b]	16.4±0.9[b]

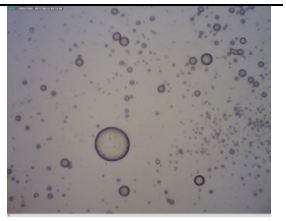
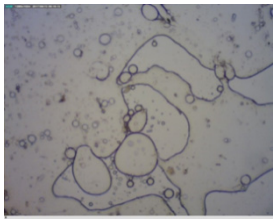
The results are expressed as mean from 3 repetitions ±SD; [a, b] Different letters after the values in a column mean statistical difference (Tukey's HSD test, $p < 0.05$).

Table 2. Monosaccharide composition of lavender by-products polysaccharides

	GlcA	GalA	Gal	Rha	Ara	Xyl	Man
	(μg/mg polysaccharide)						
CO2-L	11.91±0.19[a]	672.44±4.89[a]	172.32±1.56[a]	49.21±1.17[a]	44.28±1.06[a]	14.47±0.95[b]	29.19±1.05[a]
SD-L	10.25±0.18[a]	619.17±5.23[b]	150.35±1.48[b]	51.07±1.24[a]	37.64±1.25[b]	19.63±1.14[a]	28.36±1.24[a]

[a, b] Different letters in a column indicate significant differences (one-way ANOVA – Tukey's HSD test, $p < 0.05$)

Table 3. Physico-chemical properties of lavender by-products polysaccharides

Poly-saccharide	FF, %	σ, mN m^{-1}	ν, mm^2 s	Stability of emulsion		Microphotograms of emuslions
				EAI, m^2 g^{-1}	ESI, min	
SD-L	26.7±0.5[b]	58.5±1.2[b]	1.65±0.10[a]	8.6±0.5[b]	8.5±0.2[b]	
CO2-L	16.7±0.8[a]	63.2±1.4[a]	0.79±0.01[b]	11.3±0.6[a]	34.0±0.8[a]	

The results are expressed as mean from 3 repetitions ±SD; [a, b] Different letters after the values in a column mean statistical difference (Tukey's HSD test, $p < 0.05$).

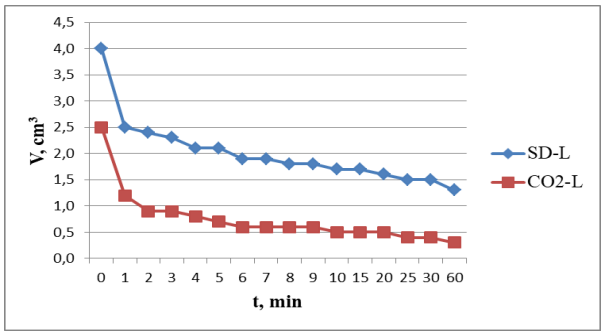


Figure 1. Foam-forming and foam-stabilizing properties of 0.6% polysaccharides solutions

In the next experiments the monosaccharide composition of the isolated acid-soluble polysaccharides was accessed by HPLC after hydrolysis with trifluoroacetic acid. The prevailing monosaccharide building the CO2-L (672.44±4.89 µg/mg polysaccharide) and SD-L (619.17±5.23 µg/mg polysaccharide) polysaccharides were statistically different and along with the other neutral monosaccharides found: galactose, rhamnose and arabinose) suggested that both polysaccharides were pectic type polysaccharides. Furthermore, several important physico-chemical properties of the polysaccharides were investigated. Firstly, the polysaccharides were dissolved in distilled water at 0.6% and foam-forming abilities (FF), surface tension (σ) and kinematic viscosity (ν) were determined (Table 2). The data in Table 3 imply that SD-L polysaccharide has the lowest surface tension (58.5±2.2 mN m^{-1}) of 0.6% aqueous solutions, higher kinematic viscosity: 1.65 mm^2 s and as expected from the data the SD-L solution has better foam-forming capabilities. For food, cosmetic and pharmaceutical applications it is important to utilize polysaccharides capable of increasing the viscosity of a disperse system (emulsion, suspension, foam), thus acting as stabilizers. The experimental results suggested that better foam-forming and foam-stabilizing properties showed 0.6% aqueous solutions of SD-L polysaccharides: 26.7% compared to 16.7% for CO2-L polysaccharides.

From the results it could be concluded that the SD-L acid-extractable polysaccharide showed better foam-forming and foam-stabilizing capabilities compared to CO2-L. This might be due to the higher amounts of proteins in SD-L and to the lower kinematic viscosity. In order to study the emulsifying capabilities of lavender polysaccharides emulsions of type oil in water (50% 0.6% polysaccharides in water and 50% oil) were prepared. The quality of the emulsions was studied by determining the index of emulsifiying activity (EAI, m^2 g^{-1}) and the stability index (ESI, min) – Table 3. The results from the analysis suggested that SD-L showed better emulsifying and emulsion-stability properties than the solution of CO2-L polysaccharide. The emulsions made with CO2-L were non-uniform, diffusive and merged oil droplets.

In the next experiments the emulsions of both polysaccharides were studied for their stability at four different temperatures (Figures 2 and 3).

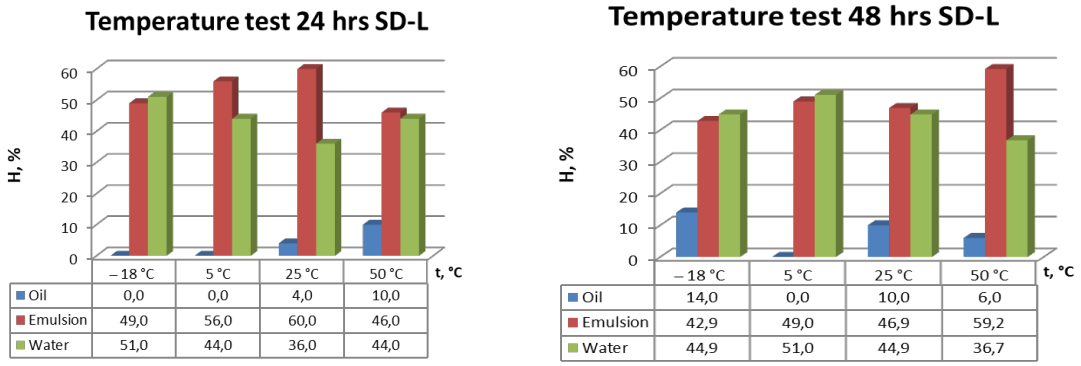


Figure 2. Temperature test for emulsion stability of SD-L at four temperatures

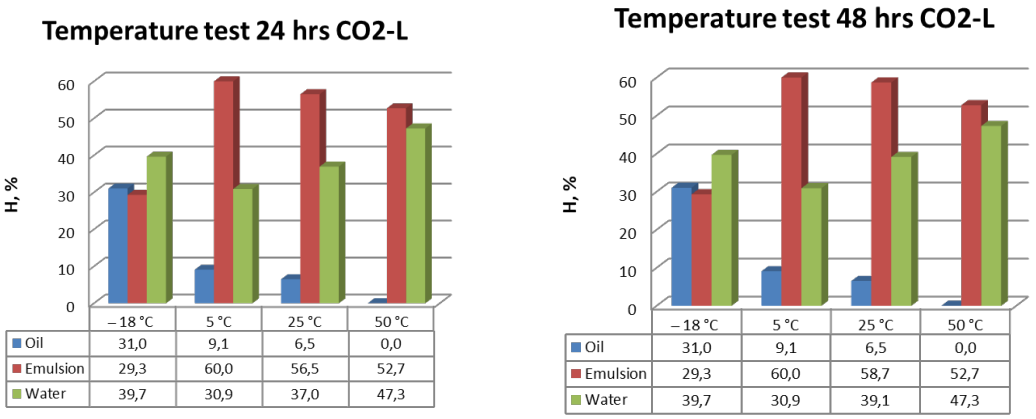


Figure 3. Temperature test for emulsion stability of CO2-L at four temperatures

The temperatures were chosen with regard to imitate the most common regimens of food preservation and keeping. Storage of the emulsions made with both polysaccharides at -18°C (freezing conditions) lead to destruction of the emulsions to a large extent: the amount of water measured was higher than the retained emulsion. At 5°C (storage in a fridge for foodstuffs) the emulsions made with CO2-L were more stable than the emulsions prepared with SD-L. For C2-L after 24- and 48-hour storage the emulsion retained was around 60% from the initial value. Furthermore, the emulsions were investigated for mechanical stability by performing centrifugal test (Figure 4).

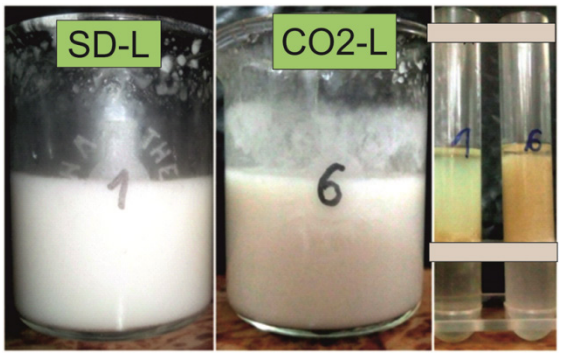


Figure 4. Centrifugal test for emulsion stability of SD-L and CO2-L. Left – 50% oil in water emulsion with 0.6% aqueous solutions of SD-L and CO2-L immediately after emulsification; To the right – after centrifugation at 5000 rpm for 20 min

The results from the centrifugal test (which imitates mechanical destabilization of the emulsions) suggested that both emulsions were destroyed at the tested conditions. The aqueous phases from both CO2-L and SD-L emulsions were comparable. The emulsion phase retained was visually higher for CO2-L emulsion but the conclusion which can be made from the test is that both emulsions were not stable and could be destroyed when mechanically disturbed.

Nevertheless, the polysaccharides extracted by dilute HCl acid from two industrial lavender wastes could be successfully utilized in food systems as viscosity modifiers and for formulation of new functionalized products.

CONCLUSIONS

The present study focuses on two plant wastes from essential oil industry: one obtained from steam-distilled lavender (SD-L) and one from subcritical CO_2 extraction of lavender (CO2-L). The lavender wastes were utilized as a source of polysaccharides. The physico-chemical properties of the polysaccharides were investigated and it was suggested that SD-L polysaccharides had better foam-forming abilities than CO2-L. The surface tension of SD-L 58.5±1.2 mN m^{-1} was lower compared to CO2-L (63.2±1.4 mN m^{-1}) which suggests that SD-L polysaccharides have better emulsification properties. The monosaccharide profile determined by HPLC revealed presence predominantly of galacturonic acid: 672.44±4.89 µg/mg and 619.17±5.23 µg/mg polysaccharide for CO2-L and SD-L, respectively. For the first time lavender residues from CO_2-extraction were investigated as a source of acid-extractable polysaccharides, and comparison with SD-L polysaccharides was performed. In general, the results suggested that the lavender waste could be utilized as a source of pectic polysaccharides and has the potential for application in the food industry as additives for foams, emulsions and viscosity modifiers.

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REFERENCES

1. L. Lesage-Meessen, M. Bou, J.C. Sigoillot, C.B. Faulds, A. Lomascolo, *Appl. Microbiol. Biotechnol.,* **99**, 3375 (2015).
2. P. P. Daramwar, R. Rincy, S. Niloferjahan, R. Krithika, A. Gulati, A. Yadav, R. Sharma, H. V. Thulasiram, *Bioresour. Technol.,* **115**, 70 (2012).
3. G. O. Elguea-Culebras, R. Sanchez-Vioque, O. Santana-Meridas, D. Herraiz-Penalver, M. Carmona, M. I. Berruga, *LWT*, **73**, 226 (2016).
4. S. Marumoto, M. Miyazawa, *Tetrahedron*, **67**, 495 (2011).
5. Y. Noma, Y. Asakawa, in: Handbook of essential oils: science, technology and applications, K. H. C. Baser, G. Buchbauer (eds.), Taylor & Francis Group, Boca Raton, 2010, p. 585.
6. I. Méndez-Tovar, B. Herrero, S. Pérez-Magariño, J. A. Pereira, M. C. Asensio-S.-Manzanera, *J. Food Drug Anal.*, **23**, 225 (2015).
7. A. Slavov, M. J. Crépeau, S. Durand, C. Garnier, J.-F. Thibault, E. Bonnin, in: Pectins and Pectinases, H. A. Schols, R. G. F. Visser, A. G. J. Voragen (eds.), Wageningen Academic Publishers, Wageningen, 2009, p. 127.
8. Y. N. Georgiev, M. H. Ognyanov, H. Kiyohara, T. G. Batsalova, B. M. Dzhambazov, M. Ciz, P. N. Denev, H. Yamada, B. S. Paulsen, O. Vasicek, A. Lojek, H. Barsett, D. Antonova, M. G Kratchanova, *Int. J. Biol. Macromol.*, **105**, 730 (2017).
9. Y. N. Georgiev, B. S. Paulsen, H. Kiyohara, M. Ciz, M. H. Ognyanov, O. Vasicek, F. Rise, H. Yamada, P. N. Denev, A. Lojek, V. Kussovski, H. Barsett, A. I. Krastanov, I. Z. Yanakieva, M. G Kratchanova, *Carbohydr. Polym.*, **174**, 948 (2017).
10. A. Slavov, P. Denev, I. Panchev, V. Shikov, N. Nenov, N. Yantcheva, I. Vasileva, *Ind. Crops Prod.,* **100**, 85 (2017).
11. E. A. McComb, R.M. McCready, *Anal. Chem.*, **29(5)**, 819 (1957).
12. A. Slavov, M. Ognyanov, I. Vasileva, *Food Hydrocoll.*, **101**, 105545 (2020).
13. A. Slavov, I. Panchev, D. Kovacheva, I. Vasileva, *Food Hydrocoll.*, **61**, 469 (2016).
14. N. Yancheva, D. Markova, D. Murdzheva, I. Vasileva, A. Slavov, *Acta Sci. Nat.*, **3(1)**, 7 (2016).
15. A. Slavov, N. Yantcheva, I. Vasileva, *Waste Biomass Valor.*, **10(9)**, 2583 (2019).
16. A. Zykwinska, M.H. Boiffard, H. Kontkanen, J. Buchert, J.-F. Thibault, E. Bonnin, *J. Agric. Food Chem.*, **56**, 8926 (2008).

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Biosynthesis and potential application of silver and gold nanoparticles to the electroanalysis of hydrogen peroxide and nitrite

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Recently, nanotechnology has become one of the most active research fields in the areas of material science, chemistry and electrocatalysis. Metal nanoparticles (NPs) are of great scientific interest because of their unique properties. Due to their extremely small size and large specific surface area, metal NPs exhibit unusual physicochemical and electronic properties, compared to those of bulk metals. Nowadays, the development of NPs-based electrochemical sensor devices has drawn great attention in the field of industrial, environmental, clinical and food analysis owing to their attractive performances: fast response, convenient operation, high sensitivity and selectivity. The electrochemical sensors offer possibility of miniaturization and potential for development of portable hand-held devices for real time monitoring. Recently, extensive studies have been performed to explore the electrochemical behavior of biosynthesized metal NPs and their potential in sensor design. Current review article features recent advancements in the plant-mediated synthesis of silver and gold nanoparticles (AgNPs and AuNPs), applicable in the electrochemical sensing of hydrogen peroxide (H_2O_2) and nitrite (NO_2^-). Additionally, perspectives and challenges for promoting the development of electrochemical sensors based on bio-mediated nanostructured materials are commented.

Keywords: electrochemical sensor, green synthesis, plant-mediated synthesis, metal nanoparticles, hydrogen peroxide, nitrite.

INTRODUCTION

Electroanalytical chemistry, also known as electroanalysis, lies at the interface between analytical science and electrochemistry. It is concerned with the development, characterisation and application of chemical analysis methods employing electrochemical phenomena [1]. The electrochemical sensor devices are rapid and precise analytical tools that play an essential role in the fields of industrial analysis, environmental monitoring, and clinical analysis [2, 3]. Electrochemical sensors have great impact because of their relatively simple design, low cost, simplicity of use, high sensitivity, low detection limits, fast response time, long-term stability, miniaturization potential for development into portable/handheld devices, and possibility of on-site monitoring.

Although electrochemical techniques are versatile, voltammetry and amperometry are favored in sensing application. Typical voltammetry methods used in electrochemical sensing include cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV). The current signal is produced by the oxidation/or reduction of electroactive species (analyte) at the surface of the working electrode and the value of current magnitude is proportional to the concentration of the analyte present in the sample.

Carbon-based electrode materials (glassy carbon, graphites, etc.) have been widely explored in electroanalytical applications, especially in electrochemical sensor design because of their excellent properties such as chemical inertness, wide potential window and low cost. The unmodified carbon surfaces exhibit slow electron transfer and often lack the sensitivity and selectivity required for the electrochemical detection. In order to overcome this shortcoming, nanomaterials have been incorporated within the electrode modifiers. Thus, the current research is mainly focused on the electrode modifications in order to increase the electron transfer kinetics and to decrease high overpotentials required for redox reactions of the target analyte on unmodified carbon electrode materials. The use of nano-sized materials (metal NPs, metal oxide NPs, alloy NPs, etc.) provide an enhanced sensor response, lower detection limit, better selectivity and sensitivity toward the detection of specific analyte due to increased surface area, enhanced electrocatalytic activity and faster electron transfer kinetics. The most commonly used metal NPs as electron-transfer mediators are AgNPs and AuNPs due to their unique physicochemical properties, good conductivity and electrocatalytic activity.

Recently, development of novel eco-friendly and cost-effective methods for synthesis of metal

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NPs has remained in the focus of researchers. Plant-mediated green synthesis of metal NPs involves the use of extracts of plant parts (leaves, flowers, fruits, roots) for the bioreduction of metal ions into their zero-valent elemental form in the size range 1–100 nm. Plant-mediated synthesis of metal NPs is distinguished by ecologic effectiveness (uses aqueous solvents, easily available plant material, biocompatible plant extracts, normal temperature and pressure), simple one-step formation and stabilization of metal NPs, as well as a possibility for waste biomass valorization.

Considerable efforts have been devoted to developing green strategies for facile synthesis of AgNPs and AuNPs. There is convincing evidence that green synthesis of metal NPs has potential to provide a new direction in the fabrication of cheap and highly effective electrode-catalysts. This review is aimed to summarize recent development of plant-mediated strategies for the sustainable production of AgNPs and AuNPs as alternative efficient green methods. Then, the application of biosynthesized AgNPs and AuNPs as electrocalysts in constructing non-enzymatic electrochemical sensors for H_2O_2 and nitrite is discussed in detail.

Green synthesis of metal nanoparticles by plant extracts

Using plant extracts for the production assembly of metal NPs has drawn attention because its rapid, eco-friendly, non-pathogenic, and economic protocol provides a single-step technique for the biosynthetic process [4, 5]. The data show that the synthesis of metallic NPs using plant extracts is simpler, easier to scale up, and less expensive than that using bacteria or fungi. A wide range of molecules present in the plant extracts, ranging from proteins to various low-molecular weight compounds as flavonoids, polyphenols, terpenoids, alkaloids, amino acids, organic acids have been reported to play a role in the bioreduction of metal salts. Generally, during the synthesis, the reducing agents donate electrons to the metal ions and convert them to NPs. These NPs exist at a high-surface energy state and tend to convert to their low-surface energy conformations by aggregating against each other. Thus, the presence of higher amounts of reducing and stabilizing agents prevents the aggregation of NPs and promotes production of smaller NPs. Additionally, proteins can trap metal ions on their surface and convert them to their corresponding nuclei, which could further aggregate and, consequently, form metal NPs [6].

A number of experiments have shown that the source of the plant extract affects the characteristics of metal NPs because different extracts may contain different combinations and concentrations of organic reducing agents. Therefore, the size and shape of biosynthesized NPs can be controlled by modifying the nature and concentration of the plant extract, concentration of the precursor (metal salt), temperature, pH and reaction time. Fig. 1(A) shows the basic steps in the plant-mediated synthesis of metal NPs and the subsequent modification of electrode surface through a dropwise addition of the resulting colloidal solution.

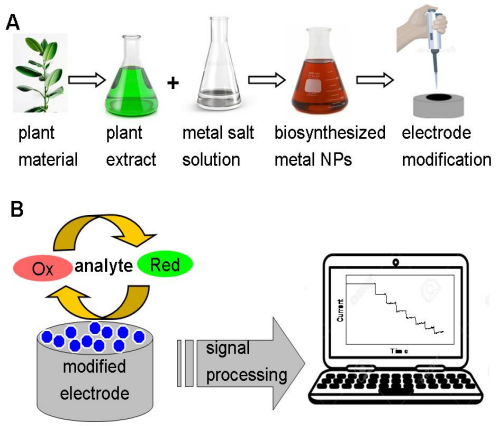

Fig. 1. Illustrative representation of: A) preparation of electrode modified with biosynthesized metal NPs; B) general principle of electrochemical detection.

The sensing system uses a three-electrode system connected to a potentiostat. The general principle of electrochemical detection is illustrated on Fig. 1(B). The electrochemical reactions take place at the electrode interface by means of a heterogeneous electron transfer. Briefly, at the surface of the working electrode the analyte participates in a redox reaction catalyzed by the electrode modifier (biosynthesized metal NPs acting as an electron transfer mediator between the electrode and a reaction substrate). The current generated in the process is converted into a signal that could be processed and displayed easily. Analyzing the signal magnitude we can obtain information about the concentration of the substance being analyzed.

Electrochemical H_2O_2 sensors based on biosynthesized silver nanoparticles

Fast, reliable and accurate detection of H_2O_2 is one of the topical problems in analytical chemistry, since H_2O_2 is an important analytical target in the field of environmental chemistry, industry, clinical analysis, food chemistry and biochemistry. In the past decades, enzyme-free electrochemical sensors have been studied increasingly because of their high performance for H_2O_2 detection. Today, the development of newly advanced greenly synthesized electrode materials for reliable H_2O_2

detection is essential. Considerable research interest has been focused on the subject how to modify the electrode surface using biosynthesized metal NPs for the purpose of obtaining a sensor with high sensitivity, selectivity and stability.

Salve *et al.* have used *Tagates erecta* (Marigold) flowers extract for AgNPs synthesis by a green route [7]. The morphology and crystal structure of the prepared nanomaterial was characterized by field emission scanning electron microscopy (FESEM), ultraviolet–visible spectroscopy (UV–Vis), elemental dispersive X-Ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS). The UV-Vis studies showed the occurrence of an absorption band at 430 nm which is specific for AgNPs. FESEM analysis indicated that the biosynthesized AgNPs have a homogenous size distribution and XRD patterns reflected that the particles are crystalline in nature with a face-centered cubic structure. Using the biosynthesized AgNPs and chitosan (CS), modified pencil graphite electrode (PGE) was fabricated by a drop-casting method and the as-prepared hybrid material AgNPs/CS/PGE was used for supercapacitator and electrochemical sensing applications. It has been shown that the modified electrode AgNPs/CS/PGE possesses remarkable catalytic activity towards electrochemical reduction of H_2O_2. Cyclic voltammetry measurements in electrolyte 0.1 M HCl/KCl (pH 2.0) demonstrated that effective electroreduction of H_2O_2 was realized and the sensor based on biosynthesized AgNPs exhibited a high sensing performance for H_2O_2 detection in the concentration range 1.0 – 10.0 μM with a detection limit of 0.52 μM and sensitivity of 0.129 mA μM^{-1}. Furthermore, the proposed sensor was used to detect H_2O_2 in cosmetic as well as in medical samples with high accuracy and selectivity, making it a good choice in the development of a disposable, low-cost device for H_2O_2 detection. The result obtained gave appreciable recovery value suggesting high sensitivity of the electrode towards H_2O_2.

The essential oil industry wastes are rich in non-volatile polar metabolites (flavonoids, organic acids, carbohydrates, amino acids, etc.) that have reduction properties and could influence the synthesis and stabilization of AgNPs. Dodevska *et al.* have reported for the first time utilization of *Rosa damascena* waste for synthesis of AgNPs and demonstated the applicability of the biosynthesized AgNPs for development of electrochemical sensors for H_2O_2 and vanillin [8]. TEM micrographs showed that using an aqueous extract of *Rosa damascena* AgNPs were obtained as sphere-like particles with an average size calculated to be 25.8 ± 11.5 nm. Biosynthesized AgNPs were deposited onto a spectroscopic graphite (Gr) electrode and the electroactive layer was stabilized by applying a thin film of chitosan onto the modified electrode surface. Chitosan is a preferable low-cost material in designing electrochemical sensors and biosensors. It is a natural linear amine-rich polysaccharide, biocompatible polymer distinguished by its membrane-forming ability, good adhesion and mechanical strength. Chitosan adsorption on the surfaces of metal NPs can stabilize and protect the nanoparticles. In the present case, the coverage of chitosan on the newly generated AgNPs will prevent it from growing further, and also stabilize the size and surface property of the AgNPs in the cluster during its applications. The electrochemical behaviour of the modified electrode AgNPs/CS/Gr was studied by means of CV, DPV and chronoamperometry in neutral medium and its applicability for quantitative detection of H_2O_2 and vanillin was investigated. Vanillin (4-hydroxy-3-methoxybenzaldehyde) has a specific aroma and it is one of the most commonly used food supplements. For adults the permissible daily intake of vanillin is less than 10 mg kg^{-1}; the addition of vanillin in baby formula and infant food is not permited. The overweight content of vanillin in food products, as the excessive ingestion via the dietary intake has potential toxic effect. Therefore, the reliable and accurate detection of vanillin has become an important research topic in food safety control. Electrochemical studies suggested that graphite electrode modified with AgNPs, biosynthesized using *Rosa damascena* waste, possesses a stable response to vanillin up to 0.5 mM with a detection limit of 8.4 μM at an applied potential of 0.58 V (vs. Ag/AgCl, 3 M KCl). Additionally, the developed electrode exhibited a sensitive and reproducible response for quantitative determination of H_2O_2. Amperometric measurements at a constant potential of –0.3 V showed a highly sensitive response to H_2O_2 up to 6.6 mM.

Electrochemical studies with AgNPs biosynthesized using flower aqueous extracts of *Achillea millefolium* and *Lavandula angustifolia* wastes as reducing agents, which is a novel simple approach, inexpensive and eco-friendly in nature, also were reported [9]. The representative electron micrographs of AgNPs showed that the nanoparticles grew very tiny with spherical shape. From the presented histograms it can be seen the

size distribution of AgNPs and their mean sizes were 2.8 nm for AgNPs/*Achillea millefolium* and 3.1 nm for AgNPs/*Lavandula angustifolia*, respectively. Selected area electron diffraction (SAED) pattern represents the (111), (220) and (222) crystal planes of the cubic structure of AgNPs in both samples. Biosynthesized AgNPs were deposited onto a spectroscopic graphite surface, applying two different procedures, and stabilized using chitosan to build new electrocatalysts. The electrochemical performance of the modified electrodes was studied by means of CV and chronoamperometry and their applicability for amperometric detection of H_2O_2 was demonstrated. The modified electrodes showed a remarkable activity at applied potentials of –0.3 V and –0.2 V (vs. Ag/AgCl, 3 M KCl), rapid, stable and reproducible amperometric response. It was stated that amperometry at a constant potential of –0.3 V is distinguished by extremely high sensitivity of 533.5 μA mM^{-1} cm^{-2} up to 4.3 mM H_2O_2. In order to study the selectivity, the amperometric response was examined in the presence of common interfering species (nitrate, glucose, uric acid, ascorbic acid, citric acid). The presented authentic record of the electrode signal clearly shows that the tested species have no effect on the H_2O_2 detection – no response was observed in the presence of the above mentioned substances and the current response for H_2O_2, registered after adding the substances, corresponds to the one determined in the calibration study. The results prove that the modified electrode has good selectivity for H_2O_2 and reveal the application potential of the so-biosynthesized AgNPs for electrochemical sensing of H_2O_2 in real samples.

Integrating graphene materials with metal NPs can offer synergistic effects, which could effectively enhance the catalytic performance of sensor systems [10]. Salazar *et al.* reported a one-step green strategy to obtain nanocomposite rGO/AgNPs using green tea extract for reducing both silver ions (Ag^+) and graphene oxide (GO) sheets [11]. TEM analysis showed that the biosynthesized AgNPs have a quasi-spherical shape and an average size of 25 nm. Glassy carbon (GC) electrode was conveniently modified with rGO/AgNPs nanohybrid and electrochemical tests were carried out to study the properties of rGO/AgNPs/GC towards H_2O_2 detection. Amperometric studies at an applied potential of –0.4 V (*vs*. Ag/AgCl) in 0.1 M PBS (pH 8.0), revealed that the rGO/AgNPs/GC sensor possesses high sensitivity (236 μA mM^{-1} cm^{-2}, $R^2 = 0.999$) in a wide linear range of 0.002 – 20 mM, rapid response (~ 2 s) and low detection limit of 0.73 μM H_2O_2 (signal-to-noise S/N = 3). The selectivity of the modified electrode was tested against different biological interferences including dopamine, glutamate, glucose and ascorbic acid with promising results. In the presented study, rGO introduces interfacial phenomena that greatly improve sensor sensitivity, selectivity, response time and limit of detection. The improved electrical conductivity, as well as the high density of edge-plane defect sites on rGO are valuable for accelerating electron transfer between the electrode and H_2O_2 molecules, leading to the superior electrocatalytic activity and selectivity towards H_2O_2 detection. The practical applicability of the developed rGO/AgNPs/GC sensor to detect selectively H_2O_2 in real samples (antiseptic solutions, commercial milk and urine) was tested and satisfactory results were obtained. After 7-month storage it was established that rGO/AgNPs/GC has no significant loss of sensitivity.

Other researchers have also shown that the biomediated approach provides a promising platform for the application of rGO-based composites in the non-enzymatic amperometric sensor field [12-14]. Kumar *et al.* have developed an Ag–Au–rGO composite through a facile and green reduction process using *Azadirachta indica* extract [12]. The morphological and structural characterization revealed that the prepared composite exhibited the unique features of uniformly distributed nanoparticles with alloy structure over the rGO sheets. The authors have proven that the electron transfer kinetics were enhanced for Ag–Au–rGO/GC over the GO/GC, rGO/GC and Ag–rGO/GC. The rGO supported Ag–Au bimetallic nanoparticle based non-enzymatic H_2O_2 sensor exhibited good selectivity at an applied potential of –0.4 V (*vs*. Ag/AgCl). Compared to the work [11] cited above, Kumar *et al.* have reported a shorter linear range (0.1 – 5 mM) and a higher detection limit (1 μM) towards the amperometric detection of H_2O_2. Unfortunately, the authors did not provide data on the sensitivity and stability of the Ag–Au–rGO/GC electrode, as well as on the sensing ability of the prepared Ag–Au–rGO composite towards H_2O_2 in real samples.

A facile synthesis of reduced graphene oxide-silver nanocomposite (rGO-Ag) was carried out from *Plectranthus amboinicus* leaf extract [13]. TEM observation revealed that the AgNPs formed are in spherical shape with diameter of 22.5 nm and uniformly decorated themselves on the rGO surface. In contrast to both articles [11, 12]

commented above, Zheng *et al.* did not use the hazardous reagent dimethyl formamide (DMF) during the electrode surface modification. At an applied potential of –0.32 V (*vs*. Ag/AgCl) the modified rGO-Ag/GC attains a steady-state current within 5 s, suggesting that the fabricated sensor has a rapid response towards H_2O_2. A linear relationship between the current response and H_2O_2 concentration (0.1804 μA μM^{-1}) was observed in the range of 1 – 800 μM; the detection limit was calculated to be 0.312 μM. The amperometric records presented in both commented articles [12, 13] clearly show that organic species such as ascorbic acid, dopamine, glucose, uric acid, etc., have no effect on the quantitative H_2O_2 determination. The results reveal the application potential of the modified electrodes Ag–Au–rGO/GC [12] and rGO-Ag/GC [13] for precise sensing of H_2O_2 in real samples.

The electrochemical enzyme-free H_2O_2 sensors based on biosynthesized AgNPs and the corresponding sensing performances are summarized in Table 1.

Electrochemical nitrite sensors based on biosynthesized silver and gold nanoparticles

Eletrochemical detection of nitrite ion (NO_2^-) is significant due to its environmental and biological-related issues. Nitrite is frequently found in industrial waste water and fertilizers. Potassium nitrite (KNO_2) and sodium nitrite ($NaNO_2$) are listed as permitted food additives (E249 and E250, respectively). KNO_2 and $NaNO_2$ are widely used as preservatives in the preparation of cured meat products – nitrite has a pronounced antimicrobial activity, acts as a color fixative and inhibits lipid oxidation, thereby slowing meat spoiling. Nitrite intake is associated with a higher relative risk of gastric cancer and colorectal cancer, since NO_2^- is a precursor of carcinogenic nitrosamine. Nitrite is toxic and mutagenic to both humans and animals. Therefore, NO_2^- detection is important for environmental security, public health and food quality control. Owing to the rapid response and simple use, electrochemical techniques are favorable for nitrite detection [16]. To date, very few data on the nitrite sensors based on biosynthesized AgNPs and AuNPs, are available. Shivakumar *et al.* reported on a facile, cost effective, green synthesis method of silver nanospheres (AgNS) by using pre-hydrolyzed liquor (PHL) from the *Nilgiri* wood generated by pulp industry [17]. XRD pattern of AgNS evidences face-centered cubic crystalline structure of metallic silver; the average crystallite size of AgNS calculated from Scherrer equation was found to be ~30 nm. Authors suggested that hemicelluloses present in PHL were responsible for the reduction of Ag^+ and the subsequent stabilization of biosynthesized AgNS. Repeated synthesis and characterization of AgNS from different batches of the PHL demonstrated the reproducibility with the current material and method. Glassy carbon electrode modified with the so-produced AgNS exhibits excellent electrocatalytic activity towards nitrite oxidation – low detection limit of 31 nM and high electrode sensitivity of 580 μA mM^{-1} cm^{-2} in the concentration range 0.1 – 8.0 μM. The modified electrode is selective for NO_2^- and most of the common interferents do not affect the quantitative nitrite determination.

Table 1. Operational characteristics of non-enzymatic H_2O_2 electrochemical sensors based on biosynthesized AgNPs.

Modified electrode	Reducing agent	Sensitivity, (μA mM^{-1} cm^{-2})	Linear range, (mM)	LOD, (μM)	Ref.
AgNPs/CS/Gr	*Achillea millefolium*	533.5	up to 4.3	–	9
AgNPs/CS/Gr	*Lavandula angustifolia*	374.7	up to 3.5	–	9
AgNPs/CS/PGE	*Tagetes erecta*	0.129 mA μM^{-1}	0.001 – 0.01	0.52	7
AgNPs/rGO/GC	*Green tea*	236	0.002 – 20	0.73	11
AgNPs/CS/Gr	*Rosa damascena*	115.2	up to 6.6	–	8
AgNPs/Gr	*Rosa damascena*	214.7	up to 3.9	–	8
AgAu/rGO/GC	*Azadirachta indica*	–	0.1 – 5	1	12
AgNPs/rGO/GC	*Plectranthus amboinicus*	–	0.001 – 0.8	0.312	13
AgNPs/GO/GC	*Callicarpa maingayi*	–	0.005 – 0.7	0.6	14
AgNPs/rGO/GC	*Rumex roseus*	64 mA mM^{-1}	0.035 – 1.95	1.1	15

LOD (limit of detection); CS (chitosan); GC (glassy carbon); Gr (graphite); GO (graphene oxide); rGO (reduced graphene oxide) PGE (pencil graphite electrode).

The here presented electrode retains up to 98 % of its initial activity after a period of 1 month. The sensor demonstrates successful nitrite detection in real samples with good stability and reproducibility. The feasibility of employing the sensing system for real sample (tap water) analysis was explored by the standard-additions method. Satisfactory recoveries (average recovery ranging from 98 – 106 %, n = 3) of nitrite concentrations were obtained indicating the viability of employing the sensor for real sample analysis. Reproducibility of the catalytic activity of AgNS/GC was tested by carrying out the nitrite oxidation with four different GC electrodes loaded with the same amount of AgNS. The oxidation currents for nitrite at 0.86 V at these four electrodes show a very small change with a RSD of 3.6 % (three repeated experiments for each electrode). The authors concluded that AgNS/GCE can serve as a reliable platform for long-term application towards electrochemical nitrite sensing.

A facile and environmentally benign method exploiting *Piper betle* biomass as a reducing and stabilizing agent was proposed for the green preparation of AgNPs by Ramachandran *et al.* [18]. AgNPs biosynthesized from dried *Piper betle* leaves extract exhibited face-centered cubic structure with preferred (111) orientation and average particle size of 20 nm associated with homogeneous distribution. The fabricated AgNPs/GC sensor possesses electrocatalytic activity toward nitrite oxidation with a response time of 10 s, high sensitivity of 1642.27 μA mM^{-1} cm^{-2} and detection limit of 0.046 μM. The amperometric current response exhibited linearity over a wide range of nitrite concentrations from 1 μM to 6 mM. However, in that paper, a significantly higher applied potential (1.0 V *vs.* Ag/AgCl) was necessary for achieving effective detection of NO_2^-. The operational stability of the fabricated AgNPs/GC was evaluated for 30 days with an interval of a day (the electrode was stored in normal atmospheric conditions, when not in use) and was found to be 91.2 % of its original response after one month. Although the constructed sensor exhibited long-term stability and good anti-interference properties, quantitative determination of nitrite in real samples was not reported.

AuNPs have attracted much attention due to their remarkable properties including high mechanical stability, unique tunable optical and electronic properties, high electrical conductivity, and catalytic activity. Mohd Taib *et al.* have described a new, reliable, environmentally friendly and cost-effective green procedure for synthesis of AuNPs using an aqueous extract of *Hibiscus sabdariffa* leaves [19]. The authors suggested that chlorogenic acid (an ester of caffeic acid and quinic acid) in *H. sabdariffa* extract is the major compound involved in the reduction of Au^{3+} to Au^0. TEM analysis confirmed that the biosynthesized AuNPs were formed with a narrow distribution and an average particle size of 7 ± 2 nm. In order to fabricate a modified electrode-catalyst, the dispersed solution containing AuNPs was sonicated for 30 min before immobilizing the NPs on the surface of the GC electrode by a casting method. The so-prepared electrode AuNPs/GC was left in an oven at 55 °C for 6 h and then was tested as a catalyst in the electrooxidation of nitrite. At a voltage of 0.8 V, the electrode detects nitrite in the range of 0.37 to 10 mM (LOD = 0.11 mM) and the sensitivity is 917 ± 30 μA mM^{-1} cm^{-2}. The obtained results confirmed that the prepared AuNPs/GC has adequate stability, repeatability and reproducibility and could be used for determination of nitrite. After 3-week storage the prepared electrode possesses around 80 % of its initial response. Analysis of ten sequentially prepared electrodes showed an RSD of 4.27 % which confirmed the repeatability of AuNPs/GC. The sensor-to-sensor reproducibility was investigated by measuring the current responses of five diverse electrodes prepared independently by the same procedure. The results showed that the response produced by different electrodes had a good reproducibility with RSD of 4.21 % and the authors concluded that the sensor fabrication methodology was reliable. The sensor performance towards real samples was studied through recovery studies by adding known concentrations of nitrite to tap water samples and mineral water. According to the data presented and considering the RSD values, as well as calculated recoveries, it can be concluded that the AuNPs/GC holds possible applications for evaluating specific concentration range of nitrite ions.

Table 2 provides an overview of the nitrite electrochemical sensors based on biosynthesized AgNPs and AuNPs. Unpublished results of our work in progress were also included. Extensive studies have been performed to explore the electrochemical behavior of biosynthesized AgNPs and AuNPs (*Rosa damascena* – mediated synthesis) and their potential applications for electrochemical quantitative detection of nitrite.

Table 2. Operational characteristics of enzyme-free NO_2^- electrochemical sensors based on biosynthesized AgNPs and AuNPs.

Modified electrode	Reducing agent	Sensitivity, ($\mu A\ mM^{-1}\ cm^{-2}$)	Linear range, (mM)	LOD, (μM)	Ref.
AgNS/GC	*Nilgiri* wood	580	0.0001 – 0.008	0.031	17
AgNPs/GC	*Piper betle*	1642	0.001 – 6	0.046	18
AuNPs/GC	*Hibiscus sabdariffa*	917	0.37 – 10	110	19
AgNPs/Ch/Gr	*Rosa damascena*	224.4	0.02 – 1.7	10	work in
AuNPs/Ch/Gr	*Rosa damascena*	156.8	0.05 – 3.7	20	progress

CLOSING REMARKS

Green development of metallic nanoparticles, particularly AgNPs and AuNPs, is important to avoid the adverse effects on the environment that are commonly associated with chemical synthesis of metallic nanoparticles. Adhering to the principles of green chemistry, a number of research groups have used plant extracts for safe and fast synthesis of stable metal NPs. This approach completely avoids the hazardous solvents/ surfactants, does not require external stabilizing agents because biogenic components of plants themselves act as stabilising, as well as capping agents; thus enables a clean and sustainable synthesis. Biomediated metal NPs are receiving increasing interest for sensor construction in recent years. In this review article we highlighted the application of biosynthesized AgNPs and AuNPs as electrocalysts for non-enzymatic electrochemical quantitative detection of H_2O_2 and nitrite. According to Web of Science database (by end-November, 2021) the number of original research articles reporting on AgNPs-based non-enzymatic electrochemical sensors for H_2O_2 is 41 (publication years 2015-2021). Only 8 studies on electrochemical sensing platforms based on biosynthesized AgNPs were identified by conducting a search of current literature. Although relatively few studies have appeared on these issues to date, the initial results are promising. The experimental data reveal that the sensors exhibit fast amperometric sensing, low detection limit, wide linear range, high sensitivity and good selectivity. Therefore, we can conclude that bio-mediated nanostructured electrode materials offer encouraging electrochemical results paving new dimensions for future research in this field.

However, there are still some obstacles to overcome compared to the conventional synthesis methods. Generally, the main challenges encountered in the development of electrochemical sensors based on biosynthesized metal NPs can be listed as follows:

1/ Optimization of plant-mediated synthesis protocols. Every plant extract varies in its capabilities to supply metallic NPs. It is well known that some properties of metal NPs that are critical to their specific applications may vary significantly, depending on the green production method used. For research works on the specific applications of NPs in electroanalysis, it is important to select a reproducibile synthesis technique based on the properties required for the targeted electrochemical reaction. A main limitation has been the control over crystal phase, sizes and shapes of greenly produced metal NPs that are mostly predetermined by different phytochemical compositions in the plant. Therefore, researchers should refine the plant-mediated synthesis protocols.

2/ Optimization of electrode modification protocols to give an improved electrochemical response. For electrochemical sensing purposes, electrode modification procedure should provide stable, reproducible, sensitive and selective analytical response. Also sensor-to-sensor reproducibility is extremely important. However, using various plant extracts it is difficult to control the bio-mediated synthesis and immobilization of the resulting metal NPs with varying populations of size and shape. Hence, the surface morphology of these nanomaterials might differ between each modified electrode. Moreover, when using nanomaterials, operational and long-term stability can become a major concern due to issues related to aggregation and flaking of NPs-modified layers on the electrode surface.

Furthermore, efforts should be devoted to optimization of electrode modification procedures to improve the electron transfer rate and to enhance the anti-interference ability of the sensors, suppressing the non-specific adsorption of interfering species. It is important for researchers to keep on developing innovative solutions in order to create more reliable sensors.

Further studies should be performed to investigate the potential of biogenic AgNPs and AuNPs in design of electrochemical sensors with advanced properties and emerging practical applications.

REFERENCES

1. A. C. Power, A. Morrin, Electroanalytical Sensor Technology, Electrochemistry, Mohammed A. A. Khalid (ed.), IntechOpen, DOI: 10.5772/51480 (2013).
2. R. Ramachandran, T.-W. Chen, S.-M. Chen, T. Baskar, R. Kannan, P. Elumalai, P. Raja, T. Jeyapragasam, K. Dinakaran, G. Gnana kumar, *Inorg. Chem. Front*., **6**, 3418 (2019).
3. K. Nemčeková, J. Labuda, *Mater. Sci. Eng. C*, **120**, 111751 (2021).
4. S. Ahmed, M. Ahmad, B. L. Swami, S. Ikram, *J. Adv. Res*., **7**, 17 (2016).
5. S. Ahmed, Annu, S. Ikram, S. Yudha, *J. Photochem. Photobiol. B: Biology*, **161**, 141 (2016).
6. G. Marslin, K. Siram, Q. Maqbool, R. K. Selvakesavan, D. Kruszka, P. Kachlicki, G. Franklin, *Materials*, **11**, 940 (2018).
7. M. Salve, A. Mandal, K. Amreen, K. P. Pattnaik, S. Goel, *Microchem. J.*, **157,** 104973 (2020).
8. T. Dodevska, I. Vasileva, P. Denev, D. Karashanova, B. Georgieva, D. Kovacheva, N. Yantcheva, A. Slavov*, Mater. Chem. Phys.,* **231**, 335 (2019).
9. Y. Lazarova, T. Dodevska, A. Slavov, D. Karashanova, B. Georgieva, *Bulg. Chem. Commun.,* **51** (Special Issue D), 192 (2019).
10. P. T. Yin, T. H. Kim, J. W. Choi, K. B. Lee, *Phys. Chem. Chem. Phys.*, **15**, 12785 (2013).
11. P. Salazar, I. Fernández, M. C. Rodríguez, A. Hernandez-Creus, J. L. Gonzalez-Mora*, J. Electroanal. Chem.,* **855**, 113638 (2019).
12. G. G. Kumar, K. J. Babu, K. S. Nahm, Y. J. Hwang, *RSCAdv.,* **4**, 7944 (2014).
13. Y. Zheng, A. Wang, W. Cai, Z. Wang, F. Peng, Z. Liu, L. Fu, *Enzyme Microb. Technol.,* **95**, 112 (2016).
14. F. Chekin, S. Bagheri, S. Abd Hamid, *J. Chin. Chem. Soc.,* **61**, 631 (2014).
15. M. Chelly, S. Chelly, R. Zribi, H. Bouaziz-Ketata, R. Gdoura, N. Lavanya, G. Veerapandi, C. Sekar, G. Neri, *Nanomaterials*, **11**, 739 (2021).
16. Z. Yang, X. Zhou, Y. Yin, W. Fang, *Anal. Lett*., **54**, 2826 (2021).
17. M. Shivakumar, K. L. Nagashree, S. Manjappa, M. S. Dharmaprakash*, Electroanalysis,* **29**, 1434 (2017).
18. K. Ramachandran, D. Kalpana, Y.Sathishkumar,Y. S. Lee, K. Ravichandran, G. G. Kumar, *J. Ind. Eng. Chem.,* **35**, 29 (2016).
19. S. H. Mohd Taib, K. Shameli, P. M. Nia, M. Etesami, M. Miyake, R. R. Ali, E. Abouzari-Lotf, Z. Izadiyan*, J. Taiwan Inst. Chem. Eng.,* **95**, 616 (2019).

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Antimicrobial activity of plant extracts of rose by-products from the essential oil industry against saprophytic and pathogenic microorganisms

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In a series of experiments, the inhibitory effect of extracts derived from by-products of rose-oil industry against pathogenic microorganisms, causing food toxicoinfection and intoxication, and saprophytic bacteria, yeasts and fungi, causing food spoilage, were determined. The lowest antimicrobial activity was established for aqueous extracts of the rose waste material while the highest antimicrobial activity was demonstrated by the aqueous-alcoholic (70%) extracts. The latter inhibited the growth of the test-pathogenic bacteria and yeast to varying degrees, with minimum inhibitory concentration (MIC) ranging from 6 ppm to 600 ppm. In determining the effect of the extracts on saprophytic microorganisms, all the extracts were found to inhibit the growth of spore-forming bacteria *Bacillus subtilis*, yeast *Candida utilis* and fungi *Rhizopus arrhizus*. The growth of the remaining fungi in the study was suppressed only by the 70% alcoholic extracts, with a MIC = 600 ppm for all. Gram-positive bacteria were more sensitive to the plant extracts tested (IZ=8-22.5 mm), with a MIC of 60 ppm. The Gram-negative bacteria tested were less sensitive (IZ=8-12.5 mm) with a MIC of 600 ppm. This was due to the difference in the structure and composition of the cell wall of the two bacterial groups. Therefore, alone or in combination with other extracts, they can be used for bio-preservation of foodstuffs.

Keywords: antimicrobial activity; waste valorization; rose species.

INTRODUCTION

There are 4 types of oil-bearing rose species in the world: *Rosa damascena* Mill.*, Rosa centifolia* L.*, Rosa gallica* L. and *Rosa alba* L. Distillation is the main method for extraction of most of the essential oils [1] along with extraction with various solvents. Hydrodistillation of *R. damascena* produces aromatic products such as rose oil and rose water. The essential oils in the flowers are widely used in pharmacy, perfumery, food industry, etc. A large amount of waste rose flowers remains as a by-product in the production of rose oil and rose water.

Many possibilities for utilization of the waste rose biomass have been investigated: obtaining aromatic products and increasing the essential oil yield [2]; utilization of waste rose biomass as a source of feed or feed additives [3]; use for compost [4]; biogas production; bio-adsorption of heavy metals [5]; isolation of bioactive substances and their application in the food industry or medicine [6]. The extracts from rose by-products were found to be rich in biologically-active substances [7-9]. Hence, the aim of the present study was to investigate the antimicrobial activity of rose by-products against saprophytic and pathogenic test microorganisms.

MATERIALS AND METHODS

Rose by-products investigated

	Designation	Species	Industrial processing	Location
1	RD/SD	*Rosa damascena*	Hydro-distillation	Mirkovo, Sofia
2	RD/CO_2	*Rosa damascena*	Extraction with supercritical CO_2	Mirkovo, Sofia
3	RD/H	*Rosa damascena*	Extraction with n-hexane	Zelenikovo, Brezovo
4	RD/F	*Rosa damascena*	Extraction with freon	Plovdiv
6	RA/SD	*Rosa alba*	Steam distillation	Tarnichane, Kazanlak
7	RA/CO_2	*Rosa alba*	Extraction with super-critical CO_2	Mirkovo, Sofia

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The extracts using water, 30% and 70% ethanol were prepared as described [7]. Briefly, 150 g of dry rose waste was treated with 1000 mL of water or ethanol solution for 1 h at 60°C with constant stirring, and then left for 24 h at room temperature. The mass was filtered, and the insoluble residue was returned for a second extraction with 500 mL at the same conditions. After filtration, the collected extracts were combined.

Determination of the antimicrobial activity against pathogenic and saprophytic micro-organisms.

Pathogenic test-microorganisms at concentrations (in brackets, cfu/mL) were used as follows: *Escherichia coli* ATCC 25922 (1.0×10^{12}), *Salmonella abony* NCTC 6017 (2.0×10^{8}), *Staphylococcus aureus* ATCC 25923 (4.0×10^{8}), *Listeria monocytogenes* ATCC 19111 (4.6×10^{9}), *Proteus vulgaris* ATCC 6380 (5.0×10^{11}), *Enterococcus faecalis* ATCC 19433 (1.2×10^{11}), *Candida albicans* NBIMCC 74 (2.1×10^{11}), *Pseudomonas aeruginosa* NBIMCC 1390, (1.0×10^{12}); saprophytic test-microorganisms at concentrations (cfu/mL in brackets): *Bacillus subtilis* ATCC 19659 (5.0×10^{9}), *Penicillium chrysogenum* ATCC 28089 (2.0×10^{7}), *Fusarium moniliforme* ATCC 38932 (1.0×10^{6}), *Aspergillus niger* ATCC 1015 (1.2×10^{7}), *A. flavus* ATCC 9643 (2.8×10^{7}), *Rhizopus arrhizus* ATCC 11145 (8.0×10^{6}), *Candida utilis* ATCC 42402 (4.6×10^{8}). All strains were deposited in the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria.

Preparation of the suspensions of the test pathogenic or saprophytic microorganisms: The test pathogenic or saprophytic microorganisms were cultured on Luria Bertani medium with glucose (LBG) agar medium (LB Broth, Miller-Novagen, Merck, Germany) at 37 ± 1ºC for 24–48 h for the pathogenic test-microorganisms and at 30 ± 1ºC for 24–48 h for the saprophytic microorganisms. Using a sterile loop biomass of the well-grown pathogenic or saprophytic microorganisms was suspended in sterile saline solution in order to obtain suspensions of the corresponding pathogenic or saprophytic microorganisms. The concentrations of the suspensions of the microorganisms were estimated after counting the single colonies formed after spread plating of the corresponding test-microorganism, followed by incubation of the inoculated Petri dishes at 37 ± 1ºC for 24–48 h for the pathogenic test-microorganisms and at 30 ± 1ºC for 24–48 h for the saprophytic test-microorganisms.

The antimicrobial activity was studied by the disc-diffusion method: sterile melted LBG agar medium (LB Broth, Miller Novagen, Merck, Germany) was poured in Petri dishes and after the hardening of the agar, the dishes were spread-plated with suspensions of the respective pathogenic test-microorganism. Sterile melted LBG agar medium was mixed with the respective saprophytic microorganism suspension and the mixture was poured in empty Petri dishes and was left to solidify. Decimal dilutions of the extracts in saline solution were prepared. Ampicillin paper discs (BB-NCIPD Ltd.) were used as positive control for *E. coli* ATCC 25922, *S. abony* NCTC 6017, *S. aureus* ATCC 25923, *P. aeruginosa* NBIMCC 1390, *B. subtilis* ATCC 6633; nystatin paper discs (BB-NCIPD Ltd.) were used as positive control for *C. albicans* NBIMCC 74*, P. chrysogenum* ATCC 10106, *F. moniliforme* ATCC 38932, *A. niger* ATCC 9029, *A. flavus* ATCC 9643; and actidione paper discs (BB-NCIPD Ltd.) were used as positive control for *S. cerevisiae* ATCC 7754. Saline solution containing 1% (v/v) Tween® 80 was used as negative control. The experiments were conducted with dilutions 10^{0}, 10^{-1}, and 10^{-2} in order to determine the minimum inhibitory concentration (MIC). The used paper discs were 6 mm in diameter. Six μL of the corresponding dilution were pipetted on the corresponding paper discs. The results were recorded as diameters of the clear zones around the paper discs, in millimeters, after 24–48 h of incubation of the Petri dishes at an optimal temperature for the growth of the corresponding test-microorganism − 37 ± 1ºC for 24–48 h for the Petri dishes with the pathogenic microorganisms and at 30 ± 1ºC for 24–48 h for the Petri dishes with the saprophytic microorganisms. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth [10]. The MICs, in ppm, were calculated based on the obtained results [11].

Statistical analysis

Data from quadruplicate experiments were processed with MS Office Excel 2013 software, using statistical functions to determine the standard deviation and the maximum estimate error at significance level $p<0.05$.

RESULTS AND DISCUSSION

The results of the experimental studies on the determination of the antimicrobial action of aqueous extracts, extracts (30% and 70% ethanol) are presented in Tables 1, 2 and 3. The type and amount of bioactive substances in the extracts with

antimicrobial effect varied depending on the industrial treatment. Aqueous extracts of rose waste materials contained significant amounts of phenylethyl alcohol, β-citronellol, nerol, geraniol, nonadecane, ferulic acid, gallic acid, 3,4-dihydroxy benzoic acid, neochlorogenic acid, and other substances with inhibitory action [7-9], with the highest content being determined in extract 3 (3RD/H) obtained from *R. damascena* waste, extracted with n-hexane, which also demonstrated the highest antimicrobial activity against the pathogenic microorganisms causing food poisoning and intoxication, included in the study (Table 1).

The MIC for this rose extract varied from 60 ppm to 600 ppm. The results for extracts 1 and 2 of waste raw materials of *R. damascena* were similar. The inhibitory activity of extract 4 of this rose species was lower (Table 1). The aqueous extracts obtained from waste materials of *R. alba* had weak effect on the growth of pathogenic bacteria and yeasts (Table 1).

The data for the experimental studies of water-alcohol extracts (30% ethanol) from rose waste raw materials were similar to the results discussed above (Table 2). The change in the type and concentration of the substances with inhibitory action was weak, therefore the influence of the obtained extracts on the growth of the pathogenic test-microorganisms was similar (Table 2).

The highest antimicrobial activity against the pathogens included in the studies was determined in water-alcohol (70% ethanol) extracts from rose waste materials (Table 3). All aqueous-alcoholic (70% ethanol) extracts from waste raw materials of *R. damascena* inhibited the growth of all pathogenic bacteria and yeast, with MIC values being less than 60 ppm or equal to 600 ppm. The largest inhibition zones (IZ) were determined in the aqueous-alcoholic extracts 3 and 4 of *R. damascena* (Table 3). The inhibitory effect of the aqueous-alcoholic extracts from waste materials of *R. alba* was lower.

Gram-positive bacteria were more sensitive to the plant extracts tested (IZ=8-22.5 mm), with a minimum inhibitory concentration of 60 ppm. The Gram-negative bacteria tested were less sensitive (IZ=8-12.5 mm) with a minimum bactericidal concentration of 600 ppm. This was due to the difference in the structure and composition of the cell wall of the two bacterial groups.

The higher inhibitory activity of the aqueous-alcoholic extracts of roses was a result of the extraction of higher concentrations of geranyl acetate, β-bourbonene, β-cubebene, trans-nerolidol, etc., as well as the increased percentage of phenylethyl alcohol [7]. Their content was high in all rose extracts, regardless of the industrial processing.

It is noteworthy that with increasing the amount of alcohol in the extracts decreased the concentration of organic acids demonstrating inhibitory effect on the growth of the test-microorganisms (Tables 2, 3, 5, 6). This weakly affected the antimicrobial activity and was at the expense of the higher concentrations of the extracted substances with antimicrobial action. The obtained results confirmed the data of studies of other authors for the presence of antimicrobial activity in alcoholic extracts of rose blossoms against *E. coli, Salmonella* sp., *S. aureus, P. vulgaris, P. aeruginosa* [12, 13].

The inhibitory effect of aqueous and aqueous-alcoholic extracts obtained from rose waste raw materials against saprophytic bacteria, yeasts and fungi causing microbial spoilage of food and beverages, was determined in a series of experiments (Tables 4-6). The obtained results confirmed the data obtained in the examination of the antimicrobial activity of the extracts against the pathogenic test-microorganisms. The aqueous extracts from rose waste materials showed a weak antimicrobial effect against spore-forming bacteria, yeasts and fungi (Table 4). 30% aqueous-alcoholic extracts followed in terms of activity (Table 5) and the highest inhibitory effect was determined in 70% aqueous-alcoholic extracts (Table 6). The aqueous-alcoholic extracts of *R. damascena* (1) had the highest antimicrobial action. It should be noted that all 70% aqueous-alcoholic extracts inhibited the growth of spore-forming bacteria of *B. subtilis* ATCC 19659, and the MIC ranged from 60 ppm to less than 600 ppm (Table 6). In the saprophytic yeast *C. utilis* ATCC 42402 the MIC was 60 ppm to 600 ppm. Differences were observed within the fungal test-microorganisms. The effect of the extracts on the spores of the *Aspergillus* species was weaker (Tables 5 and 6). The obtained results confirmed the data from the studies of other authors for the antimicrobial activity of aqueous and aqueous-alcoholic rose extracts against *B. subtilis, Aspergillus* sp., *P. chrysogenum* [13].

The determined high antimicrobial activity of the studied extracts from rose waste materials is a prerequisite for the application of the obtained extracts in food and beverage production as potential bio-preservatives.

Table 1. Antimicrobial activity and MIC of different water plant extracts against pathogenic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} =6 mm.

Test-MO / Sample	*E. .coli* ATCC 25922		*S. abony* NCTC 6017		*S. aureus* ATCC 25923		*L. monocytogenes* ATCC 19111		*P.vulgaris* ATCC 6380		*E. faecalis* ATCC 19433		*P. aeruginosa* NBIMCC 1390		*C. albicans* NBIMCC 74	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/H_2O 2018	-	-	-	-	9.17±0.47^{a}	600	9.17±0.47^{a}	600	10.17±0.24^{a}	600	10.17±0.24^{b}	600	10.17±0.24^{c}	600	9.17±0.47^{c}	600
2/H_2O 2018	-	-	9.67±0.47^{a}	600	9.17±0.47^{a}	600	9.17±0.47^{a}	600	11.17±0.47^{a}	>60	9.17±0.47^{c}	600	11.17±0.47^{b}	>60	10.17±0.47^{b}	600
3//H_2O 2018	9.17±0.24^{a}	600	14.67±0.47^{b}	60	-	-	12.17±0.24^{b}	60	10.17±0.24^{a}	600	12.17±0.47^{a}	60	13.17±0.47^{a}	60	12.67±0.47^{a}	60
4/H_2O 2018	-	-	9.17±0.24^{a}	600	-	-	15.17±0.47^{c}	60	10.17±0.47^{a}	600	-	-	12.67±0.47^{a}	60	11.67±0.47^{a}	>60
6/H_2O 2018	-	-	-	-	-	-	-	-	12.17±0.47^{b}	60	-	-	9.17±0.47^{d}	600	-	-
7/H_2O 2018	-	-	9.17±0.47^{a}	600	-	-	-	-	10.17±0.47^{a}	600	-	-	-	-	-	-

$^{a, b, c, d}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

Table 2. Antimicrobial activity and MIC of different plant extracts (in 30% ethanol) against pathogenic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} =6 mm.

Test-MO / Sample	*E. coli* ATCC 25922		*S. abony* NCTC 6017		*S. aureus* ATCC 25923		*L. monocytogenes* ATCC 19111		*P. vulgaris* ATCC 6380		*E. faecalis* ATCC 19433		*P. aeruginosa* NBIMCC 1390		*C. albicans* NBIMCC 74	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/30% 2018	10.17±0.47^{a}	600	12.17±0.47^{a}	>60	9.17±0.47^{a}	600	9.17±0.47^{a}	600	11.17±0.47^{a}	>60	9.17±0.47^{b}	600	9.17±0.47^{b}	600	10.17±0.47^{d}	600
2/30% 2018	10.17±0.24^{a}	600	10.17±0.47^{b,d}	600	9.17±0.47^{a}	600	9.17±0.47^{a}	600	17.67±0.47^{b}	60	10.17±0.47^{a}	600	9.67±0.47^{a,b}	600	15.17±0.47^{a}	60
3/30% 2018	10.17±0.47^{a}	600	16.17±0.47^{c}	<60	-	-	17.67±0.47^{b}	<60	17.67±0.47^{b}	<60	-	-	10.17±0.47^{a}	600	12.17±0.47^{c}	60
4/30% 2018	9.17±0.47^{a}	600	15.17±0.47^{c}	<60	-	-	15.17±0.47^{c}	<60	16.67±0.47^{b}	<60	-	-	9.17±0.47b	600	14.17±0.47^{b}	<60
6/30% 2018	-	-	-	-	-	-	9.17±0.47^{a}	600	-	-	-	-	-	-	-	-
7/30% 2018	-	-	9.17±0.47^{b}	600	-	-	10.17±0.24^{d}	600	-	-	-	-	-	-	-	-

$^{a, b, c, d}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

Table 3. Antimicrobial activity and MIC of different plant extracts (in 70% ethanol) against pathogenic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} =6 mm.

Test-MO / Sample	*E. coli* ATCC 25922		*S. abony* NCTC 6017		*S. aureus* ATCC 25923		*L. monocytogenes* ATCC 19111		*P. vulgaris* ATCC 6380		*E. faecalis* ATCC 19433		*P. aeruginosa* NBIMCC 1390		*C. albicans* NBIMCC 74	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/70% 2018	12.67±0.47^{a}	60	11.67±0.47^{a}	>60	10.67±0.24^{a}	600	18.17±0.47^{a}	60	12.17±0.47^{a}	60	9.67±0.47^{a}	600	10.17±0.24^{a}	600	12.67±0.47^{d}	60
2/70% 2018	12.67±0.47^{a}	60	11.67±0.47^{a}	>60	10.17±0.24^{a}	600	9.17±0.47^{b}	600	15.17±0.47^{b}	60	9.67±0.47^{a}	600	11.67±0.47^{a}	>60	13.67±0.47^{c}	60
3/70% 2018	10.17±0.47^{b}	600	15.17±0.47^{b}	<60	10.17±0.47^{a}	600	17.17±0.47^{a}	<600	9.67±0.47^{c}	600	10.17±0.47^{a}	600	10.17±0.47^{a}	600	15.17±0.47^{b}	<60
4/70% 2018	10.17±0.47^{b}	600	13.67±0.47^{c}	60	10.17±0.47^{a}	600	15.17±0.47^{c}	<600	14.17±0.47^{b}	<60	10.17±0.47^{a}	600	10.17±0.47^{a}	600	16.67±0.47^{a}	<60
6/70% 2018	10.17±0.47^{b}	600	10.17±0.47^{a}	600	10.17±0.47^{a}	600	9.17±0.47^{b}	600	9.17±0.47^{c}	600	-	-	10.17±0.47^{a}	600	9.17±0.47^{f}	600
7/70% 2018	9.67±0.47^{b}	600	10.17±0.24^{a}	600	10.17±0.24^{a}	600	9.67±0.47^{b}	600	9.67±0.47^{c}	600	10.17±0.24^{a}	600	10.17±0.24^{a}	600	10.17±0.24^{e}	600

$^{a, b, c, d, e, f}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

Table 4. Antimicrobial activity and minimum inhibitory concentration (MIC) of different water plant extracts against saprophytic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} =6 mm.

Test-MO / Sample	*B. subtilis* ATCC 19659		*C. utilis* ATCC 42402		*A. niger* ATCC 1015		*P. chrysogenum* ATCC 28089		*F. moniliforme* ATCC 38932		*R. arrhizus* ATCC 11145		*A. flavus* ATCC 9643	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/H_2O 2018	-	-	9.17±0.24^{a}	600	-	-	-	-	11.00±0.00^{c}	60	9.17±0.24^{b}	600	-	-
2/H_2O 2018	9.17±0.47^{a}	600	9.17±0.24^{a}	600	9.17±0.24	600	-	-	9.17±0.70^{d}	600	9.17±0.47^{b}	600	-	-
3//H_2O 2018	9.17±0.47^{a}	600	13.17±0.47^{b}	60	-	-	-	-	15.17±0.47^{a}	60	10.17±0.47^{a}	600	-	-
4/H_2O 2018	-	-	14.17±0.47^{b}	<600	-	-	-	-	-	-	9.67±0.47^{a,b}	600	15.17±0.24^{a}	60
5/H_2O 2018	-	-	-	-	-	-	-	-	10.17±0.47^{c}	600	10.17±0.24^{a}	600	-	-
6/H_2O 2018	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7/H_2O 2018	-	-	9.17±0.24^{a}	600	-	-	-	-	13.67±0.47^{b}	60	-	-	10.17±0.24^{b}	600

$^{a, b, c, d}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

Table 5. Antimicrobial activity and MIC of different plant extracts (in 30% ethanol) against saprophytic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} =6 mm.

Test-MO / Sample	*B. subtilis* ATCC 19659		*C. utilis* ATCC 42402		*A. niger* ATCC 1015		*P. chrysogenum* ATCC 28089		*F. moniliforme* ATCC 38932		*R. arrhizus* ATCC 11145		*A. flavus* ATCC 9643	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/30% 2018	9.17±0.24^{a}	600	13.17±0.24^{a}	<600	15.00±0.10^{a}	>60	-	-	9.17±0.24^{b}	600	9.67±0.47^{c,d}	600	-	-
2/30% 2018	10.17±0.47^{b}	600	9.17±0.24^{b}	600	14.17±0.24^{b}	60	-	-	9.17±0.24^{b}	600	9.17±0.47^{d}	600	9.33±0.47	600
3/30% 2018	10.17±0.24^{b}	>60	16.17±0.47^{c}	60	-	-	-	-	-	-	11.17±0.47^{a}	<600	-	-
4/30% 2018	11.17±0.47^{b}	60	14.17±0.47^{d}	60	-	-	-	-	-	-	10.17±0.47^{b,c}	600	-	-
5/30% 2018	-	-	-	-	-	-	9.17±0.47	600	16.17±0.47^{a}	60	-	-	-	-
6/30% 2018	9.17±0.47^{a}	600	-	-	-	-	-	-	-	-	-	-	-	-
7/30% 2018	9.17±0.24^{a}	600	13.17±0.24^{a}	<600	15.00±0.10^{a}	>60	-	-	9.67±0.47^{b}	600	10.67±0.47^{a,b}	600	-	-

$^{a, b, c, d}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

Table 6. Antimicrobial activity and MIC of different plant extracts (prepared with 70% ethanol) against saprophytic microorganisms (MO). Inhibition zones (IZ) in mm. d_{disc} = 6 mm.

Test-MO / Sample	*B. subtilis* ATCC 19659		*C. utilis* ATCC 42402		*A. niger* ATCC 1015		*P. chrysogenum* ATCC 28089		*F. moniliforme* ATCC 38932		*R. arrhizus* ATCC 11145		*A. flavus* ATCC 9643	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
1/70% 2018	13.17±0.47^{a}	<600	14.17±1.24^{a}	60	9.17±0.47^{a}	600	9.17±0.47^{a}	600	13.33±0.47^{a}	60	13.33±0.47^{a}	60	9.00±0,00	600
2/70% 2018	14.17±0.47^{a}	<600	10.17±0.47^{b}	600	14.17±0.47^{b}	60	12.17±0.24^{b}	600	10.17±0.47^{b}	600	10.17±0.47^{d}	600	-	-
3/70% 2018	11.17±0.24^{b}	60	15.67±0.47^{a}	60	9.17±0.47^{a}	600	10.17±0.47^{a,c}	600	10.17±0.47^{b}	600	9.67±0.47^{d}	600	-	-
4/70% 2018	10.17±0.47^{c}	600	17.17±0.47^{c}	60	-	-	10.67±0.47^{c}	600	13.17±0.24^{a}	60	11.17±0.47^{c}	<600	-	-
5/70% 2018	9.67±0.47^{c}	600	9.17±0.47^{b}	600	9.17±0.47^{a}	600	9.67±0.47^{a}	600	-	-	9.67±0.47^{d}	600	-	-
6/70% 2018	10.67±0.47^{c}	600	9.67±0.47^{b}	600	9.17±0.47^{a}	600	9.17±0.47^{a}	600	9.17±0.47^{c}	600	9.67±0.47^{d}	600	-	-
7/70% 2018	13.17±0.47^{a}	<600	14.17±1.24^{a}	60	9.17±0.47^{a}	600	9.17±0.47^{a}	600	-	-	12.17±0.47^{b}	>60	-	-

$^{a, b, c, d}$ – the different letters show statistically different results in a column (one-way ANOVA (Tukey's criterion; $p < 0.05$).

CONCLUSION

The inhibitory effect of plant extracts derived from waste raw materials from the rose essential oil industry against pathogenic microorganisms, causing food toxicoinfection and intoxication, and saprophytic bacteria, yeasts and fungi, causing food spoilage, were determined. The lowest antimicrobial activity was established for aqueous extracts of the rose waste material while the highest antimicrobial activity was demonstrated by the aqueous-alcoholic (70%) extracts. The latter inhibited the growth of the test-pathogenic bacteria and yeast to varying degrees, with MICs ranging from 6 ppm to 600 ppm. In determining the effect of the extracts on saprophytic microorganisms, all the extracts were found to inhibit the growth of spore-forming bacteria *B. subtilis*, yeast *C. utilis* and fungi *R. arrhizus*. The growth of the remaining fungi in the study was suppressed only by the 70% alcoholic extracts, with a MIC = 600 ppm for all. Gram-positive bacteria were more sensitive to the plant extracts tested (IZ=8-22.5 mm), with a minimum inhibitory concentration of 60 ppm. The Gram-negative bacteria tested were less sensitive (IZ=8-12.5 mm) with a minimum bactericidal concentration of 600 ppm. This was due to the difference in the structure and composition of the cell wall of the two bacterial groups. Therefore, alone or in combination with other extracts, they can be used for the biopreservation of food and beverages.

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REFERENCES

1. E. Georgiev, L. Kupenov, G. Ganchev, B. Konovska, *Rivista Italiana Ess.,* **54** (6), 422 (1972).
2. N. Nedkov, K. Kanev, N. Kovacheva, S. Stanev, A. Djurmanski, K. Seikova, C. Lambev, A. Dobreva, Handbook of the main essential oils and medicinal plants cultivation, Helikon, Kazanlak, 2005.
3. D. Balev, D. Vlahova-Vangelova, K. Mihalev, V. Shikov, S. Dragoev, V. Nikolov, *J. Mt. Agric. Balkans.*, **18** (2), 224 (2015).
4. E. Onursal, K. Ekinci, *Waste Manag. Res.*, **33** (4), 332 (2015).
5. M. J Iqbal, F. Cecil, K. Ahmad, M. Iqbal, M. Mushtaq, M. A. Naeem, T. H. Bokhari, *Asian J. Chem.*, **25** (4), 2099 (2013).
6. G. Ozkan, O. Sagdic, N. G. Baydar, H. Baydar, *Food Sci. Technol. Int.*, **10**, 277 (2004).
7. N. Yantcheva, PhD Thesis, University of Food Technologies, Plovdiv (2021). Available online on demand at https://researchgate.net author Nikoleta Yantcheva.
8. A. Slavov, I. Vasileva, P. Denev, R. Dinkova, D. Teneva, M. Ognyanov, Y. Georgiev, *Bulg. Chem. Commun.*, **52** (Special Issue D), 78 (2020).
9. A. Slavov, P. Denev, I. Panchev, V. Shikov, N. Nenov, N. Yantcheva, I. Vasileva, *Ind. Crops Prod.,* **100**, 85–94 (2017).
10. D. Teneva, R. Denkova-Kostova, B. Goranov, Y. Hristova-Ivanova, A. Slavchev, Z. Denkova, G. Kostov, *Z. Naturfosch. C Biosci.*, *74* (5-6), 105 (2019).
11. R. Randrianarivelo, S. Sarter, E. Odoux, P. Brat, M. Lebrun, B. Romestand, C. Menut, H. S. Andrianoelisoa, M. Raherimandimby, P. Danthu, *Food Chem.*, **114**, 680, (2009).
12. N. Hirulkar, M. Agrawal*, Int. J. Pharm. Biol. Arch.*, **1(5)**, 478 (2010).
13. M. Shohayeb, El-S. S. Abdel-Hameed, S. A. Bazaid, I. Maghrabi, *Glob. J. Pharmacol.*, **8(1)**, 1 (2014).

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Synthesis of 2,5-furandicarboxylic acid using biosynthesized silver and gold nanoparticles as catalysts

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In the recent years there is a constant search for replacement of fossil fuel-derived chemicals and polymers produced on their basis. The furan derivatives and especially 2,5-furandixarboxylic acid (FDCA) obtained from renewable resources (carbohydrates) are among the most promising substances. Therefore, the present study focused on the possibility to synthesize FDCA employing bio-synthesized gold and silver nanoparticles. One-pot two-step synthesis starting from mucic acid was considered. In the first step mucic acid was dehydrated in the presence of *p*-toluenesulfonic acid through intermediate 5-hydroxymethyl-furfural (5-HMF) and FDCA was obtained with yield 28±0.5% and the 5-HMF conversion was 75±0.9%. In the next step the mixture was alkalized and bio-synthesized gold (SiO_2/AuNPs) or silver (SiO_2/AgNPs) nanoparticles loaded on silica at 2.5, 5, 7.5 and 10% (w/w) were added. The final yield of FDCA increased to 33±0.4% for the SiO_2/AuNPs experiments (at 5% load). For SiO_2/AgNPs (at 5% load) added as catalyst the total yield of FDCA increased insignificantly (29±0.3%) due to formation of 5-hydroxymethyl-2-furancarboxylic acid (partially oxidized product) as main compound and lower yield of FDCA.

Keywords: bio-synthesis; gold nanoparticles; silver nanoparticles; mucic acid; 2,5-furandicarboxylic acid.

INTRODUCTION

Nowadays our everyday life relies on vast amounts of plastic polymeric materials. These polymers are exclusively prepared from fossil resources. However, with the rising prices of fossil-based starting materials, diminishing quantities and the recycling problems with the plastic wastes there is an urgent need for searching alternatives and switching to a more sustainable model of development. In this regard, bio-based raw materials and, in particular carbohydrates from undervalorized biomass wastes, are promising starting feedstocks for the production of added-value chemicals, such as: esters, diols, hydroxyl-acids and esters, lactones, carbonyl compounds, cyclic ethers, di-amines, amino acids, lactams, alkenes, acrylics and conjugated dienes [1]. Among them very promising precursors for biopolymer synthesis are considered the derivatives of furan and in particular 2,5-furandicarboxylic acid (FDCA) [2]. Polyesters made using FDCA had found various applications but the most promising one is for replacing polyethylene terephthalate-based products. However, there are many difficulties in front of the successful production, application and utilization of FDCA as promising bio-based material. Attempts on FDCA-based materials development were diffusive in individual scientific investigations and besides cooperation between scientists and manufacturers has also been occasional, incapacitating and troubling their successful industrialization and market introduction. In order to shed more light and facilitate the FDCA synthesis, applications and economic viability, various initiatives and joint actions were established (for example COST Action CA18220 European network of FURan based chemicals and materials FOR a Sustainable development – FUR4SUSTAIN), which are intended to gather the individual efforts to elaborate novel paths for FDCA and its derivatives synthesis.

Various routes and approaches for obtaining FDCA as a starting material for bio-based polymers were suggested depending on the reactants, catalysts, temperature, pressure, etc. [2–5]. In general, two major approaches are employed: dehydration and oxidation of hexoses (mainly fructose and glucose) with strong acids and selective oxidation of 5-hydroxymethyl furfural (5-HMF). Combined, one-pot syntheses were also reported as alternatives [3], as well as photocatalytic oxidation [6] and microorganisms' assisted bio-synthesis [7]. The main problem in the field is the price of the obtained furan-based monomers and respectively their bio-based polymers. Therefore, new synthetic routes, catalysts and conditions are constantly investigated. The present study employs mucic acid which could be easily produced from the respective hexose by

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dehydration, as a starting material for synthesis of 2,5-furandicarboxylic acid. The reaction was carried out in two steps: acid-catalyzed dehydration (first step) and mild heat treatment in basic medium using as catalysts bio-synthesized silver or gold nanoparticles obtained by reduction with an extract from by-products of rose oil industry (second step).

MATERIALS AND METHODS

Materials

The industrial by-products from steam-water distillation of *Rosa damascena* (RD_SWD) were provided by ECOMAAT distillery (Mirkovo, Bulgaria, 2021). After treatment of the fresh rose flowers, the RD_SWD were cooled down, inspected for impurities, dried at 50°C and stored at -18°C until further treatment. The mucic acid was obtained from Riedel-de Haën-Honeywell International Inc. (Germany); silver nitrate ($AgNO_3$), chloroauric acid ($HAuCl_4.3H_2O$), silica (nanopowder with specific surface area 175-225 m^2 g^{-1} (BET), *p*-toluenesulfonic acid monohydrate (*p*-TSA.H_2O, 98%), 2,5-furandicarboxylic acid (FDCA), 5-hydroxymethyl-2-furancarboxylic acid (HFCA), levulinic acid and 5-hydroxymethyl furfural (5-HMF) were from Sigma Aldrich Chemie GmbH (Germany).

Methods

The bio-synthesized silver nanoparticles (AgNPs) were obtained by mixing 0.4 mL of RD_SWD water extract (obtained according to Georgieva *et al*. [8]), 0.4 mL of deionized water and 1.2 mL of 0.01M $AgNO_3$. The bio-synthesized gold (AuNPs) nanoparticles were obtained by mixing 0.3 mL of RD_SWD water extract with 1.2 mL of 0.01M $HAulCl_4$.

The mixtures of AgNPs and AuNPs were left in dark for two hours, centrifuged for 15 min (6000 rpm, 4ºC) and the supernatant was removed by decantation. The nanoparticles were washed three times with deionized water and dried at 50ºC.

The silica-supported AgNPs (SiO_2/AgNPs) or AuNPs (SiO_2/AuNPs) were prepared as follows: the dried AgNPs or AuNPs were suspended in deionized water at 2.5%, 5%, 7.5% and 10% (w/v) and silica was added at 25% (w/v). The suspension was stirred overnight at room temperature and then heated at 50ºC for one hour. After that the mixture was centrifuged for 20 min (12000 rpm, 4ºC) and the supernatant was removed. The pellets were dried at 80ºC and then heated at 200ºC for 3 hours.

The FDCA was synthesized in two steps as a one-pot synthesis: a mixture of mucic acid (420 mg, 2 mmol) and *p*-TSA (768.8 mg, 4 mmol) was heated in an oil bath at 100ºC for 5 min until suspension appeared and then the temperature was raised to 160ºC. The mixture was heated for 30 min under constant stirring. The temperature was lowered to 40ºC and the pH was brought to 9.5 with 2.5M NaOH. The catalysts were added (SiO_2/AgNPs – 85 mg or SiO_2/AuNPs – 94 mg) and the mixture was transferred to a laboratory home-made autoclave (max. temperature 250ºC, max. pressure 60 bars, Teflon inlets), purged three times with air and heated at 90ºC, 4 bar air pressure for 3 hours. At the end of the reaction the mixture was cooled down and the pH was lowered to 5 using 1M HCl.

In order to follow the reaction progress a sample was taken at the end of the first step (acid-catalyzed dehydration) and every 30 min during the second step, filtered through a 0.45 μm cellulose acetate CA syringe filter (IsoLab, Germany) and properly diluted for HPLC analysis on ELITE LaChrome Hitachi equipped with a VWR Hitachi Chromaster 5450 refractive index detector. The FDCA and HFCA were determined using Bio-Rad Aminex HPX-85H column (250 × 4.6 mm × 5 μm particle size), solvent 5 mM H_2SO_4 at an elution rate of 0.5 mL min^{1}, column temperature 50ºC, and detector temperature 35ºC. The 5-HMF was determined on a Supelco Discovery HS C18 RP column (250 × 4.6 mm × 5 μm particle size), solvent CH_3OH:H_20=90:10 (v/), at an elution rate of 1 mL min^{-1}, column temperature 25ºC and VWR Hitachi L-2455 diode array detector at 284 nm wavelength.

Statistical analysis

The experiments and analyses were run three times, and the data were given as mean values. Statistical significance was detected by analysis of variance (ANOVA, Tukey's test; value of $p<0.05$ indicated statistical difference).

RESULTS AND DISCUSSION

The dried biomass obtained after fresh *Rosa damascena* industrial processing by water-steam distillation, was used to prepare water extracts. The extracts were found to be rich in carbohydrates and polyphenolic compounds which are the main responsible substances for reduction of Ag^+ and Au^{3+} to nanoparticles [8, 9]. The synthesis of AgNPs and AuNPs started immediately after the mixture was prepared and visually after the 10^{th} minute the solutions turned grey (for the AgNPs) and dark blue (for the AuNPs). The process of synthesis was followed by UV-Vis measurements: Figure 1 (for the AgNPs) and Figure 2 (for the AuNPs). It can be seen that after two hours the NPs

were synthesized and no further increase of the absorption (for the AuNPs) was observed. For the AgNPs a process of agglomeration of the NPs was established and at the 24th hour the absorption was out of the range. The lack of absorption maximum in the spectra could be due to the heterogeneous and polydisperse character of the AgNPs and AuNPs. The bio-synthesized nanoparticles (NPs) by this method were previously utilized for assembling of sensors for electrochemical determination of hydrogen peroxide and vanillin [10] and it was found that the prepared electrodes could be successfully utilized for analyses. The other usual application of noble metal NPs is in the field of catalysis and for this reason they were further utilized in this direction.

The preliminary experiments suggested that the bio-synthesized NPs are difficult to be utilized as catalysts directly, as they are obtained *in situ* with plant extract and other substances are present in the medium, for conversion of aldaric acids or 5-HMF to FDCA. For this reason, the metal NPs were isolated and then were loaded on silica in order to obtain silica-supported AgNPs and AuNPs.

The conversion of hexoses, their respective aldaric acids or 5-HMF to furan derivatives could be successfully performed by heating in the presence of strong acid. The reaction usually takes place at temperatures above 100ºC and as an intermediate 5-HMF is obtained (Scheme 1).

The action of strong acids on mucic acid is leading to formation firstly of 5-HMF. The reaction continues to formation of FDCA by additional loss of water.

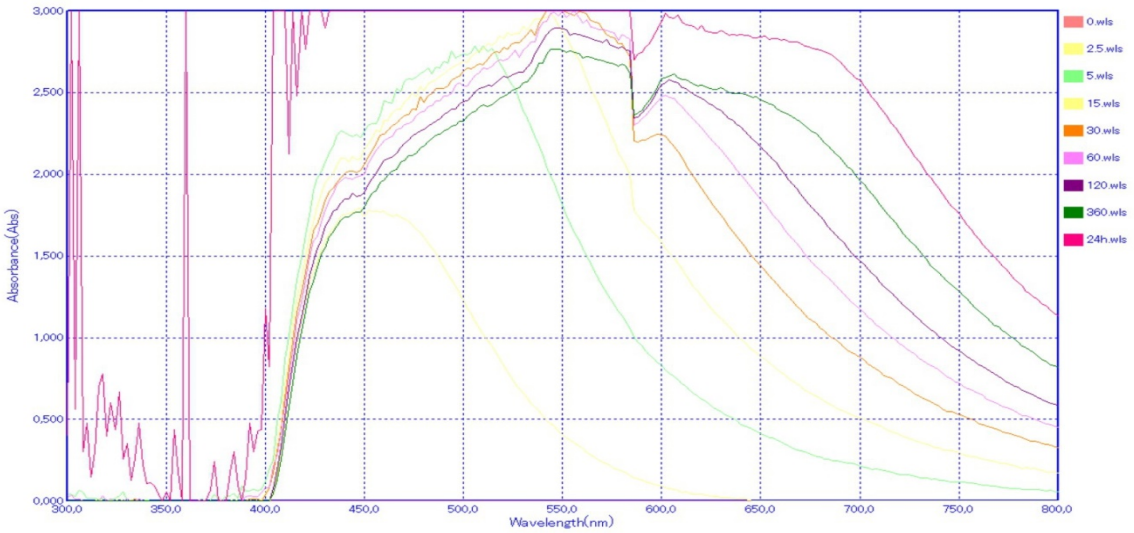


Figure 1. UV-Vis spectra of AgNPs formation with RD_SWD water extract (legend: minute of taking the spectra from the beginning of the AgNPs synthesis)

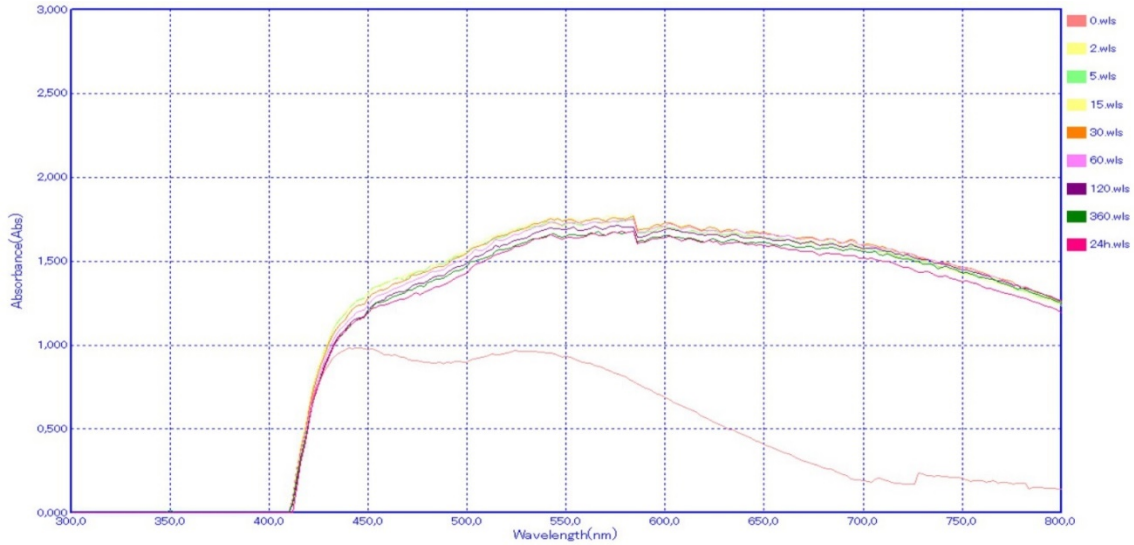
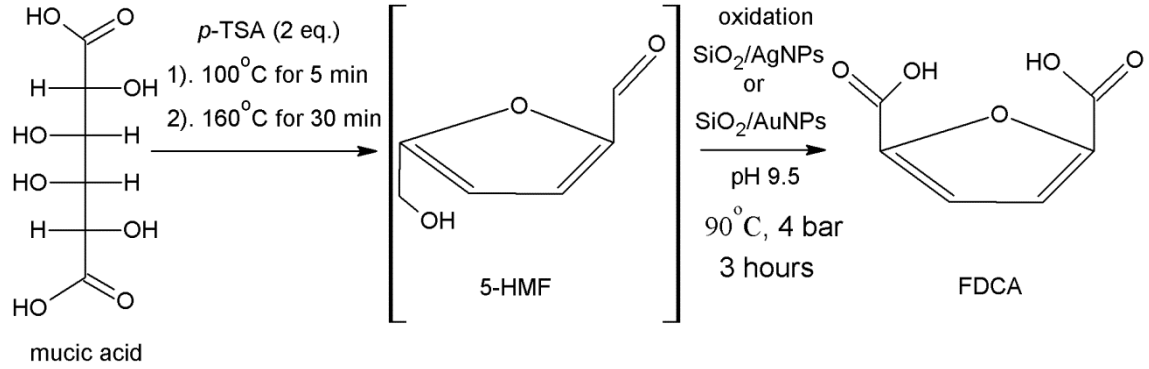


Figure 2. UV-Vis spectra of AuNPs formation with RD_SWD water extract (legend: minute of taking the spectra from the beginning of the AuNPs synthesis)



Scheme 1. Synthesis of FDCA from mucic acid in two steps

Table 1. Catalysts SiO_2/AgNPs or SiO_2/AuNPs application for oxidation of 5-HMF formed in the first step of mucic acid treatment. Reaction conditions: pH 9.5; AgNPs – 85 mg or AuNPs – 94 mg; 90ºC for 3 h.

Catalyst	№	Load, %	5-HMF conversion, %	Relative yield[1], %		Productivity[2] ($mol_{product}$ h^{-1} mol_{metal}^{-1})
				HFCA	FDCA	
SiO_2/AgNPs	1	2.5	93.1±0.2[c]	7.0±0.1[c]	2.0±0.2[b]	29.3±0.7[c]
	2	5	97.2±0.3[a]	10.1±0.1[a]	2.9±0.1[a]	35.6±0.5[a]
	3	7.5	93.6±0.2[c]	7.6±0.2[b]	1.9±0.1[b]	30.4±0.4[b,c]
	4	10	95.3±0.4[b]	7.8±0.2[b]	2.7±0.2[a]	31.3±0.6[b]
SiO_2/AuNPs	5	2.5	92.0±0.5[d]	3.1±0.1[c]	8.2±0.1[c]	28.5±0.3[d]
	6	5	96.6±0.3[a]	5.4±0.2[b]	10.8±0.2[a]	34.8±0.4[a]
	7	7.5	93.3±0.4[c]	5.6±0.3[b]	9.2±0.1[b]	30.2±0.8[c]
	8	10	94.2±0.3[b]	7.7±0.2[a]	9.4±0.2[b]	32.4±0.3[b]

[1]Relative yield was estimated as the product obtained from the mixture from the first step of the reaction;

[2]Productivity is given as moles of product formed per mole of noble metal and time. For SiO_2/AgNPs or SiO_2/AuNPs catalysts, the productivity is given for the major products FDCA and HFCA synthesized, respectively;

[a, b, c] – Values with different letters in a column are statistically significant (ANOVA, Tuckey's post hoc test, $p < 0.05$). Compared were values in the № 1-4 (SiO_2/AgNPs) and № 5-8 (SiO_2/AuNPs) experiments.

Disadvantage of this reaction is the formation of side products: levulinic acid, 5-hydroxymethyl-2-furancarboxylic acid, products of 5-HMF degradation and polymeric products (humins) [2, 5]. Moreover, 5-HMF is relatively unstable in acidic medium [5] and it is not completely oxidized to FDCA.

Employing only the first approach in our work (acidic conversion of mucic acid to FDCA) the yield of the final product was found to be 28±0.5% and the 5-HMF conversion was 75±0.9%. Similarly, Zhao *et al*. [3], investigating the process using different acids, found around 30% FDCA yield employing *p*-TSA and heating the mixture for 30 min at 160ºC. The authors were able to increase the final yield up to 38-37% increasing the time of treatment. In our studies the time was also increased in order to try to augment the yield but it was observed that significant amounts of humins started to be formed after 45 minutes of heating.

For this reason, different approaches were investigated. The acid-catalyzed dehydration was followed by addition of SiO_2/ AgNPs or SiO_2/AuNPs in basic medium. The results of the second step conversion are presented in Table 1.

The addition of silver or gold silica-supported NPs catalysts led to additional formation of FDCA and from 28±0.5% yield employing only *p*-TSA as catalyst to 33±0.4% for the subsequent SiO_2/AuNPs experiments (at 5% load). For SiO_2/AgNPs added as catalyst the total yield of FDCA increased insignificantly (29±0.3%) due to formation of HFCA (partially oxidized product) as main compound and lower yield of FDCA. In both experiments using SiO_2/AgNPs or SiO_2/AuNPs as catalysts the optimal load with NPs was at 5%.

CONCLUSIONS

The present study focuses on the synthesis of FDCA by combining the two major approaches employed nowadays: acid-catalyzed dehydration and catalytic (metals, alloys, nanoparticles, organic catalysts, etc.) conversion of 5-HMF. Employing *p*-TSA treatment of mucic acid for 30 min at 160ºC the yield of FDCA was 28±0.5% and the 5-HMF conversion was 75±0.9%. Additional second step utilizing silica supported AuNPs led to increase of the final yield of FDCA to 33±0.4% but for utilization of SiO_2/AgNPs as catalyst no significant increase of the final yield was observed. As a side

product partially oxidized HFCA was determined. In both experiments using SiO_2/AgNPs or SiO_2/AuNPs as catalysts, the optimal load of NPs on silica was 5%. Optimization of the NPs support and search for alternative approaches is ongoing. The results from the work suggested that bio-synthesized gold nanoparticles could be successfully employed for catalytic conversion of mucic acid and synthesis of FDCA.

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REFERENCES

1. B. M. Stadler, C. Wulf, T. Werner, S. Tin, J. G. de Vries, *ACS Catal.,* **9**, 8012 (2019).
2. A. F. Sousa, C. Vilela, A. C. Fonseca, M. Matos, C. S. R. Freire, G.-J. M. Gruter, J. F. J. Coelho, A. J. D. Silvestre, *Polym. Chem.*, **6**, 5961 (2015).
3. D. Zhao, F. Delbecq, C. Len, *Molecules*, **24**, 1030 (2019).
4. E. Kang, Y. Hong, D. Chae, B. Kim, B. Kim, Y. Kim, J. Cho, Y. Kim, *Chem. Sus. Chem.*, **8** (7), 1179 (2015).
5. N. van Strien, S. Rautiainen, M. Asikainen, D. Thomas, J. Linnekoski, K. Niemelä, A. Harlin, *Green Chem.*, **22**, 8271 (2020).
6. I. Krivtsov, E. I. García-López, G Marcì, L. Palmisano, Z. Amghouz, J. R. García, S. Ordóñeze, E. Díaz, *Appl. Catal. B*, **204**, 430 (2017).
7. R. O. Rajesh, T. K. Godan, A. K. Rai, D. Sahoo, A. Pandey, P. Binod, *Bioresour. Technol.*, **284**, 155 (2019).
8. B. Georgieva, D. Karashanova, R. Angelov, A. Slavov, I. Vasileva, T. Dodevska, *Bulg. Chem. Commun.*, **50** (F), 158 (2018).
9. N. S. Yantcheva, I. N. Vasileva, P. N. Denev, H. N. Fidan, R. S. Denkova, A. M. Slavov, *Bulg. Chem. Commun.*, **51** (D), 178 (2019).
10. T. Dodevska, I. Vasileva, P. Denev, D. Karashanova, B. Georgieva, D. Kovacheva, N. Yantcheva, A. Slavov, *Mater. Chem. Phys.*, **231**, 335 (2019).

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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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