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SUMMARY

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STUDIES ON SOME BIOACTIVE COMPOUNDS FROM COLORED WHEAT

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Abstract

The varieties of colored wheat registered are very limited, and those available have a low agronomic value. The anthocyanin compounds present in colored wheat vary depending on the wheat variety as well as the growing conditions. Due to their antioxidant activity, anthocyanins are of increasing interest to nutritionists, food researchers and plant breeders. The objective of this work was to determine some bioactive compounds from wheat genotypes H171-I with purple grains and H171-III - with red-purple grains. The samples were evaluated by UV-VIS spectrophotometry for anthocyanins, phenols, enzymes and total antioxidant capacity. The determinations showed that the highest level of anthocyanins was found in the genotype H171-I, followed by Pitar and H171-III; phenolic compounds were present in all wheat genotypes, but unlike the other samples, they were synthesized at higher levels in the genotype H171-I. This genotype also had the highest total antioxidant capacity. Data on the activity of polyphenol oxidase and peroxidase enzymes showed a high level for both enzymes in the red grains Pitar genotype. The analyzed compounds varied according to the investigated wheat variety.

Key words: *anthocyanins; antioxidant capacity; colored wheat; enzymes; phenols*

INTRODUCTION

Wheat (*Triticum aestivum*) is the second most consumed cereal type worldwide, after rice (Arendt and Zannini, 2013). White and red wheat varieties are the most commonly consumed. Colored wheat varieties, which contain anthocyanins, are either not widely grown or are grown as special crops. To date, colored wheat species have been studied to obtain information on the exact composition of anthocyanins in these grains, but this is still largely unknown (Abdel-Aal et al., 2006). This would mainly be due to differences in the extraction methods applied. Wheat species containing high amounts of anthocyanins in the outer layers of the pericarp are those with blue, purple or black grains. Anthocyanins are phenolic compounds classified as flavonoids, along with flavones, flavanols, flavanones and iso-flavonoids (Liu, 2004). Grain seeds are not typical sources of anthocyanins, but blue, red and purple seeds are attractive to consumers, food producers and plant breeders (Guo et al., 2012), therefore relevant knowledge has also been accumulated about maize, wheat, barley, oats and rice, which are the most important food sources in the world. There is still a need for wheat varieties with good genetic stability, excellent stress resistance and high yield (Guo et al., 2012). Anthocyanins are generally defined as bioactive, non-nutritional compounds responsible for antioxidant and UV/photoprotective functions (Ryan et al., 2001) and also play a role in plant reproduction (Kong et al., 2003). Anthocyanins participate in the formation of non-specific disease resistance in plants (Treutter, 2006), such as pre-harvest germination, where the red pigment of red-bean wheat is synthesized via the flavonoid biosynthetic pathway, in which the dihydroflavonol-4-reductase (DFR) gene is one of the genes involved in anthocyanin synthesis (Bi et al., 2014).

In purple-grained wheat, anthocyanins were found to be located in the pericarp, whereas in blue wheat they are found in the aleurone layer. In black wheat (referred to as "deep purple"), anthocyanins were found in both the pericarp and the aleurone layer (Abdel-Aal et al., 2012).

In the case of heat-treated grains, the distribution of anthocyanins in the outer layers of the grain may affect the stability of these compounds in the extraction process. Purple wheat has, on average, a lower total anthocyanin content than blue and black wheat varieties. Colored wheat seeds are a natural source of pigments as phytochemicals and can impart desirable color and stability to commercial food products.
The objective of this work was to determine biochemical compounds with antioxidant properties in four wheat genotypes with different colored pericarp, including purple.

MATERIALS AND METHODS

The biological material was constituted of the wheat genotypes "Pitar", H171-I and H171-III from the National Agricultural Research and Development Institute, Fundulea (Figure 1).

Figure 1. Wheat genotypes investigated

"Pitar" is a Romanian winter wheat variety, registered in 2015, with a high quality and red grains. The genotypes H171-I and H171-III are experimental lines with purple (H171-I) and purple-red (H171-III) pericarp respectively, but the grains still have irregular color, obtained by crossing the wheat variety "Favorit" with the couch grass species *Thinopyrum bessarabicum.*

Extracts preparation
For each sample 4 g of material was weighed to which 40 ml of methanol was added. The mixtures were incubated in a shaking incubator at 120 rpm for 48 hours. For experiments, the mixtures were centrifuged at 10000 rpm for 5 minutes. The obtained filtrate was stored at 40^0C until use (24 hours). Extracts were then analyzed for biochemical compounds.

Determination of total anthocyanins. The total anthocyanin content of diluted plant material extracts was estimated by the pH differential method (Giusti and Wrolstad, 2001). Extracts were diluted twice: once with potassium chloride buffer (pH 1.0) and then with sodium acetate buffer (pH 4.5). The dilutions were allowed to equilibrate for 15 min before measuring their absorbance in the spectrophotometer at two wavelengths, at 520 nm and 700 nm respectively. The dilution factor (10) was obtained by dividing the final sample volume by the initial volume. The dilutions were allowed to equilibrate for 15 minutes before measuring their absorbance in the spectrophotometer. Absorbance (A) was measured at 520 nm and 700 nm in both buffers respectively:

$$\mathbf{A= (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}}$$

The following equation were applied to estimate the total anthocyanin content:

$$\text{TAC (mg/L)} = \frac{A\ x\ MW\ x\ DF\ x\ 10^3}{\varepsilon\ x\ l}$$

Where: TAC=Total Anthocyanins Content in mg/L; DF is the dilution factor (10); MW is the molecular weight of cyanidin-3-O-glucoside (Cy3G) (MW = 449.2 g/mol), and ε its molar absorptivity (29,900 L / mol $^{-1}$ & cm-1 for cyd-3-glu); 10^3 = the conversion factor from g to mg.

Determination of total phenolic content. The spectrophotometric determination of total phenol content was carried out according to the Folin-Ciocalteu method (Singleton, 1999). The method consists of chemically reducing the Folin-Ciocalteu reagent (a mixture of tungsten and molybdenum oxides) and measuring the absorbance at 765 nm. Total phenol values were expressed as mg gallic acid equivalent (GAE)/L sample. Measurements were obtained using an Eppendorf UV/Vis spectrophotometer. The concentration of polyphenols (mg GAE/L) in the samples was determined from the standard calibration curve (y = 0.0012x + 0.0102; R2 = 0.9996) obtained for different gallic acid concentrations (50 - 500 mg/L) (Figure 2).

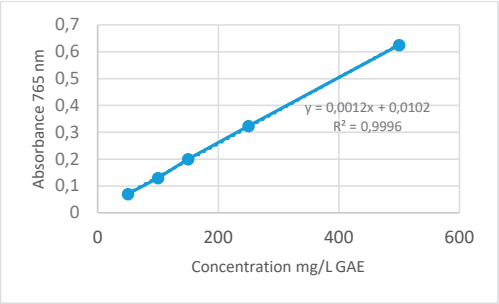

Figure 2. Standard curve of Gallic acid

Determination of polyphenol oxidase (PPO) and peroxidase (POD)

PPO activity was determined by the method of Liu et al. (Liu et al., 2005). The standard reaction mixture contained: 1.5 ml of 40 mmol/l catechol and 2.3 ml of 0.1 mol/l phosphate buffer (pH 6.5). The reaction mixture was incubated at 25°C for 5 min. Then 0.2 ml of sample was added to the test tube over the mixture with shaking. Immediately, the absorbance increase was measured at 420 nm with a UV-VIS spectrophotometer (Eppendorf).

Peroxidase activity was determined spectrophotometrically at 470 nm using the method described by Bergmeyer (1974) with minor modifications. The reaction mixture contained: 2.8 ml 0.1 M potassium phosphate buffer pH 7.0; 0.05 ml 30 mM guaiacol solution; 0.05 ml 3% H2O2 and 0.1 ml protein extract. The samples thus prepared are read in the spectrophotometer at OD 470 nm (for each sample - 1 reading/minute for 2 minutes). In both cases, the optical density variation was studied for two minutes. Enzyme activities were calculated according to the formula:

$$\text{U/ml} = \frac{\Delta OD \; x \; Vt \; x \; D}{\Delta t \; x \; \varepsilon \; x \; Vs}$$

Where: ΔOD = optical density; Vt = total volume of reaction mixture; Vs = sample volume; ε = extinction coefficient of catechol (3400 mM/cm) and guaiacol (26.6 mM/cm) respectively; Δt = reaction time.

Total antioxidant activity

Total antioxidant activity of the samples was assessed by the phosphomolybdate method (Prieto et al., 1999), using ascorbic acid as a standard (Garrat, 1964). Results were expressed in ascorbic acid equivalents based on the standard calibration curve (y = 0.0059x + 0.0409; R=0.993) obtained for different ascorbic acid concentrations (10 - 200 µg/ml) (Figure 3).

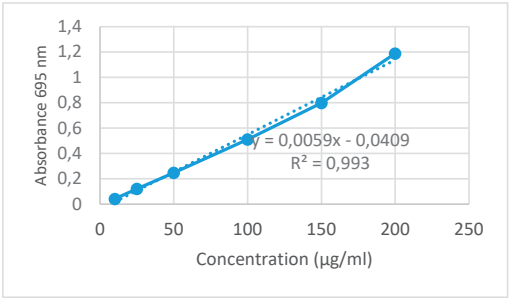

Figure 3. Standard curve of Ascorbic Acid

The method is based on the reduction of Mo (VI)-Mo(V) by extracts and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The reaction mixture consisted of: 0.3 ml of extract combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm relative to the control using a spectrophotometer. Methanol (0.3 ml) was used as a control. The higher the absorbance values, the higher the total antioxidant capacity. Ascorbic acid equivalents were calculated using a standard graph. Results were expressed in ascorbic acid equivalents based on the standard calibration curve (see Figure 6) obtained for different ascorbic acid concentrations (10 - 200 µg/ml).

Statistical analysis

All experiments were performed in 3 replicates. Results were expressed as mean values and standard deviation (±SD). Statistical analyses were performed using Microsoft Office Excel 2019.

RESULTS AND DISCUSSIONS

Wheat genotypes "Pitar", H171-I and H171-III were evaluated by UV-VIS spectrophotometry for their total anthocyanins, phenols and enzymes composition (polyphenol-oxidase - PPO and peroxidase - POD).

Determination of total anthocyanins content (TAC) showed that the highest level of anthocyanins was recorded in genotype H171-I (24.58±0.208 mg/L), followed by "Pitar" (16.96±0.095 mg/L) and H171-III (12.19±0.089 mg/L) (Figure 4).

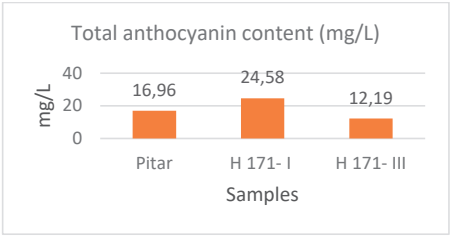

Figure 4. Anthocyanin content in grains

The anthocyanin compounds present in colored wheat depend on the wheat variety, growing conditions and maturity of the grain. These factors can sometimes make it difficult to identify and quantify anthocyanins (Rao et al., 2018). Due to their potential health-enhancing attributes through their antioxidant activity, anthocyanins are of interest to the food industry. At present, in the world, there is some cultivars with purple pericarp as Laval, Laval 19, Indigo with the TAC range between 72 and 96 mg/kg (Francavilla and Joye, 2020).

Selection of the wheat lines with intense purple pericarp, from H171-I, could increase the total anthocyanin content.

Total phenol content was determined from the standard calibration curve (see Figure 2).

The data obtained showed that the highest level of total phenols was recorded in genotype H 171-I (69.59±0.810 mg GAE/L), followed by wheat genotype 'Pitar' (50.4 ±0.483 mg GAE/L). The lowest concentrations of polyphenols were recorded in H-171-III (37.4 ± 0.813 mg GAE/L) (Figure 5).

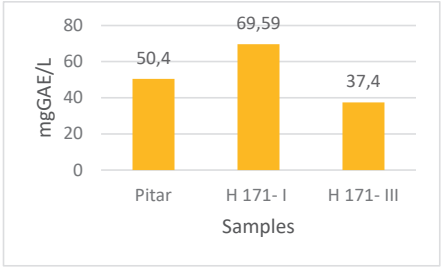

Figure 5. Total phenols content in wheat grains

Biosynthesis of phenolic compounds is not yet well understood, but has been observed to depend on genotype and growth temperature (Ma et al., 2016; Wu et al., 2016). Moisture level has also been shown to affect the concentration of total phenolics in cereal grains (Abdel-Aal et al., 2003).

Determination of enzyme activity.

Data on the enzyme activity of polyphenol-oxidase (PPO) and peroxidase (POD) showed that it was maximum for both enzymes in the red grain wheat genotype "Pitar" (0.206±0.009 U/ml PPO and 0.055 ± 0.002 POD U/ml, respectively). The results suggest that these enzymes are among those responsible for the oxidation of catechol in wheat. The wheat lines H171-I and H171-III showed similar levels of activity, but slightly higher for genotype H171-I (0.156±0.002 U/ml PPO and 0.026±0.001POD) compared with genotype H171–III (0.144 ± 0.009 PPO and 0.022 ± 0.002 POD) (Figure 6).

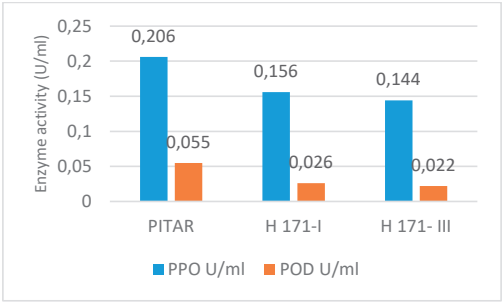

Figure 6. Polyphenol-oxidase (PPO) and peroxidase (POD) activities in wheat grains

Polyphenol oxidase (PPO) is predominantly found in the outer layers of wheat kernel (Baik et al., 1994). High levels of PPO have been associated with discoloration of wheat end-use products (Singh & Sheoran 1972; Edwards et al., 1989). In wheat, polyphenol oxidase (PPO) is responsible for the oxidation of polyphenolic compounds to quinones, which, following further reactions, leads to the production of brown melanin (Stauffer, 1987). Peroxidase (POD), the phenol-oxidizing enzyme, has been studied in many plant-pathogen interactions because of its significant role in the biosynthesis of plant cell wall components (Grisebach, 1981; Lamport, 1986). In wheat grains, peroxidase is present in the epidermis, seed coat, embryo and

endosperm. POD can oxidize ferulic acid and other major phenolic acids, producing chromophoric groups and brown substances (Fraignier et al., 2000). A high activity of POD can influence the quality of the flour and consequently can cause an undesirable color of the paste (Taha and Sagi, 1987; Kobrehel et al., 1974). In addition, peroxidase has been reported to play a key role in wheat resistance to leaf rust (Johnson and Lee, 1978).

Total antioxidant capacity. Total antioxidant activity was expressed in ascorbic acid equivalents.
Table 1 shows the results of the total antioxidant activity of the tested samples.

Table 1. Total antioxidant activity of the samples

Genotype	µg/ml
PITAR	24.86 ± 0.294
H171 I	29.13 ± 0.191
H171 III	22.23 ± 0.350

Results were expressed as mean values and standard deviation (±SD).

These results showed that total antioxidant activity was recorded in the genotype H171- I (29.13±0.19 1µg/ml), followed by "Pitar" (24.86±0.294 µg/ml) and H171-III (22.23±0.350 µg/ml).
A number of other reports have observed certain correlations between total anthocyanin content, total phenolic content and antioxidant potential (Lee et al., 2013; Harakotr et al., 2014).

CONCLUSIONS

The results obtained highlighted the presence of anthocyanins and phenolic compounds in all investigated wheat genotypes, but at higher levels in the case of the H171-I line with purple pericarp; Data on polyphenol-oxidase (PPO) and peroxidase (POD) enzyme activity showed that it was higher, for both enzymes, in the red "Pitar" grain genotype; The genotypes H171-I with purple grains and H171-III with red-purple grains recorded similar enzyme activity levels, but slightly higher in the H171-I genotype. Phenolic compounds can promote the total antioxidant capacity in the plant.

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STUDY ON THE EVALUATION OF POMOLOGICAL, BIOCHEMICAL, AND ORGANOLEPTIC PARAMETERS OF VARIETIES APRICOT

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Abstract

Fruit production influences people's health due to its biochemical compounds. Apricot fruit is rich in potassium, magnesium, and calcium and contains sufficient glucose, sucrose, fructose, vitamins A and E, antioxidant compounds, beta-carotene, and lycopene. Cultivars are an essential factor in determining the final yield of the product, the quality of the fruits produced, the vegetative growth of the plant, and the stress resistance. Several studies have been conducted to increase fruit quality in important fruit indicators such as color, taste, carbohydrate content, titratable acidity, fruit size, firmness, time of fruit harvest, and ripening time. The present study presents the results for more than 25 cultivars of fruit quality parameters correlated to canopy shape or rootstock.

Key words*: titratable acidity, total soluble solids, dry matter.*

INTRODUCTION

Besides satisfying people's nutritional needs, fruit production also influences people's health due to its biochemical compounds (Akin et al., 2008). Apricot is used as fresh, dried, and processed fruit (Altindag et al., 2006; Özdoğru et al., 2015). Apricots are rich in potassium, magnesium, and calcium, and it has been explained that some organic acids, sugar, and phenolic compounds are also present in them (Muradoğlu et al., 2011; Ali et al., 2011). In addition, they contain sufficient glucose, sucrose, fructose, vitamins A and E, antioxidant compounds, beta-carotene, and lycopene (Muradoğlu et al., 2011). The lycopene in apricots is effective against cancer and protects the body from the dangers of high cholesterol followed by heart disease (Iordanescu et al., 2018).

The taste and color of apricots are influenced by the amount of carotenoids and phenolic compounds (Huang et al., 2013). It has been found that the amount of organic acids and sugar effectively determines the taste and smell of the fruit in apricots (Miloševic et al., 2016). In a study conducted by Gundogdu (2019), it was found that using different rootstocks affects some of the phytochemical fruit parameters. Cultivars are essential in determining the final yield in fruit trees and orchards (Akin et al., 2008). The selection of the suitable rootstock influences the final yield of the product and the quality of the fruits produced, the vegetative growth of the plant, as well as the stress resistance (Son & Küden, 2003; Şahiner et al., 2013; Milosevic et al., 2011; Arıcı, 2008).

Selecting the suitable rootstock has to be done according to the growing conditions (Milošević & Milošević, 2019b). Among its objectives are the optimal development of the plant, the increase of the quality parameters of the fruits, the improvement of the yield, and the resistance to biotic and abiotic stress (Zhebentyayeva et al., 2012). In other research, the importance of the cultivar (Mratinic et al., 2011; Iordanescu et al., 2012), geographical conditions of the region (Milosevic et al., 2010; Campbell et al., 2011), cultivation system (Leccese et al., 2010), ripening process (Ayour et al., 2017), and protection and processing technologies (Hussain et al., 2013) fruit related indicators were also discussed.

Several studies have been conducted to increase fruit quality in important fruit indicators such as color, taste, carbohydrate content, titratable acidity, fruit size, firmness, time of fruit harvest, and ripening time. (Oprita et al., 2020).

MATERIALS AND METHODS

The study was conducted for 30 apricot cultivars planted in the Experimental Fruit Field of the Faculty of Horticulture in Bucharest, established in 2017, with two canopy shapes – Trident and Bi-Baum® (Al-Suwaid et al., 2023).

From the sample of 20 fruits, each fruit was measured with the digital caliper, determining the length and the two diameters.

The shape index was determined using the formula: i = l/ [(d1+d2)/2].

The share of the weight of the pulp, respectively of the seeds, from the total average weight of the fruit was determined. Total acidity (expressed in g malic acid/100 g fresh product), dry matter (%), and total soluble solids (º Brix) were determined.

RESULTS AND DISCUSSIONS

The series of cultivars cultivated in the Trident system

Fruit length study for different cultivars in two consecutive years showed that the most extended fruit length in the first year belonged to Primaya/SJA, Anegat/M29C, and Faralia/M29C cultivars. In the second year, it belonged to Farbali/M29C cultivars. The values varied between 45.94 and 55.95 mm in 2021 and 38.12 and 55.48 mm in 2022 (Table 1).

Table 1. Fruit length in different cultivars in the period 2021-2022 (cm) at the Trident system

Variant	2021	2022
Anegat/M29C	55.95a	45.16f
Faralia/M29C	55.73a	52.83bc
Farbali/M29C	46.45b	55.48a
Farbali/SJA	48.35b	54.72ab
Farbela/M29C	47.28b	52.51bc
Farclo/M29C	48.18b	48.32e
Farclo/SJA	48.53b	50.24df
Farely/SJA	45.94b	45.35f
Farlis/M29C	47.78b	44.30f
Fartoly/SJA	46.01b	38.12g
Primaya/SJA	55.16a	52.24cd
Average	49.47b	49.02e

Studying the **average fruit diameter** of the different varieties analyzed, it was found that the fruits produced by the Primaya/SJA cultivar had the highest value of this index compared to other varieties in both years of the study. In addition, it was found that in the second year, the fruits of Farbali/M29C and Farbali/SJA had values similar to Primaya/SJA, higher compared to other varieties. The values varied between 41.55 and 53.26 mm in 2021 and 38.47 and 51.87 mm in 2022, respectively.

The fruit shape index had values generally above unity, the fruits having an ovoid shape, Fartoly/SJA being the only variety with round/symmetrical fruits.

Absorption difference index (IAD)

The fruits were harvested after evaluating the absorption difference index determined with the DA-meter. This index was determined for each fruit and at the time of analysis. The values varied between 0.07 and 0.36 in 2021 and between 0.09 and 0.39 in 2022, indicating the degree of ripening of the fruits (Table 2).

Table 2. Index of absorbance difference (AD index) at the time of analysis in cultivars in the period 2021-2022 at the Trident system

Variant	2021	2022
Anegat/M29C	0.19c	0.14e
Faralia/M29C	0.26bc	0.28bc
Farbali/M29C	0.36a	0.33ab
Farbali/SJA	0.28abc	0.17de
Farbela/M29C	0.09d	0.11e
Farclo/M29C	0.06d	0.23cd
Farclo/SJA	0.22c	0.38a
Farely/SJA	0.31ab	0.31abc
Farlis/M29C	0.26bc	0.11e
Fartoly/SJA	0.26bc	0.39a
Primaya/SJA	0.28abc	0.09e
Average	0.23e	0.23cd

Weight pulp

In the first and second years of the study, it was found that the Primaya/SJA cultivar presented the highest comparative values with the others. In the second year, Farbali/SJA and Farbali/M29C had significantly similar values. Values ranged between 41.02 and 82.60 g in 2021, respectively, and between 28.83 and 77.51 g in 2022.

Kernel weight

Values ranged between 2.49 and 4.77 g in 2021 and 2.46 and 4.96 g in 2022, respectively. Fartoly/SJA and Farlis/M29C showed the highest values in 2021; in the second year of study, the highest value was observed in Farbali/M29C. In the second year, the other cultivars did not show significant differences in kernel weight.

Total soluble solids content. The highest value in the first year was observed at Fartoly/SJA, and the lowest was observed at Primaya/SJA. In the second year, the highest amount was observed in the cultivars Farely/SJA, Farbali/SJA, and Farbela/M29C, and the lowest amount was observed in the cultivar Primaya/SJA (Table 3). Values ranged between 8.19 and 14.30 Brix in 2021 and between 8.1 and 17.06 Brix in 2022.

Table 3. Total soluble solids content in different cultivars in the Trident system (2021-2022)

Variant	2021	2022
Anegat/M29C	9.63fg	15.50ab
Faralia/M29C	11.51cde	13.46c
Farbali/M29C	8.90gh	16.43a
Farbali/SJA	10.42ef	14.03bc
Farbela/M29C	12.40bcd	15.98a
Farclo/M29C	12.61bc	12.90c
Farclo/SJA	13.06ab	13.63c
Farely/SJA	11.00def	17.06a
Farlis/M29C	11.83bcde	9.82d
Fartoly/SJA	14.30a	13.63c
Primaya/SJA	8.19h	8.10e
Average	11.23cdef	13.63c

Total acidity

Examining the changes in total titratable acidity values in different cultivars cultivated in the Trident system showed that the highest value of this parameter in the first year was observed in the cultivars Primaya/SJA, Farbela/M29C, and Farbali/M29C (1.7567 g acid malic/100 g fw)) and in the second year to the cultivar Farbela/M29C (1.980 g acid malic/100 g fw). The lowest values were at Fartoly/SJA (0.530 g acid malic/100 g fw) and Faralia/M29C (0.660 g acid malic/100 g fw) in the first year, and Farlis/M29C in the second year (0.590 g acid malic/100 g fw) (Table 4).

Table 4. Tritable total acidity in different cultivars in the Trident system (2021-2022)

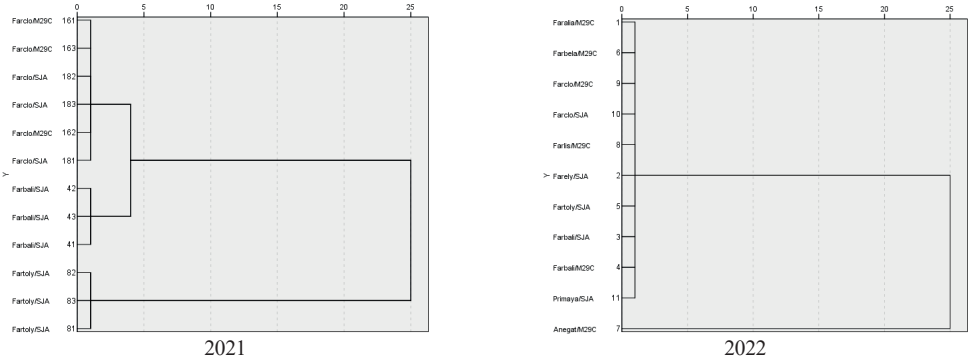
Variant	2021	2022
Anegat/M29C	0.8167bc	1.2900d
Faralia/M29C	0.6600c	0.9175e
Farbali/M29C	1.7567a	1.1833d
Farbali/SJA	0.8067	1.4500c
Farbela/M29C	1.8100a	1.9800a
Farclo/M29C	0.8867bc	1.1867d
Farclo/SJA	1.1067b	1.1833d
Farely/SJA	1.1267	1.0250e
Farlis/M29C	0.8167bc	0.5900f
Fartoly/SJA	0.5300c	1.2767d
Primaya/SJA	1.9200a	1.7800b
Average	1.1124b	1.2570d

Total dry matter

The analysis of the content in the total dry matter found that the cultivar Farclo/M29C had the highest value compared to other cultivars in both years. In the first year of study, the cultivars Farclo/SJA and Fartoly/SJA had similar values. In the years 2021, values ranged between 7.92% (Primaya/SJA) and 16.50% (Fartoly/SJA), and in 2022, between 9.95% (Anegat/M29C) and 18.72% (Farclo/M29C).

Cluster analyze

In the analysis of the cultivars in the first and second years, according to the studied parameters, the results showed that the studied cultivars in the first year were divided into three different groups. In contrast, they were divided into two groups in the second year. In 2022, the Anegat/M29C cultivar was placed in one group, and other cultivars in another group (Figure 1).



2021 2022

Figure. 1. Cluster analysis of different cultivars in the Trident system

The series of cultivars grown in the Bi-Baum system, 2.0 m per row

Fruit Length

The study of the fruit length index showed that the highest fruit length in the first year was observed in cultivars Delice/M29C (53.75 mm) and Medflo/M29C (51.65 mm). In the second year of the study, the average length of the fruits of the cultivars Wonder Cot/M29C (51.42 mm) and Lady Cot/M29C (50.95 mm) was significantly higher compared to other cultivars.

Average fruit diameter

In the investigation of the average diameter index, it was found that, in the first year of the study, the highest value of this index was observed at Delice/M29C (52.33 mm), while in the second year at Delice/M29C (49.92 mm) and Lady Cot/M29C (50.86 mm) (Table 5).

Table. 5. Average diameter in the Bi-Baum system, 2.0 m (mm) (2021-2022)

Variant	2021	2022
Congat	43.02c	43.55c
Delice	52.33a	49.92a
Flopria	43.29c	42.24c
Lady Cot	47.95b	50.86a
Lido	37.07d	45.676b
Lilly Cot	48.04b	39.79d
Medflo	46.26b	47.29b
Mikado	0	46.32b
Milord	46.59b	46.83b
Swired	42.46c	42.46c
Wonder Cot	48.00b	43.02c
Average	45.50bc	45.27b

Absorbance difference index (IAD)

In 2021, the IAD values at the time of the analysis were between 0.11 and 0.56; in 2022, they were between 0.05 and 0.27. In the analysis of this index in different apricot cultivars, it was found that Delice/M29C, Lady Cot/M29C, Lilly Cot/M29C, Medflo/M29C, and Wonder Cot/M29C had similar values in the first year compared to other cultivars.

The value of this index was the lowest in the cultivars Flopria/M29C, Lido/M29C, and Milord/M29C in the first year. In the second year, the highest value of this index was observed in the cultivar Milord/M29C, the lowest at Flopria/M29C and Lido/M29C (Table 6). The ADI values reflect the fruits' ripening degree, respectively, a comparative scale between cultivars.

Table 6. IAD in different cultivars at the analyses moment (2021-2022)

Variant	2021	2022
Congat	0.28b	0.21b
Delice	0.53a	0.17bc
Flopria	0.15c	0.07ef
Lady Cot	0.56a	0.15cd
Lido	0.36ab	0.04f
Lilly Cot	0.55a	0.09ef
Medflo	0.55a	0.12de
Mikado	0	0.07ef
Milord	0.10c	0.27a
Swired	0.20bc	0.20bc
Wonder Cot	0.47a	0.06f
Average	0.38	0.13cd

Weight pulp

According to the research results, it was found that the highest value was observed and recorded in the first year in the cultivar Delice/M29C (80.57 g) and the second year at the cultivar Lady Cot/M29C (77.84 g). The values varied in 2021 between 27.69 and 80.57 g and 2022 between 37.55 and 77.84 g.

Kernel weight

In the study of the differences in kernel weight in different cultivars in the Bi-Baum planting system, 2.0 m, it was found that in the first year, the cultivars Congat/M29C and Wonder Cot/M29C had the highest values. The other varieties had lower values and no significant differences between them in the first year of the study. In the second year of the study, it was found that the cultivars Delice/M29C, Lady Cot/M29C, and Milorod/M29C had the highest kernel weight compared to other cultivars studied. Values ranged between 1.89 g (Lilly Cot/M29C) and 3.90 g (Delice/M29C) in 2021, respectively between 2.01 g (Wonder Cot/M29C) and 4.01 g (Lady Cot/M29C) in 2022.

Total soluble solids content

In the analysis of the content of total soluble solids (TSS) content in different cultivars, it was found that in the first year, the TSS amount was significantly higher in the cultivars Congat/M29C and Delice/M29C compared to other cultivars. In the second year of research, the highest TSS amount was observed and recorded in the cultivar Congat/M29C (Table 7). The values were

between 7.21 and 12.52 °Brix in 2021, respectively between 8.13 and 16.91 Brix in 2022.

Table 7. Total soluble solids content in different cultivars in the Bi-Baum system, 2.0 m (°Brix) (2021-2022)

Variant	2021	2022
Congat	12.52a	16.91a
Delice	11.89a	10.84c
Flopria	7.60de	9.25def
Lady Cot	8.92bcd	10.86c
Lido	7.21e	10.35cd
Lilly Cot	7.49de	8.13f
Medflo	7.49de	9.36def
Mikado	0	8.61ef
Milord	11.89b	13.57b
Swired	9.74bc	9.74cde
Wonder Cot	8.60cde	8.79ef
Average	9.17bc	10.58cd

Total acidity

The highest value was observed in cultivars Lido/M29C and Lilly Cot/M29C in the first year of research, respectively, in the cultivar Lady Cot/M29C for the second year. Congat/M29C presented the lowest value in both years compared to other cultivars. Values ranged between (Congat /M29C) 1.05 g acid malic/100 g fw and (Lilly Cot/M29C) 1.97 g acid malic/100 g fw in 2021, respectively between (Congat/M29C) 0.72 g acid malic/100 g fw and (Lady Cot/M29C) 2.08 g acid malic/100 g fw) in 2022.

Soluble dry matter

The content values ranged between 7.20% (Lido/M29C) and 12.50% (Congat /M29C) in 2021, respectively between 8.51% (Wonder Cot/M29C) and 17.00% (Congat/M29C) in 2022. The highest value of this index was observed and recorded in the cultivar Congat/M29C in both years of study. In addition, the Swired cultivar had a similar value in the second year.

Cluster analyze

The cluster analysis found that the cultivars were divided into several groups in the first year. The similarities were between Wonder Cot/M29C and Medflo/M29C, Lady Cot/M29C, Delice/M29C, Swired/M29C, and Lilly Cot/M29C (Figure 2).

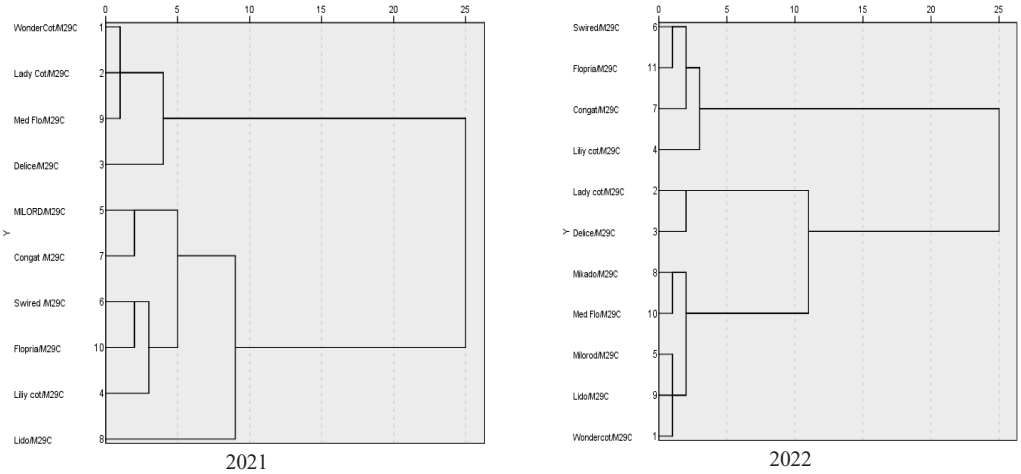


Figure 2. Custer analyzes different cultivars in Bi-Baum, 2.0 m

The series of cultivars grown in the Bi-Baum system, 1.5 m per row

Fruit Length

The results related to the length of the fruits showed that in the first year of the study, maximum values were observed in the fruits of the variety Pisana/M29C (59.42 mm), followed by cultivars Vitillo/M29C (54.99 mm) and Primaya/SJA (53.63 mm). In the second year of the study, the most extended fruit length was observed in the cultivar Vitillo/M29C (57.84 mm), followed by Primaya/SJA (53.39 mm). The most petite fruit length was recorded in the cultivars Congat/GF677 in both years of study (42.45 mm in 2021, 38.68 mm in 2022).

Average fruit diameter

In the examination of the average diameter of the fruits, it was found that, in the first year, the values

varied from 41.08 mm (Boccuccia Liscia/M29C) to 53.88 mm (Pisana/M29C). In the second year, the values ranged between 36.73 mm (Congat/GF677) and 55.37 mm (Vitillo/M29C).

Absorption difference index (IAD)

The values of the absorption difference index varied between 0.08 (Boccuccia Liscia/M29C) and 0.39 (Rubista/M29C) in 2021, respectively between 0.05 (Boccuccia Liscia/M29C) and 0.41 (Congat/M29C) in 2022, indicating the level of ripening of the fruit (Table 8).

Table 8. IAD in different cultivars in the Bi-Baum system, 1.5 m (2021-2022)

Variant	2021	2022
Bergeron/M29C	0.17bc	0.09cde
BoccucciaLiscia/M29C	0.08d	0.05e
CMBU/M29C	0.27b	0.16bc
Congat/GF677	0.13cd	0.41a
Pisana/M29C	0.23bc	0.18b
Portici/M29C	0.18bc	0.09de
Primando/SJA	0	0.12bcd
Primaya/SJA	0.23bc	0.13bcd
Rubista/M29C	0.39a	0.14bcd
Vitillo/M29C	0.15cd	0.13bcd
Average	0.20bc	0.15bcd

Weight pulp

The research results showed that the cultivars studied showed significant differences in pulp weight in both years studied. The highest value in the first year was observed at Pisana/M29C (81.85 g), followed by Congat/GF677 (77.98 g), Primaya/SJA (70.08 g), and Vitilo/M29C (73.46 g). Cultivars Boccuccia Liscia/M29C (40.78 g) and Rubista/M29C (20.41 g) had the lowest pulp weight in the first year of the study. In the second year of the study, the highest pulp weight was observed in Vitillo/M29C (93.35 g), followed by Primaya/SJA (81.09 g).

It should be noted that pulp weight in other cultivars studied in the second year was generally less than 50 g.

The **kernel weight** results at the plants in the Bi-Baum system, 1.5 m per row, showed that the cultivar Vitillo/M29C had the highest kernel weight in both years compared to the other cultivars. Kernel weight in this cultivar was four times greater in the second year compared to cultivars Primando/SJA and Rubista/M29C, which had the lowest values. This year, the kernel weight of other cultivars was less than 4 g.

Total soluble solids content

Analyzing the content in total soluble solids (TSS), it was found that in the studied years, the cultivars Boccuccia Liscia/M29C and Congat/GF677 had the highest values compared to other cultivars. Pisana/M29C and Primaya/SJA showed the lowest values in 2021, and Primando/SJA, Primaya/SJA, and Vitlilo/M29C in 2022 (Table 31). Values ranged between 7.13 °Brix and 15.47 °Brix in 2021, respectively between 8.50 °Brix and 17.69 °Brix in 2022.

Total acidity

The titratable acidity measurement showed that the highest value was observed in the cultivar Rubista/M29C, followed by Primaya/SJA in the first year. In the second year of the study, it was also found that the titratable acidity of the cultivar Primando/SJA was significantly higher than other cultivars studied.

Dry matter content

Data related to the total dry matter in the first year of the study showed that the cultivars Boccuccia Liscia/M29C, Congat/GF677, and Primaya/SJA showed higher values than other varieties. The lowest value was observed at CMBU/M29C in the first year. In the second year, the values increased in most of the varieties studied compared to the first year. In the second year, the highest total dry matter was recorded in the cultivar Congat/GF677 (17.01%).

Cluster analysis

Cluster analysis of cultivars showed that apricot cultivars were generally placed in two groups according to the traits studied in the two years. Cultivars Portici/M29C, CMBU/M29C, Bergeron/M29C, Bocuccio/M29C, Pisana/M29C, and Vitilo/M29C were classified in a single group, and the cultivars Congat/GF677, Primaya/SJA, and Rubista/M29C were classified in another separate group (Figure 3). Note the similar profiles for the following cultivars: Portici/M29C, CMBU/M29C, Bergeron/M29C, and Pisana/M29C; Boccuccia Liscia/M29C and Vitillo/M29C; Congat/GF677and Primaya/SJA.

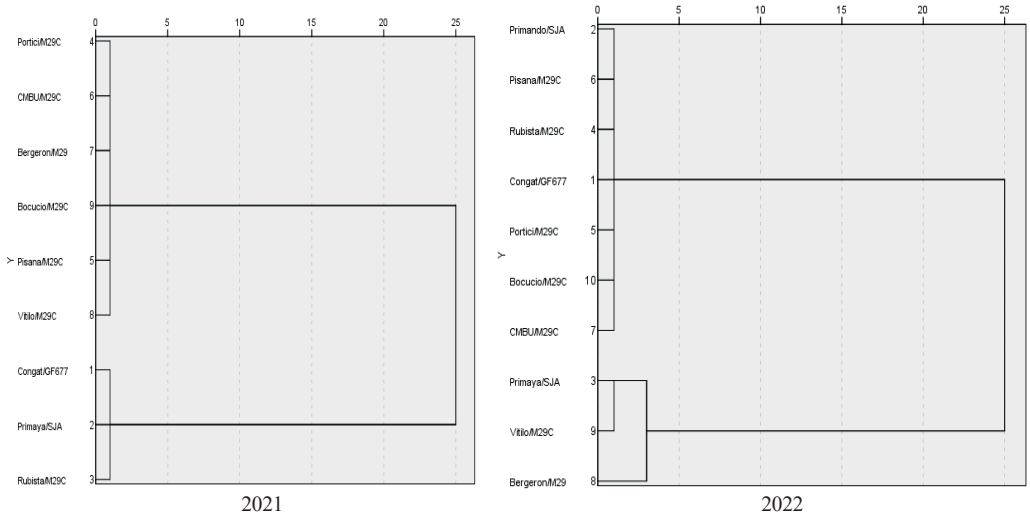


Figure 3. Cluster analysis in different cultivars in the Bi-Baum system, 1.5 m

The Influence of rootstock or canopy shape on fruit quality parameters

Comparing the quality parameters to the cultivar Primaya/SJA in the two planting systems, Trident and Bi-Baum, significant differences were observed between the parameters, as with the Congat cultivar on two different rootstocks (M29C, GF677) in the planting system, Bi-Baum.

Discussion

The research results showed that the different cultivars in the study, depending on the rootstock and canopy shape, responded differently to the indices studied in the evaluation. The difference between the studied parameters comes from the differences between the cultivars, the planting system, and the climatic and geographical conditions of the place where the plants are grown (Pfeiffer & Hegedus, 2011).

Fruit quality parameters have always been important in trade. The quality of the fruit is correlated with the balance between sugar and acid content and its unique flavor (Molaie et al., 2016).

The high amount of sugar and the low amount of organic acids in the fruit cause a sweet taste (Akin et al., 2008). In addition, firmness and fruit size are two important and influential factors in the increase in economic value and fruit sales (Rousos et al., 2011). The color of apricot fruit is affected by flavonoid glucosides. It should be noted that the synthesis of these compounds in the plant is influenced by sunlight. Therefore, environmental factors such as light and temperature play an essential role in apricot fruit coloring (Pfeiffer & Hegedus, 2011).

This issue can effectively choose the right canopy shape of the plant. Jannatizadeh et al. (2010) studied 39 cultivars and genotypes of Iranian apricots using morphological traits; their results showed that the coefficient of variation of the ratio of total soluble solids (TSS) to titratable acidity (TA), kernel weight, fruit flesh weight, and fruit weight had high values, which indicate the possibility of selection for improvement and modification in breeding programs.

Mohhamadzadeh et al. (2005) evaluated 21 pomological and morphological traits in research to compare, group, and select the superior genotypes from 32 native apricot cultivars and genotypes.

The results showed large variations between cultivars, and significant positive and negative correlations were observed between some of their traits. Primaya/SJA had greater fruit length, average fruit diameter, kernel length, pulp weight, total titratable acidity, and total titratable acidity/TSS ratio than other cultivars. However, the TSS and glucose content were the lowest compared to other cultivars.

Cultivar Anegat/M29C also had higher fruit and kernel lengths than other apricot cultivars.

This study showed that the Farbali cultivar grafted on M29C and SJA also showed acceptable performance about fruit-related indicators. The study of different cultivars grafted on M29C in

the Bi-Baum system, 2.0 m per row, showed that the cultivar significantly affects the biochemical traits of the fruit. Delice, Wonder Cot, and Congat varieties showed acceptable results among the studied cultivars.

In examining cultivars in the Bi-Baum system (1.5 m on the raw), it was also found that Primaya/SJA, even in this system, showed good performance in traits related to the plant's fruit. This issue shows the importance of choosing the proper cultivar more than before. According to the purpose of cultivation of the apricot cultivars (fresh, dried, and processed consumption), selecting suitable cultivars from the studied cultivars is possible.

CONCLUSIONS

It can be explained that the different cultivars studied in the research have shown different behaviors about the indicators related to the fruit. In some cases, regardless of the shape of the canopy, the cultivated cultivar has provided appropriate responses.

This issue indicates the great importance of a suitable cultivar during cultivation. In addition, choosing the suitable rootstock in the plant is also an essential and influential factor in the plant's growth. Therefore, it can be explained that choosing the suitable rootstock and cultivar according to the ecological conditions of the region has, in some cases, resulted in producing a good quality product that can compete with native cultivars that are sometimes susceptible to certain diseases or biological stress.

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THE CHALLENGES AND ALTERNATIVES OF FOOD SUSTAINABILITY: MEAT ANALOGUES & CULTURED MEAT

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Abstract

The present paper is based on a bibliographic study of over 100 articles published between 2001 and 2023 with general aim of the identification of the most causes that led to the need to replace meat of animal origin with meat analogues and/or cultured meat. The identification of new protein sources and the characterization of the nutritional profile, textural behaviour, sensorial attributes, etc. of meat analogues open new research horizons. The review of the most relevant studies on how to obtain plant-based meat analogues brings to the attention of researchers various pretreatments such as extrusion, hydrogenation, hydrolysis, as well as other technological challenges in improving the quality of plant-based meat analogues. Current research on the new food products development highlights the need regarding the risks and benefits analysis of plant-based and cultured meat analogues, which require in-depth studies in many directions.

Key words*: food sustainability, meat analogues, cultured meat, processing methods.*

INTRODUCTION

The food sector represents approximately 26% of global greenhouse gas emissions (Zioga et al., 2022). Concerns about the health of the planet are increasingly prominent meat alternatives being a potential alternative (Gbejewoh et al., 2022).

The harmful effects of animal production led to the development of technologies and the need to find alternatives, such as vegetable proteins (Szpicer et al., 2022; Estel et al., 2021; Lai et al., 2017). These are textured food products made from plant-derived proteins that mimic or replace meat (Wang et al., 2022; Lee et al., 2020).

The aim of these analogues is to imitate the physical and organoleptic properties of animal products through the fibrous composition and the mixture of ingredients from plant sources, using appropriate technology, which allows providing a similar texture and flavors. (Lima et al., 2022).

The demand for innovative meat analogs is a relevant issue in the food sector (Szpicer et. al., 2022), the perspectives of food science and technology providing industrial challenges in identifying innovative technological solutions is the one that provide new products with patent possibilities (Tyndall et al., 2022). Innovative technologies and alternative protein sources have been associated with sustainable food systems as well as improved nutritional quality and safety of the food products (Hassoun et al., 2022).

For humanitarian reasons, vegans and vegetarians avoid animal products and enjoy the nutritional benefits by using alternative foods (Kazir & Livney, 2021). Plant-based diets are beneficial for health due to reduced risk of obesity, tumors and cardiovascular diseases (Hassoun A. et al., 2022; Craig et al., 2021; Samtya et al., 2021), these changes being promoted by the Commission European within the Farm-to-Fork-Strategy (Prache et al., 2022).

In this context, this review provides an in-depth documentation of the characteristics of artificial meat from different sources, analyzes current trends, materials and methods used, and consumer perception of meat analogs.

CLASSIFICATION OF ARTIFICIAL MEAT

Food researchers are currently analyzing two types of artificial meats: plant-based meat (He et al., 2020; Joshi & Kumar, 2015; Wild et al., 2014) and cultured meat (He et al., 2020; Hocquette, 2016; Bhat & Fayaz, 2011).

Meat alternatives can be classified in turn: plant-based (soy, pea, gluten, etc.), cell-based (*in vitro* or cultured meat) and fermentation-based (microproteins or microalgae extracted from Spirulina and isolated proteins from insects), the figure (Figure 1) presented below highlighting the concept of artificial meat classification (Sha & Xiong, 2020).

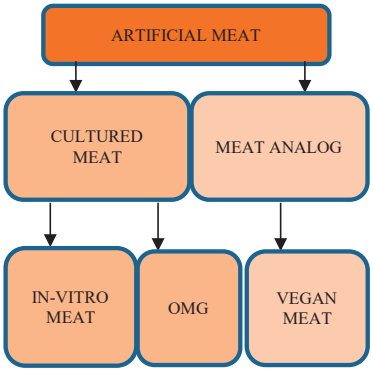

Figure 1. Classification of artificial meat *(in vitro* meat, OMG-genetically modified organisms, vegan meat) Source: (Mateti et al., 2022)

CHARACTERIZATION OF ARTIFICIAL MEAT SOURCES

The representative meat alternatives on the market are analogs derived from vegetable proteins, for various socio-economic, nutritional and technological considerations (Huang et al., 2022). A transition from animal to vegetal proteins would be beneficial for biodiversity, land use, water use, climate, and people, also for animal health and welfare (Hartmann et al., 2017; Aiking et al., 2011; Leip et al., 2015). Plant proteins mixture may have a well-balanced amino acid composition and excellent potential to replace meat by developing healthy meat-like and nutritionally similar products (Sun et al., 2021).

One of the key components for the formation of the structure and nutritional value of meat analogs is plant proteins (Zhang et al., 2021), which can come from various plant sources such as soybeans, peas, beans, lentils, cereals, algae and microalgae, etc., each possessing its own characteristics (McClements & Grossmann, 2021a; McClements & Grossmann, 2021b). However, proteins from plant products are deficient in at least one of the essential amino acids, such as lysine, methionine, or cysteine (Xie et al., 2022). Meat analogs used to obtain vegan meat mainly come from soybean derivatives rich in carbohydrates, proteins, fats, fibers, vitamins, micro and macronutrients (Ishaq et al., 2022), fermented products, etc., (Mateti et al., 2022). Although soy protein has good functional properties in terms of appearance, texture, structure and taste (such as emulsification, foaming, water and oil absorption, viscosification ability and gelation), it also has limitations such as undesirable grassy bean flavor, high allergenicity and methionine as a limiting amino acid (Lee, Choi, Han, 2022). Compared to soy, cereals (wheat, rice, barley and oats) are rich in carbohydrates and have a lower protein content, and from a functional point of view, the structure of wheat proteins gives consistency and texture similar to meat products (Bohrer, 2019).

Mushrooms have a high protein content, comparable to that of animals or poultry, close to that of soybean and pea protein, and higher than that of wheat (Wang & Zhao, 2022). In the case of meat analogs, the organoleptic properties must be as close as possible to those of meat, and can be stimulated by adding mineral and vegetable spices, food colorings, etc. (Flores & Piornos, 2021).

The first insect-based product approved by the European Union for human consumption (using yellow mealworms) was granted in May 2021 (Wood & Tavan M, 2022), however the consumption of insect-based foods in Europe is relatively low, due to social and contextual factors (House et al., 2016). The nutritional profile of insects (mealworms, crickets, grasshoppers), shows that they are rich in protein (60% for crickets), fats, minerals and vitamins (Wood & Tavan, 2022), their edibility representing a high potential to become a major source of human nutrition that can be produced more efficiently (with lower levels of gas emissions and water consumption) than conventional animals, (Alexander et al., 2017; Onwezen et al., 2019).

Microalgae or microproteins are a rich source of numerous nutrients and components beneficial to health, including vitamins, minerals, proteins containing essential amino acids, polyunsaturated fatty acids, antioxidants and dietary fiber (Bernaerts et al., 2019). The yield

of microalgae can reach between 15-30 tons of dry biomass/area unit per year, while soybean yield can reach 1.5-3.0 tons/area unit per year (Fu et al., 2021).

Various studies have indicated the production of microprotein biomass using agro-industrial wastes such as industrial peas and pineapple peas (Ahmad et al., 2022).

Cereal polysaccharides are an important source of dietary fiber, studies highlighting their exploitation in different food matrices. Their positive role as an antioxidant, antitumor, anti-inflammatory, antimicrobial agent being proven by in vitro and in vivo chemical research (Kaur & Sharma, 2019)

Cultured meat is part of the field of cell agriculture, a promising technology with key challenges and techniques including cell source, culture medium, mimicking animal-derived in vivo myogenesis medium, and bioprocessing for industrial-scale production (Stephens et al., 2018).

MATERIALS, METHODS AND TECHNIQUES

Relevant technology studies for vegetable protein-based meat analogues & cultured meat

The new generation of plant-based textured meat analogs is trying to boost dietary fiber consumption. (Diaz et al., 2022). Meat analogs usually contain more than 20 ingredients: fats, sugars, vitamins, minerals, genetically modified pigments, phosphates, organic acids, etc. (Nagapo, 2022). These products are obtained using extrusion technology or other methods (Shaghaghian et al., 2022). Textured plant proteins are the most common ingredients in plant-based meat analogs (Lin et al., 2022). Pea proteins have an unpleasant flavor similar to beans, and to eliminate it, modern microbiologists use fermentation with the help of microorganisms, which also has other benefits such as restoring the intestinal microflora and repairing damage to the intestinal epithelium caused by food additives (Tao et al., 2022). Soy cakes contain proteins, fats, dietary fibers, but also a lot of anti-nutrients. The reduction of antinutrients is achieved by solid state fermentation with lactic acid bacteria, the results obtained, as well as the sensory properties of the meat analogues obtained, recommend them for the use of pressed soybean cake in meat analogues (Razavizadeh et al., 2022). Structuring methods such as cell extrusion and shearing techniques have been widely studied (He et. al., 2020). Currently cell extrusion and shearing technologies have advanced, providing an optimal combination of scalability and efficiency in the approach to structured proteins (Herz et al., 2021). During extrusion, complex physicochemical reactions occur such as denaturation and aggregation of proteins, gelatinization and degradation of carbohydrates, inactivation of enzymes, microorganisms and antinutritional factors (Zhang et al., 2023). Various studies have exploited the optimal processing conditions of vegetable protein meat analogs in high moisture extrusion technology (Wang et al., 2022; Dekker et al., 2018). A lot of experiments have shown that high moisture extrusion technology presents multiple advantages: lack of waste, low costs, low energy consumption, efficiency, versatility and superior quality of textured products, representing an optimal choice for obtaining meat analogues with fibrous structures (Xia et al., 2022). Improving the sensory properties of plant-based meat analogues opens new research horizons (Tibrewal et al., 2023). The study of the structure and texture of meat and meat analogues includes mechanical, spectroscopic and imaging characterization methods (Schreuders et al., 2021) as shown in the table below (Table 1).

Table 1. Textural and structural methods used for meat (M, the color red) and meat analogues (MA, the color green)

Texture and structure Mechanical	**Meat**	**Meat analogues**
Warner-Bratzler (Distructive)	X	X
Kramer Shear Cell (Distructive)	X	
Tensile (Distructive)	X	X
Compression & puncture (Distructive)	X	X
Texture Profile Analysis (Distructive)	X	X
Texture and structure Spectroscopy	**Meat**	**Meat analogues**
FTIR (Non-destructive)	X	X
NIR (Non-destructive)	X	
MIR (Non-destructive)	X	
Raman	X	
(Non-destructive)		
Fluorescence polarization (Non-destructive)	X	X
NMR (Non-destructive)	X	X

SA(X)S (Non-destructive)	X	
(SE)SANS (Non-destructive)	X	X
Light reflectance (Nondestructive)	X	X
Texture and structure Imaging	**Meat**	**Meat analogues**
Visual (Distructive)	X	X
CLSM (Distructive)	X	X
SEM (Distructive)	X	X
TEM (Distructive)	X	
AFM (Distructive)	X	X
MRI (Non-destructive)	X	
Ultra sound imaging (Non-destructive)	X	
Hyperspectral imaging (Non-destructive)	X	
XRT (Non-destructive)	X	X

Abbreviations: NIR, Near-infrared; MIR, Mid- infrared; NMR-Nuclear Magnetic Resonance Spectroscopy; SA(X)S, Small-angle (X-ray) scattering; (SE)SANS, (Spin-echo) Small- angle neutron scattering; CLSM, Confocal laser scanning microscopy; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy; AFM, Atomic force microscopy; MRI, Magnetic resonance imaging; XRT, X- ray tomography. Source: (Schreuders et al., 2021).

The researchers studied different compositions to develop plant-based meat analogues, using pea protein and wheat protein in different proportions. The experiment highlighted the potential of plant proteins in the development of plant-based analogues (Yuliarti et al., 2021). After extrusion technology, meat analogs based on vegetable proteins are subjected to secondary processing in which food additives (flavors, dyes) are added to the composition to that they possess meat-like sensory properties (Wang et al., 2022). The wide variety of meat analogues has led some researchers to check whether the methods used to detect *Salmonella* are effective (Sampson et al., 2023). In order to btain cultured flesh there are two main methods of propagation in vitro: propagation from axillary or terminal buds and propagation by the formation of adventitious shoots or somatic embryos (Goncalves et al., 2013).

TRENDS AND PERSPECTIVES

The meat market is restricted by population growth (Thomson, 2003), animal diseases (Bonny et al., 2015), environmental problems (Aiking, 2011; Nemecek et al, 2016;), potential risks of disease such as diabetes, obesity, cardiovascular diseases (Larsson & Wolk, 2010; Mehta et al., 2015; Rohrmann et al.,2013) and production costs (Pimentel & Pimentel, 2003; Smetana et al., 2015), which inspires a tendency to find suitable meat substitutes (Xiao et al., 2022).

The transition from eating meat and other animal products to plant-based products such as meat analogs is supported by the research community (Banovic et al., 2021; Aiking et al., 2018; Bryant et al., 2019; Graça et al., 2019; He et al., 2020), because these products have the potential to meet both the nutritional needs of the population (Banovic et al., 2021; Bohrer, 2019), as well as decreasing the negative impact of food production on the environment ((Banovic et al., 2021; Kyriakopoulou et al., 2019). The transition from animal products to ecological alternatives can be achieved when consumer acceptance of herbal alternatives is high (Zhang et al., 2023).

The non-profit organization Good Food Institute reported that the total plant-based food market in the United States has grown by 27% (Hu, et al., 2022). Replacing traditional meat with plant-based and cultured meat analogues could solve the main environmental problem, namely the reduction of greenhouse gas emissions (Nezlek & Forestell, 2022). Efforts to improve the sustainability of food systems benefit from a transition towards an increased reliance on plant-based foods and a decrease in the consumption of meat and other animal products (Graça et al., 2019). Vegetarians, vegans and flexitarians have a high ethical conscience and are the main consumers of meat analogs; consisting of 78.1% vegan, 32% vegetarian, 37.1% high/added protein and 31.3% gluten-free (Ishaq et al, 2022). In order to stimulate the transition from meat consumption to plant-based meat alternatives, studies show that the textural and sensory properties of meat analogues should be improved (Dinani et al., 2023; Grossmann & McClements, 2021; Hoek et al., 2011; Michel et al., 2021).

Meat analogs have similar nutritional profiles to animal meat (Ahmad et al., 2022) and are found in many vegetarian diets in developed countries. (Mihalache et al., 2022). Proteins from legumes and plants have the highest level of acceptability among consumers (Onwezen et al., 2021), beans, oats, peas, rapeseed, soy, etc. representing a sustainable and healthy source (Banovic et al., 2022). One of the critical aspects of plant-based

meat analogue development is the selection of appropriate protein supply. It is listed that mixing wheat gluten with soy protein produced a meat analog with physical properties like animal meat (Mishal et al., 2022; Chiang et al. 2019).

Plantain-based meat analogs are becoming increasingly important, satisfying consumers' desires for meat-like products (Jia et al., 2022). Among plant-based foods, soy protein isolate is a popular ingredient due to its relatively low price and versatile properties (Peng et al., 2023). Soy protein is an excellent substitute for animal protein, which has been widely used since the 1990s due to its characteristic gelling property and ability to fabricate anisotropic fiber structure (Zhang et al., 2021; Day et al., 2013; Lan et al., 2020). Soybean protein textured with beetroot juice (obtained from fresh beetroot, cooked beetroot, beetroot powder and commercial beetroot juice) showed the same appearance as beef and pork, respectively, which is consistent with their hue values and reflectance spectra. The attractive red color of betalains and their stability at the pH value of meat analogs make beetroot juices ideal for their application as colorants in meat analogs (Fernandez-Lopez et al., 2023). Color and color variations significantly influence the quality of meat analogs (Ishaq et al., 2022).

The choice to consume meat analogs is influenced by a number of factors (Pater et al., 2022) such as price, sensory quality, health, convenience (Bryant, 2022), environmental sustainability, animal welfare (Tyndall et al., 2022) and by consumers' emotional associations with food products that can even improve the prediction of food choice (Lagast et al., 2017). One of the problems of cultured meat is the absence of myoglobin in the composition, which is responsible for the red color of meat of animal origin. To solve this problem, the following can be used in the cultured meat production process: natural dyes (sugar beet or saffron), hemoglobin isolated from the animal's blood or its derivatives (Siddiqui et al., 2022; Mateti, Laha and Shenoy, 2022).

COOKING CHARACTERISTICS OF MEAT ANALOGUES

The traditional texturing process is extrusion (Tyndall et al., 2022), and both low moisture and high moisture extrusion processes can be used (Vatansever et al., 2020). High-moisture extrusion originated in the 1980s-1990s, and low-moisture protein extrusion developed in the 1960s, giving rise to expanded products or low-moisture meat analogs (Ubbink & Muhialdin, 2022). High-moisture extrusion cooking with a novel rotary die was experimentally performed using a Clextral Evolum 25 twin-screw extruder (Clextral, Firminy, France). The extruder having a screw diameter of 25 mm and a length/diameter ratio of 40 (Snel et al., 2022). Studies have shown that different cooking methods (thermal treatments based on heat transfer) of meat analogues affect tenderness and also changes in the structure of meat analogues (Wen et al., 2022). The texture of meat analogs is correlated with moisture content (Jung et al., 2022). Rheology can be used to characterize plant protein mixtures (wheat, soy, pea) used in meat analog applications (Schreuders et al., 2021), using the closed cavity rheometer (Dinani et al., 2023).

CONFLICT OF INTEREST

Both authors declare no conflict of intertest.

CONCLUSIONS

The excessive consumption of meat of animal origin creates environmental, ethical, ideological and last but not least public health concerns. An alternative to environmental sustainability could be the analogs of meat and cultured meat. Among existing analogs, plant-based meat analogs are the most representative with high consumer acceptability.

The main sources of plant-based proteins used for the production of meat analogues are legumes (soy, peas, lentils, chickpeas), pseudocereals (buckwheat), cereals (wheat, rice), tubers (potatoes), seeds and nuts (Kazir et al., 2021).

Extrusion is the most widely used method for obtaining plant-based meat analogues. The problem of environmental sustainability could be solved much more advantageously by breeding and developing insects, mealworms, crickets, grasshoppers or the production of microalgae, but so far there is a low acceptability from consumers, mainly European consumers.

Recent studies indicate the exploitation and development of science in various research directions, such as analyzing the benefits-risks of the production of plant-based meat analogues, verifying the effectiveness of the methods used to detect *Salmonella,* finding innovative technologies. An alternative to environmental sustainability can be represented by plant-based meat analogues, contributing favorably to solving ethical, ideological and, not least, health-related problems.

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IMPACT OF HEAT TREATMENTS ON THE ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF SWEET CHESTNUTS (*Castanea sativa* MILL.)

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Abstract

Sweet chestnuts are highly regarded and widely consumed throughout Europe because of their nutritional composition and health benefits, which have become important in the human diet, for example, in gluten-free diets. This study aims to assess the impact of heat treatment using the UV-VIS spectrophotometry methods. TPC values (mg GAE/g DW - dried weight) ranged from 1.935 to 6.165 (raw flesh), 1.676 to 4.342 (boiled samples), 1.580 to 3.091 (roasted samples), 1.193 to 8.272 (microwaved samples), and 2.556 to 5.655 (steamed samples). The DPPH values (µmol TE/g DW) for raw flesh ranged from 4.232 to 5.094, for boiled samples from 3.396 to 5.147, for roasted samples from 3.185 to 4.726, for microwaved samples from 2.798 to 5.816, and for steamed samples from 4.441 to 5.171. The FRAP antioxidant activity (µmol TE/g DW) values ranged from 10.971 to 207.11 (raw flesh), 4.058 to 134.651 (boiled samples), 11.954 to 132.476 (roasted samples), 7.795 to 179.129 (microwaved samples), and 17.468 to 367.957 (steamed samples). Among the methods used, steaming and microwaving had the greatest impact on total polyphenol content and antioxidant activity (DPPH, FRAP).

Key words*: DPPH, FRAP, heat treatments, polyphenols, sweet chestnuts*

INTRODUCTION

The European chestnut (*Castanea sativa* Mill.) is part of the Fagaceae family. For many decades, chestnut nuts have been one of the major food sources in farm-based regions, and the tree is regarded as crucial in the agricultural and forestry economies. Nowadays, a significant amount of nuts is consumed fresh or processed to produce goods like flour, chestnut purée, and other items that are part of gluten-free diets (Squillaci et al., 2018).

Sweet chestnuts are rich in vitamins, minerals, proteins, lipids, free sugars, and starch - all of which are beneficial for the consumer's health. Various mono- and disaccharides, such as glucose, fructose, sucrose, and maltose, are components of carbohydrates and play a key role in determining the commercial quality of chestnut nuts. Starch made of polysaccharides is also heavily present in the entire mixture. Up to one-third of the entire amount of carbs can be attributed to the free sugar sucrose. The estimated average of total carbohydrates in 100 g of fresh chestnut fruit is 44.7 g (Ramadan, 2019; De Vasconcelos et al., 2010a; Pino-Hernández et al., 2021). Crude fat, which is high in unsaturated fatty acids and low in saturated fatty acids, is found in very small amounts in sweet chestnuts. Depending on the harvesting year and chestnut cultivar, the protein content ranges from 2 to 3%. The protein fraction from chestnuts includes 17 amino acids, but in relatively small amounts (Barreira et al., 2009; Barreira et al., 2012; Murthy et al., 2020).

Humans cannot synthesise vitamin C, also known as ascorbic acid, which is a necessary substance for good health. Vitamin C is an antioxidant in the human body's biochemistry and molecular composition. There is a correlation between vitamin E and a lower risk of cardiovascular disease and cancer. One hundred grams of chestnuts have 1.9 mg of vitamin E, or 12.7% of the recommended daily intake (RDI) for both genders. Similarly, one hundred grams of fresh chestnut fruits contain 15.6 mg of vitamin C, or 20.8% of the RDI for women and 17.3% for men (De Vasconcelos et al., 2010b).

Many factors affect the change in chemical composition and representation of individual bioactive substances in sweet chestnuts.

Significant differences are observed between species/cultivars and between chestnuts from different geographical areas. Furthermore, their quality and nutritional composition are affected by seasonal variability and climatic factors such as temperature, sunlight, amount of precipitation; environmental conditions, altitude of cultivation, methods of agriculture (soil, nutrients, minerals, cultivation, pests, diseases, and periods for storage) (Martinéz et al., 2022).
The redox effect caused by phenolic substances is responsible for chestnuts antioxidant ability. Important antioxidants and phenolic acids protect the human body from the damaging effects of free radicals (Šnirc et al., 2023). Chestnut's mineral and total polyphenolic content are best preserved by boiling. Cooked chestnuts contain a significant amount of polyphenols, hydrolysable and condensed tannins, gallic and ellagic acids, and organic acids and phenolics. Boiling and baking are the most popular heat-treatment methods. Boiling methods impact chestnuts' sensory and nutrient content, boosting their organoleptic properties, bioavailable nutrients, and shelf life (Braga et al., 2014).
This study aims to determine the ways heat treatments affect the polyphenol content and antioxidant activity (DPPH, FRAP) of sweet chestnuts from important Slovakian growing crop regions.

MATERIALS AND METHODS

Sample collection and processing

In this study, analysed samples of sweet chestnuts were collected at five sampling points in Slovakia: Rovňany, Močenok, Modrý Kameň, Jelenec, and Svätý Jur. Chestnut samples were gathered during the autumnal harvest (in September and October). Depending on the distance and amount of nuts, 1-3 trees were gathered at each sampling point. The sample's average weight was 1.5 kg. Samples of sweet chestnuts were kept at −18°C for a month following harvest. The samples were prepared, peeled, divided into seed and shell, and heat treated using four methods: boiling (30 min/100°C), roasting in the oven (20 min/180°C), microwaving (2 min/800 W), and steaming (30 min/100°C). Following heat treatment, the materials were homogenised (Grindomix GM2000 Retsch, 2000 rpm, 30 sec), extracted with 80% methanol (ratio 1:2), and conducted for 12 hours at a horizontal shaker (Heidolph Promax 1020, Heidolph Instruments GmbH, Schwabach, Germany). Following preparation, the samples were filtered using Muktell paper no. 392 (Munktell & Filtrac GmbH, Bärenstein, Germany) and kept in a vial tube at 4°C until they were subjected to TPC, DPPH, and FRAP analyses. Chemicals were acquired from Merck (Germany) and Sigma-Aldrich (Sigma Aldrich ChemieGmbH, Steiheim, Germany). The dry matter content of the samples was determined using a moisture analyzer (KERN DLB 160–3A, KERN & SOHN GmbH, Balingen, Germany).

Determination of Total Polyphenol Content (TPC) by Folin–Ciocalteu Assay

Total polyphenol content (TPC) was measured in the samples using a UV-VIS spectrophotometer T92+ (PG Instruments, Leicestershire, United Kingdom) and Folin-Ciocalteu reagent by the standard colorimetric method described by Lachman et al. (2006). 0.1 mL of sample extract was combined with 5 mL of sodium carbonate (20%), distilled water, and Folin-Ciocalteu's reagent in a volumetric flask. The calculation's standard for total polyphenol content was gallic acid. A colored complex (blue-colored solutions) forms after two hours of churning. At a wavelength of 765 nm, the absorbance of the created solutions was measured. Every sample completed a total of four runs of the measurement process. The results after conversion were expressed as mg GAE/g DW - milligram equivalents of gallic acid per gram of dry matter.

Determination of Antioxidant Activity

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity and the FRAP (ferric reducing ability of plasma) techniques were used to measure the antioxidant activity.
With minor modifications, the DPPH assay was performed using the methods of Brand-Williams et al. (1995) and Martínez et al. (2022). Trolox was used as a standard to calculate the antioxidant activity. A stock solution of 0.025 g of DPPH was prepared and

kept cold at 4°C by dissolving it in 99.8% methanol. Before analysis, the DPPH working solution was made by mixing the DPPH stock solution with methanol (1:10). The absorbance value (A0) of a 2,2-diphenyl-1-picryhydrazyl solution was measured using a UV-VIS spectrophotometer T92+ (PG Instruments, Leicestershire, United Kingdom) at a wavelength of 515.6 nm after the solution was pipetted (3.5 mL). The reaction mixture was stored in a dark environment. The DPPH radical is decreased and changes color when it interacts with an antioxidant substance. After measuring A_0, 0.1 mL of the extract was added and mixed three times. After ten minutes, the absorbance (A_{10}) was measured. The measurement of each sample was repeated four times. The percentage value of DPPH inhibition was calculated based on the following equation:

$$DPPH\ inhibbition(\%)=[(A_0-A_{10})/A_0]\times 100,$$

where A_0 control is the absorbance of the blank at time 0 and A_{10} is the absorbance of the sample after 10 minutes. The results were represented as micromole Trolox equivalent (TE) per gram in dry weight (μmol TE/g DW).

The FRAP was assessed according to Paulová et al. (2004). A master solution of the FRAP reagent was made by mixing 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3.6H_2O$). Acetate buffer (pH = 3.6) was prepared by mixing acetic acid and sodium acetate. FRAP master solution was mixed with 100/50 μL of sample. Trolox was used as a standard. The absorbance was measured at a wavelength of 593 nm using a UV-VIS spectrophotometer T92+ (PG Instruments, Leicestershire, United Kingdom) during a 30-minute incubation period at 37°C. Every sample underwent four repetitions of the measurement process. Micromole Trolox equivalent (TE) per gram in dry weight (μmol TE/g DW) was used to represent the results.

Statistical Analysis

To study the relationships between different chestnut samples depending on steaming, cooking, microwaving, and roasting, the total polyphenol content and antioxidant activity were evaluated using XLSTAT software. The analyses were carried out four times, with the findings given as mean ± standard deviation (SD). The Kruskal-Wallis (nonparametric ANOVA) and Multiple pairwise comparisons - Dunn's tests were used to determine the statistical differences ($p < 0.05$) between the tested variables. TPC, DPPH, and FRAP have been determined to be correlated using Spearman's correlation coefficient.

RESULTS AND DISCUSSIONS

Chestnuts may have a preventive effect against oxidative stress, which is a typical occurrence in degenerative disorders, because of the antioxidants included in plant foods. Polyphenols are one of the most significant antioxidant classes found in chestnuts. For human neuroblastoma cells, they provide a neuroprotective function that helps to reduce neurological issues (Pandey & Rizvi, 2009; Barros et al., 2011; Brizi et al., 2019). Based on the Folin-Ciocalteu technique, chestnuts have a significant total polyphenol content (TPC). Compared to other varieties of nuts, which have 100 mg of gallic acid equivalents (GAE/100 g), sweet chestnuts, pistachios, and pecans contain more than 1000 mg (Mustafa et al., 2021).

The obtained results of TPC in raw and heat-treated sweet chestnut samples are presented in Table 1. Samples from sampling point Jelenec showed the highest TPC content (2.497–8.272 mg GAE/g DW). Samples from sampling point Močenok showed the lowest values (1.193–2.556 mg GAE/g DW).

Based on these results, climatic conditions were the main factor affecting the differences in polyphenol levels in individual sampling points. TPC increased variably in heat-treated samples. Of the thermal treatments, the values were highest after microwaving (1.193–8.272 mg GAE/g DW) and after steaming (2.556–5.655 mg GAE/g DW). The lowest values (1.580–3.091 mg GAE/g DW) were shown by most of the samples after roasting in the oven. The exception was the sampling points Močenok and Svätý Jur, where the lowest TPC value was determined in the microwaved sample (1.193; 1.371 mg GAE/g DW).

Table 1. Contents of total phenolic content, and antioxidant activity (DPPH, FRAP) of sweet chestnuts

Sampling point	Treatment	TPC (mg GAE/g DW)	DPPH (μmol TE/g DW)	FRAP (μmol TE/g DW)
Rovňany	raw	6.165±0.058^{ab}	4.841±0.013^{bc}	87.662±0.197^{a}
	boiled	3.876±0.543^{ab}	4.712±0.029^{abc}	36.097±0.253^{a}
	roasted	3.091±0.069^{a}	4.076±0.019^{a}	44.311±0.091^{a}
	microwaved	6.311±0.232^{ab}	4.996±0.022^{ab}	97.825±0.026^{a}
	steamed	4.540±0.244^{b}	4.984±0.074^{c}	59.534±0.041^{a}
Močenok	raw	2.342±0.029^{ab}	4.232±0.074^{bc}	18.398±0.113^{a}
	boiled	1.676±0.144^{ab}	3.396±0.259^{abc}	15.955±0.056^{a}
	roasted	1.580±0.077^{a}	3.185±0.054^{a}	16.313±0.160^{a}
	microwaved	1.193±0.126^{ab}	3.017±0.054^{ab}	7.996±0.209^{a}
	steamed	2.556±0.202^{b}	4.441±0.024^{c}	22.107±0.187^{a}
Modrý Kameň	raw	5.904±0.124^{ab}	5.094±0.010^{bc}	207.110±0.127^{a}
	boiled	4.342±0.209^{ab}	5.147±0.012^{abc}	134.651±0.396^{a}
	roasted	1.702±0.094^{a}	4.726±0.026^{a}	132.476±0.181^{a}
	microwaved	6.065±0.837^{ab}	5.816±0.019^{ab}	179.129±0.412^{a}
	steamed	5.655±0.215^{b}	5.171±0.063^{c}	367.957±0.213^{a}
Jelenec	raw	2.497±0.023^{ab}	4.676±0.018^{bc}	20.233±0.633^{a}
	boiled	3.034±0.135^{ab}	4.567±0.172^{abc}	15.148±0.642^{a}
	roasted	2.774±0.098^{a}	4.111±0.018^{a}	22.618±0.141^{a}
	microwaved	8.272±0.265^{ab}	3.484±0.075^{ab}	91.597±0.170^{a}
	steamed	3.800±0.139^{b}	4.989±0.045^{c}	30.348±0.231^{a}
Svätý Jur	raw	1.935±0.086^{ab}	4.270±0.047^{bc}	10.971±0.244^{a}
	boiled	1.682±0.148^{ab}	4.112±0.028^{abc}	4.058±0.166^{a}
	roasted	1.880±0.120^{a}	3.240±0.025^{a}	11.954±0.431^{a}
	microwaved	1.371±0.096^{ab}	2.798±0.037^{ab}	7.795±0.310^{a}
	steamed	2.615±0.143^{b}	4.819±0.020^{c}	17.468±0.326^{a}

Note: mean ± standard deviation (n = 4); a–c statistically significant differences between heat treatments for each analysis (TPC, DPPH, FRAP) considered separately, p-value 0.0167 (TPC); <0.0001 (DPPH); 0.2529 (FRAP)

The obtained results about the highest value of TPC in microwaved samples are compared to Wani et al. (2017). It states that the TPC value changes during heat treatment, the highest being obtained for microwaved chestnuts samples. The degradation of hydrolyzable tannins after heating into smaller phenolic compounds can be used as an explanation for the rise in the TPC of chestnuts. It is well known that heat treatment can affect the chemical composition of certain molecules, such as proteins associated with phenolic chemicals.

High TPC in plant food following heat treatment might result from an increase in their amount (Lemos et al., 2012). However, the TPC values obtained were not higher in all heat treatments, as the authors state (Mustafa et al., 2021; Neri et al., 2010). Therefore, a more detailed analysis of the effect of processing on sweet chestnuts is needed. Statistical differences were observed in TPC between steamed and roasted samples.

There is a clear correlation between the amount of total phenolics in extracts and plant capacity to function as antioxidants. The defence mechanism against hazardous oxidative damage is aided by phenolic chemicals, which help prevent disorders linked to oxidative stress (Braga et al., 2014). Using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) techniques, the

antioxidant activity of sweet chestnuts was determined. The obtained results of antioxidant activity (DPPH, FRAP) in samples of raw and heat-treated chestnuts are presented in Table 1. Samples from sampling point Modrý Kameň showed the highest antioxidant activity of DPPH and FRAP (4.771–5.816; 132.476–367.957 µmol TE/g DW). The lowest values were shown by the samples from sampling point Svätý Jur (2.798–4.819; 4.058–17.468 µmol TE/g DW). Antioxidant activity increased variably in heat-treated samples as well as in TPC. The DPPH values were highest in heat-treated samples after microwaving (2.798–5.816 µmol TE/g DW) and after steaming (4.441–5.171 µmol TE/g DW). The FRAP values of heat-treated samples increased in the order: steamed>microwaved>raw>baked>boiled. In other studies described in the literature, the antioxidant activity of different varieties of chestnuts from different locations was measured by several different methods, and it is difficult to compare the results. Differences in antioxidant activity values are significantly influenced by the use of two individual measurement methods. Most antioxidants are found in nut skins or shells. In addition, the antioxidant activity decreases considerably when the shell is removed from the nuts. After that, less than 10% is present in nuts (Mustafa et al., 2021). Blomhoff et al. (2006) report values of 7.55 µmol per 1 g for Italian chestnuts using the FRAP antioxidant activity method. The antioxidant capacity of different types of sweet chestnuts ranges from 0.564 to 1.046 µmol TE/g, according to Barros et al. (2011). This indicates that the antioxidant capacity is crop-specific. There were statistically significant differences in DPPH between the steamed/raw, roasted, and microwaved samples. Significant statistical differences were not observed in FRAP between monitored samples.

Physiological and reproductive cycles of species are influenced by environmental conditions, which also change fruit quality and yield. Differences in the environmental conditions of the same variety of chestnuts also affect their antioxidant activity. Increased levels of specific metabolites are among the defense mechanisms against the harmful effects of external factors (Yang, et al., 2018).

Spearman's correlation coefficient showed a strong correlation between monitored parameters (TPC, DPPH, FRAP). Phenolic substances are the most important contributors to the capacity to scavenge free radicals and, consequently, antioxidant activity (Franková et al, 2022). Antioxidant activity FRAP showed the strongest correlation with TPC ($r = 0.796$) in this study. Antioxidant activity DPPH showed 0.696 correlation. The connection between antioxidant capacity and TPC in sweet chestnuts is also confirmed by previous studies by the authors (Barreira et al., 2008; Vázquez et al., 2008; Neri et al., 2010; Mustafa et al., 2021).

CONCLUSIONS

These days, people are becoming more and more interested in foods' antioxidant qualities and how they improve consumers' health. More precise information is highly desired, not only regarding the effects of heat treatment on the bioactive elements of chestnuts but also regarding the influence of cultivars in the context of the nutritional and antioxidant qualities of chestnuts. Thermal treatments associated with common consumption of chestnuts – steaming, roasting, microwave oven and cooking – affect antioxidant activity and total content of polyphenols. Steamed and microwaved chestnuts show a positive increase in antioxidant activity and total polyphenol content. However, the results of our study show that the antioxidant activity and the total polyphenol content are most influenced by the geographical and climatic conditions of Slovakia. A more detailed analysis of the given parameters is therefore necessary to clarify the results and impacts.

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THE EFFECT OF THERMAL PROCESSING ON ANTIOXIDANT ACTIVITY AND TOTAL POLYPHENOLS IN JERUSALEM ARTICHOKE (*Helianthus tuberosus* L.)

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Abstract

The Jerusalem artichoke (Helianthus tuberosus L.) is high in protein, inulin, and other bioactive ingredients. In this study, the effect of heat treatment methods as well as the effect of the variety on its total polyphenol content and antioxidant activity were investigated. The total polyphenol content ranged from 0.36 to 0.87 mg GAE.g^{-1} DW in raw flesh and from 0.41 (baking) to 1.01 mg GAE.g^{-1} DW (boiling) in heat-treated tubers. The antioxidant activity (AA) was determined by the methods of DPPH and FRAP. DPPH values ranged from 0.18 to 1.09 (raw flesh), 3.03 to 4.74 (boiled samples), 2.34 to 2.94 (baked samples), 2.88 to 3.22 (microwaved samples), and 2.25 to 6.17 (steamed samples) µmol TE.g^{-1} DW. AA values by the method FRAP ranged from 3.5 to 3.82 (raw flesh), 7.51 to 8.53 (boiled samples), 4.26 to 6.80 (baked samples), 4.98 to 6.72 (microwaved samples), and 4.89 to 8.04 (steamed samples) µmol TE.g^{-1} DW. All studied heat treatment methods had a positive effect on the TPC and AA. Our results confirm the promising potential of artichoke use in functional food preparation.

Key words*: Jerusalem artichoke, heat treatment, polyphenols, antioxidant activity.*

INTRODUCTION

Originally from North America, the Jerusalem artichoke is an invasive perennial weed that has been brought as a cultivated plant to Europe and Asia (Liava et al., 2021). The *Asteraceae* family and *Helianthus* genus include the Jerusalem artichoke (*Helianthus tuberosus* L.), which has a high level of pest and disease resistance. It can withstand freezing temperatures and drafts. This plant has been grown for many years as a valuable edible raw material with potent medicinal properties (Sawicka et al., 2020).

The tubers can be oval in shape, but they can also be rounded, oblong, pear-shaped, or thin. They can be white, red, light brown, brown, violet-brown, or dark brown. Numerous cultivation parameters, such as plant variety, available moisture, and soil texture, influence these variations. While some varieties produce small tubers on long stems, relatively large tubers are grouped close to the main root in the better varieties (Kosaric et al., 1984; Liava et al., 2021).

The tubers of Jerusalem artichokes are an excellent source of phytochemicals. Jerusalem artichokes are highly suited for the obtaining of functional foods due to their nutritional composition (Michalska-Ciechanowska et al., 2019). Tubers store 6-12% protein, amino acids, and inulin-type fructans, which are polymers of fructose molecules (Dias et al., 2016).

The most prevalent carbohydrate in Jerusalem artichoke tubers and stems is inulin. The degree of polymerization, or number of units, of this compound typically ranges from 2 to 60, and the amounts of it in tubers vary amongst genotypes (Liava et al., 2021).

The Jerusalem artichoke tuber has an inulin content ranging from 7 to 30% of its fresh weight or 60 to 85.5% of its dry weight (Samal et al., 2017). Because Jerusalem artichokes have a high inulin content, they can be used to make diabetic foods such as flour, juice, syrup, confections, and bakery goods. Dietary fibre and inulin can absorb a large quantity of glucose from food and stop it from entering the bloodstream, lowering blood sugar levels.

When Jerusalem artichokes are used, cholesterol levels are significantly lowered. Inulin stimulates the body's bifidobacteria to grow rapidly, inhibits the growth of harmful

microflora, and aids in the restoration of the gastrointestinal tract's disrupted function (Shariati et al., 2021). Jerusalem artichokes are an excellent supplier of essential minerals in sufficient quantities for human nutrition. Greater than other tubers (carrots, potatoes, turnips, beets, etc.) in terms of iron content is the Jerusalem artichoke. Jerusalem artichokes also include minerals like silicon, magnesium, calcium, potassium, sodium, fluorine, and chromium (Ishniyazova et al., 2020; Rushchitc et al., 2022).

Jerusalem artichokes are a multipurpose crop that can be used for biomass and bioenergy production (bioethanol and biogas), pharmaceutical applications, and human food consumption (directly as tubers or as a raw material for obtaining sweeteners). Furthermore, Jerusalem artichokes have potential as a crop for fodder. Tubers are used for functional food component preparation because they are high in protein, inulin, and other bioactive ingredients (Kosaric et al., 1984; Dias et al., 2016; Mahokhina et al., 2022).

Growing times vary from 110 to 240 days, depending on the region and genotype under cultivation. Harvesting should be done after stem drying if Jerusalem artichoke is grown for tuber production, while if the aerial part is the primary product, harvesting can take place while tuber bulking is underway. In order to produce bioethanol, the stems in middle-season/late clones should be harvested at the flower bud stage, and in early clones, between the flower bud and dry head stages. This is because the stem's sugar content decreases beyond these stages (Liava et al., 2021).

The primary elements influencing the bioavailability of polyphenols are the food matrix, food processing, and the initial content of polyphenols in foods. Most vegetables and fruits are eaten in processed form. Boiling, baking, steaming, and other forms of industrial or domestic heat processing affect the amount of polyphenols present as well as their bioaccessibility and bioavailability (Arfaoui, 2021).

This study analyzed how the Jerusalem artichoke variety's overall polyphenol content and antioxidant activity were affected by various heat treatment techniques, including baking, boiling, steaming, and microwave cooking.

Figure 1: Jerusalem artichoke – plant (https://as2.ftcdn.net/v2/jpg/03/53/28/33/1000_F_353283302_Maarw5jeQKpEsBg3whLkcPBbVcZcsaE4.jpg)

MATERIALS AND METHODS

Chemicals

The following materials were acquired from Sigma-Aldrich (Sigma Aldrich Chemie GmbH, Steiheim, Germany): methanol (99.8%), gallic acid (p.a.), DPPH (2,2′-diphenyl-1-picrylhydrazyl), Trolox (2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), HCl, and acetic acid. Na_2CO_3 and $FeCl_3$ were purchased from CentralChem (Slovakia); sodium acetate was supplied by Mikrochem (Slovakia); and Folin-Ciocalteu reagent was acquired from Merck (Merck KGaA, Darmstadt, Germany).

Plant Material

For analysis, three Jerusalem artichoke varieties (pink, white 1, and white 2) grown in the same location (Slovakia), one of them with a different peel colour, harvested in September 2021, were used. In total, two kilograms of fresh Jerusalem artichokes from each variety were utilized for the heat treatment and extract preparation process.

Samples preparation

After a thorough cleaning, distilled water (dH_2O) was used to wash the Jerusalem artichokes.

After that, the tubers underwent peeling. Peeled Jerusalem artichokes (flesh) were cut into slices that were roughly 3 mm thick after being repeatedly cleaned in dH_2O. A portion of the raw flesh from the tubers was homogenised and mixed using a Grindomix GM 200, Retsch, Haan, Germany, for 30 seconds. The remaining portions of the tuber underwent the following heat-treatment procedures, as described by Musilová et al. (2020): steaming (15 min, 97 ± 2 °C), boiling (10 min), baking (15 min, 200 °C), and microwaving (5 min, 800 W). Following each heat treatment, Jerusalem artichoke slices were homogenised, cooled, and mixed (using a Grindomix GM 200, Retsch, Haan, Germany; 30 seconds). The homogenised samples were prepared into extracts needed for analysis using 80% methanol.

50 mL of 80% methanol was added to 25 g of homogenised material (raw, microwaved, steamed, boiled, and baked flesh, respectively) in order to prepare the extracts. The samples were extracted using a horizontal shaker (Heidolph Promax 1020, Heidolph Instruments GmbH, Schwabach, Germany) for a duration of 12 hours. After being filtered through Muktell No. 392 paper (Munktell & Filtrac GmbH, Bärenstein, Germany), the extracts were refrigerated at 4 °C in closed 50 mL centrifuge tubes.

Analyses of samples

Determination of total polyphenol content

Using spectrophotometry (UV-VIS spectrophotometer T92+, PG Instruments, Leicestershire, United Kingdom), the total polyphenol content (TPC) was ascertained using a Folin-Ciocalteu agent according to the method by Lachman et al. (2006). To the volume of sample extract (0.1 mL) in the volumetric flask (50 mL), the Folin-Ciocalteu reagent was added. After three minutes, 5 mL of 20% sodium carbonate aqueous was added, and distilled water was added to the mark. Standard gallic acid solutions were prepared for the calibration curve using the same procedure. After mixing the prepared solutions, they were kept at room temperature for two hours. Following that, a measurement of the solutions' absorbance at 765 nm was made. Each sample's polyphenol content was reported as milligrammes of gallic acid equivalent per gramme of dry weight (mg GAE.g^{-1} DW).

Evaluation of Antioxidant Activity

The DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) assay were used to measure the antioxidant activity (AA).

DPPH Radical Scavenging Activity

According to Brand-Williams et al. (1995), the method for determining antioxidant activity (AA) was based on scavenging the stable free radical 2,2´-diphenyl-1-picrylhydrazyl (DPPH). A 100 mL flask containing 0.025 g of DPPH free radical was filled with 99.8% methanol to create a stock solution, which was then kept in the cold and dark. The stock solution for DPPH was combined with methanol (1:10) to create the DPPH working solution needed for analysis. At a wavelength of 515.6 nm, the absorbance (A0) of the DPPH working solution was measured using a UV-VIS spectrophotometer T92+ (PG Instruments, Leicestershire, United Kingdom) for the analysis. After carefully mixing 0.1 mL of the sample extract into the DPPH solution in the cuvette, it was left to stand in the dark for 10 minutes. Subsequently, the absorbance (A10) was determined. The absorbance of the DPPH solution (A0) and the absorbance after adding the sample extract at time t = 10 minutes (A10) were used to calculate the percentage values of DPPH inhibition for each sample using the following formula:

$$\% \text{ DPPH inhibition} = [(A0 - A10) / A0] \times 100$$

Trolox equivalents (μmol TE.g^{-1} DW) are units of measurement for the antioxidant activity assessed using the DPPH method.

Ferric reducing antioxidant power assay

To make the FRAP reagent, a 1:1:10 ratio of TPTZ solution (5 mmol.L^{-1} in 40 mmol.L^{-1} HCl), ferric chloride solution (10 mmol.L^{-1}), and acetate buffer (acetic acid, c = 0.1 mol.L^{-1}; sodium acetate, c = 0.1 mol.L^{-1}, pH 3.6) was used. The following procedure was used to prepare the sample solutions for determination. Test tubes were filled with 6 mL of FRAP reagent and 0.1 mL of each sample extract, then they were homogenized and closed. After preparing the samples in this way, they were placed in a dark, 37 °C water bath for 30 minutes. Using the same process as the samples, standard solutions of Trolox were created for the calibration curve. An UV-VIS spectrophotometer T92+ (PG Instruments, Leicestershire,

United Kingdom) was then used to measure the absorbance at 593 nm.

Trolox equivalents per gram of dry weight (μmol TE.g^{-1} DW), was the unit of measurement for the ferric reducing antioxidant power.

Statistical evaluation

There were four replicates of each analysis (n = 4). The arithmetic mean ± standard deviation (SD) is used to express the results. Every variable under test had a nonparametric distribution. Thus, to ascertain the statistical differences ($p < 0.05$) between varieties and heat treatment techniques, the Kruskal-Wallis test was employed. To ascertain the relationship between the three parameters under investigation (TPC, DPPH, and FRAP), Spearman's correlation coefficient was employed. The XLSTAT 2014 software package was used to carry out the computational work.

RESULTS AND DISCUSSIONS

Total polyphenol content

One significant class of secondary plant metabolites with a variety of functions in plants is represented by polyphenols. In general, genetics determines the amount and presence of polyphenols (Franková et al., 2022b). The cultivar, harvest time, and storage conditions all affect the amount of polyphenols in Jerusalem artichoke tubers. Genetics also influences variations in polyphenolic compound concentrations (Showkat et al., 2019). The raw Jerusalem artichoke flesh samples under investigation had total polyphenol content (TPC) ranging from 0.359 (White 1) to 0.868 mg GAE.g^{-1} DW (Pink). TPC in the pink-fleshed variety (Pink) was two times higher than in white-fleshed (White 1 and White 2) Jerusalem artichokes (Table 1). Varieties with coloured flesh have higher total polyphenol contents because compared to varieties with white or yellow flesh, they have higher proportions of anthocyanins (Franková et al., 2022a).

Comparisons with previous studies revealed differences in the measured TPC values. A higher total polyphenol content was determined in the Kaentawan #1 variety from Thailand (2.81 mg GAE.g^{-1} DW) (Puyanda et al., 2020). Additionally, Amarowicz et al. (2020) in their study examined, like us, two varieties of white and one variety of pink Jerusalem artichoke tubers. The authors found that the TPC content in the tubers was 7.81–9.76 mg.g^{-1}. The differences in our results may have been influenced by the different preparations of the extract because the authors prepared the extract from freeze-dried samples. In the Norwegian variety Dagnøytral, the content of polyphenols was in the range of 1.1–2.3 mg GAE.g^{-1} DW (Showkat et al., 2019). In the water extract of Jerusalem artichoke, variety Patate, grown in Algeria, the TPC content was 6.58 mg GAE.g^{-1} DW (Sarsar et al., 2021). In an additional research (Nizioł-Łukaszewska et al., 2018), the authors investigated the content of TPC in tubers and leaves of Jerusalem artichoke, and the results revealed a greater amount of TPC in the leaves of the plant (389.88 mg GAE.g^{-1}) than in the tubers (76.84 mg GAE.g^{-1}). The use of ultrasound-assisted extraction methods could have influenced the results of TPC content compared to our and other studies. Values in accordance with our results were reported by the study of Plangklang & Tangwongchai (2011), which also reported low TPC values (between 0.327 and 0.510 mg GAE.g^{-1} FW) in varieties HEL65 and JA89. Our TPC values in fresh matter ranged from 0.359 to 0.868 mg GAE.g^{-1}. TPC increased in varieties with white skin (White 1, White 2) as a result of the carefully supervised heat treatments (steaming, boiling, baking, and microwaving). In the case of tubers with pink skin (Pink), the TPC increased only after the heat treatment of boiling, while the other thermal treatments led to a decrease of TPC in this sample. Sarsar et al. (2021) cooked tubers at 70°C for 10 min, and TPC values increased up to twofold to 13.11 mg GAE.g^{-1} DW, as in the case of our white varieties (White 1, White 2). Other authors (Puyanda et al., 2020) baked tubers in an oven at 65 °C, and the TPC values increased (from 2.81 to 4.37 mg GAE.g^{-1} DW), similar to our case. After microwaving in the research of Showkat et al. (2019), the TPC content increased slightly (1.5–3.5 GAE.g^{-1} DW), and upon boiling (60 °C), TPC also increased (1.7–3.8 GAE.g^{-1} DW). Microwaving and steaming are examples of heat treatments that can disrupt cell structure and increase the efficiency of compound extraction from the cell cytoplasm (Franková et al., 2022b). Complex

carbohydrates and proteins contain phenolic compounds. The species, variety, agrotechnical, climatic, and, in particular, plant parts can all affect the composition of the phenolic fraction in natural plant sources (Nizioł-Łukaszewska et al., 2018).

In Table 1, the relationships between TPC and heat treatments are indicated by different letters (a, b). Statistically significant differences were observed between raw and boiled samples ($p = 0.0048$), as well as between heat treatment by boiling and baking ($p = 0.0015$).

Table 1. TPC and AA in raw and heat-treated Jerusalem artichoke

Variety	Heat treatment	TPC (mg GAE.g^{-1} DW)	Antioxidant activity		
			DPPH (μmol TE.g^{-1} DW)	DPPH (%)	FRAP (μmol TE.g^{-1} DW)
Pink	Raw flesh	0.868 ± 0.114^{a}	1.085 ± 0.108^{a}	13.99	3.504 ± 0.536^{a}
White 1		0.359 ± 0.113^{a}	0.183 ± 0.055^{a}	6.58	3.687 ± 0.360^{a}
White 2		0.487 ± 0.149^{a}	0.302 ± 0.297^{a}	8.74	3.821 ± 0.163^{a}
Pink	Steaming	0.816 ± 0.228^{ab}	6.168 ± 0.106^{b}	69.17	8.022 ± 0.376^{bc}
White 1		0.566 ± 0.112^{ab}	2.253 ± 0.187^{b}	31.43	4.888 ± 0.119^{bc}
White 2		0.922 ± 0.084^{ab}	5.267 ± 0.160^{b}	62.08	8.040 ± 0.535^{bc}
Pink	Boiling	1.009 ± 0.158^{b}	4.745 ± 0.300^{b}	69.53	8.533 ± 0.411^{bc}
White 1		0.860 ± 0.192^{b}	4.013 ± 0.198^{b}	45.69	8.014 ± 0.716^{bc}
White 2		0.863 ± 0.445^{b}	3.027 ± 0.183^{b}	17.02	7.507 ± 0.233^{bc}
Pink	Baking	0.515 ± 0.143^{a}	2.942 ± 0.117^{ab}	76.88	4.744 ± 0.107^{ab}
White 1		0.406 ± 0.046^{a}	2.341 ± 0.208^{ab}	55.90	4.260 ± 0.059^{ab}
White 2		0.738 ± 0.194^{a}	2.737 ± 0.078^{ab}	79.62	6.803 ± 0.719^{ab}
Pink	Microwaving	0.644 ± 0.092^{ab}	2.877 ± 0.042^{b}	75.49	4.983 ± 0.271^{b}
White 1		0.484 ± 0.135^{ab}	3.095 ± 0.208^{b}	66.24	5.162 ± 0.468^{b}
White 2		0.689 ± 0.047^{ab}	3.219 ± 0.070^{b}	74.95	6.724 ± 0.133^{b}

Legend: TPC – Total polyphenol content, GAE – gallic acid equivalent, DW – dry weight, DPPH – 2,2′-diphenyl-1-picrylhydrazyl, TE – Trolox equivalents, FRAP – Ferric reducing antioxidant power. The values are expressed as average ± SD. Different letters (a-c) indicate significant differences among heat treatments, for each method separately.

Antioxidant activity

An analysis of the antioxidant activity of natural products serves as the foundation for the evaluation and recommendation of foods with high antioxidant activity to consumers (Xu et al., 2017). The ferric reducing antioxidant power (FRAP) assay and DPPH free radical scavenging activity were used to assess the antioxidant activity (AA) of Jerusalem artichoke tubers. As individual bioactive compounds have a different affinity for different antioxidant activity tests, the sensitivity and specificity of one method of antioxidant activity determination do not provide a complete representation of all the antioxidants in the sample (Makori et al., 2020).

AA values of DPPH varied between 0.18 and 1.09 (raw flesh), 3.03 to 4.74 (boiled samples), 2.34 to 2.94 (baked samples), 2.88 to 3.22 (microwaved samples), and 2.25 to 6.17 (steamed samples) μmol TE.g^{-1} DW. The AA values obtained using the FRAP method varied from 3.5 to 3.82 (raw flesh), 7.51 to 8.53 (boiled samples), 4.26 to 6.80 (baked samples), 4.98 to 6.72 (microwaved samples), and 4.89 to 8.04 (steamed samples) μmol TE.g^{-1} DW (Table 1). The steaming heat treatment affected the antioxidant activity in the samples most significantly compared to the raw flesh. Jerusalem artichokes' total polyphenol content and antioxidant activity were positively impacted by all of the heat treatment techniques that were investigated.

The antioxidant activity investigated in a study in Poland (Amarowicz et al., 2020) showed that AA values measured by the DPPH method were in the range of 40.9–46.2 μmol.g^{-1}. Nizioł-Łukaszewska et al. (2018) reported that Jerusalem artichoke tubers had a free radical scavenging capacity of 56%. Boiled tubers at a temperature of 60°C and also raw flesh in the work of Sarsar et al. (2021) showed AA in the range of 83–99.54%. In another study, the authors (Mu et al., 2021) observed the effect of different tuber storage temperatures on AA. The tubers were kept in storage between -18 and

4°C, while the percentage of free radical absorption ranged from 39.08 to 84.04%. Raw flesh and heat-treated samples of our variants had this ability in the range of 6.58%–79.62%, so the authors report similar AA results. Other authors (Alyas et al., 2021; Puyanda et al., 2020; Plangklang & Tangwongchai, 2011; Zhang & Kim, 2015) also examined the antioxidant activity using the FRAP and DPPH techniques; however, due to the difference in the reported units, it is not possible to compare these results. According to some theories, oxidation, leaching, thermal degradation, and enzymatic or nonenzymatic conversion can all contribute to the loss of antioxidant activity that happens when fruits and vegetables are processed. The softening of the food matrix during cooking, on the other hand, has been linked to increased antioxidant activity during processing. This increases the extractability of the antioxidant constituents, leading them to further convert to more antioxidant compounds (Amagloh et al., 2022).

The Kruskal-Wallis test, a statistical analysis, confirmed that there were differences ($p < 0.05$) in all examined antioxidant activity parameters (DPPH and FRAP) between heat-treated and raw flesh. Statistically significant differences in AA by DPPH were observed between raw flesh and steaming, boiling, and microwaving, respectively. The same statistically significant differences were also found with the FRAP method, but significant differences were also found between the heat treatments of boiling and baking and boiling and microwaving.

The Spearman's correlation coefficient

Relationships between the two monitored parameters - total polyphenol content and antioxidant activity (DPPH and FRAP) - were ascertained using Spearman's correlation coefficient. According to our findings, positive correlations were found for every parameter that was examined. TPC showed a positive correlation ($r = 0.57$) with both FRAP ($r = 0.56$) and DPPH free radical scavenging activity. The two antioxidant activity methods, DPPH and FRAP, showed a very strong positive correlation ($r = 0.84$, $p < 0.0001$). Thus, it can be inferred that the predictive power of both techniques for Jerusalem artichoke's antioxidant activity is similar.

CONCLUSIONS

Our results confirm the promising potential of Jerusalem artichoke use in functional food preparation. All of the processing techniques that were investigated had a positive effect on the overall polyphenol content and antioxidant activity. The variations between the heat treatment techniques and the varieties under observation were validated through statistical analysis. Our study's data can contribute to a deeper understanding of how processing affects the bioactive compound content and antioxidant activity of Jerusalem artichokes.

This plant could be usefull for the development and production of nutritious and extremely successful therapeutic and preventive foods. A good way to expand the range of alternative uses for root vegetables is to incorporate them into different food products.

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SUBMERGED CULTIVATION OF SOME SPECIES OF EDIBLE BASIDIOMYCETES FOR THE SIMULTANEOUS BIOSYNTHESIS OF BIOMASS AND LACCASE ENZYME

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Abstract

Five species of edible basidiomycetes - Pleurotus ostreatus, Flammulina filiformis, Ganoderma lucidum, Lentinula edodes, and Polyporus squamosus - were isolated and cultivated as submerged mycelium for the biosynthesis of laccase enzymes and biomass production. The selected mushrooms were grown on different agar media to determine the average growth rate, tested on the Potato Dextrose Agar medium supplemented with guaiacol to determine the laccase enzyme index, and then grown submerged in a rotary incubator, in Erlenmeyer flasks. The development of these species was studied in a medium with sugarcane molasses and mineral salts. The dry biomass of the culture media was determined, and the laccase activity was analysed in the filtrate.

Key words*: basidiomycetes, laccase, biomass.*

INTRODUCTION

The quality, nutritional value and safety of food is an ever-present issue for the food industry and consumers. An important source of proteins and bioactive compounds is edible fungi (class Basidiomycetes), which can be cultivated as mycelium on the surface of solid substrates or submerged in liquid media, with aeration. Their medicinal and nutraceutical properties have been known for a long time, but they have not been used to their full value in human nutrition.
Unlike the traditional cultivation of edible mushrooms, which requires significant time, space and produces variable yields, mycelium cultivation is a current method, which significantly reduces growth time, external contamination and produces a biomass with valuable nutritional properties. In addition, some mushrooms have considerable therapeutic qualities, due to bioactive metabolites synthesized intra- and extracellularly: they have anti-inflammatory, antioxidant, anti-carcinogenic, hypocholesterolemic, anti-diabetic, immunomodulatory and other properties.
Also, some fungi can synthesize enzymes from the class of oxido-reductases (laccase with lignin degradation action) and hydrolases (amylases, cellulases, proteases and others), with a role in the decomposition of organic substrates. These enzymes can be recovered from liquid cultures of mycelial biomass production (Bakratsas et al, 2021; Dudekula et al., 2020; Sandargo et al., 2019; Lu et al., 2020; Zhang et al., 2019).
Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes, in the active site, which catalyse the oxidation reaction of various substrates such as phenolic and aromatic compounds (ortho- and para-diphenols, amino phenols, methoxy phenols, polyphenols), aliphatic amines and inorganic cations, resulting in water as a product of reduction of molecular oxygen (Ferdes et al., 2022).
The aim of this paper is to present an analysis of the capacities of five species of fungi belonging to the Basidiomycetes, to synthesize laccases, cellulases and other enzymes, and to produce biomass in liquid media.

MATERIALS AND METHODS

Biological material

Five species of edible mushrooms cultivated in the form of mycelium were used, namely:

Pleurotus ostreatus, Flammulina filiformis (commercially known as Enoki flower), *Ganoderma lucidum* (also known as Reishi), *Lentinula edodes* (Shiitake), and *Polyporus squamosus.*

Culture media

Potato Dextrose Agar (PDA) medium supplemented with 0.1% chloramphenicol was used to isolate fungal mycelium. The mycelium was cultured in tubes or Petri dishes on potato dextrose agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CZA) media. Cultivation of the submerged mushroom species was carried out using the liquid medium with the following composition: sugar beet molasses 100 g/L, NH_4NO_3 3 g/L, $(NH_4)_3PO_4$ 1 g/L. Enzymes were detected on specific media that allowed the visualization of a modification reaction of the agar gel: laccase was detected on PDA medium supplemented with 0.02% guaiacol; for cellulases, a medium with carboxy methyl cellulose (CMC) 10 g/L, $(NH_4)_2SO_4$ 1.4 g/L, K_2HPO_4 2 g/L, $CaCl_2$ 0.3 g/L, $MgSO_4$ 0.3 g/L, yeast extract 0.25 g/L, peptones 0.75 g/L, agar 17 g/L was used; the medium is then colored with a Congo red solution, and the producing colonies become surrounded by a colorless zone. To evaluate the synthesis of proteolytic enzymes, the fungal colonies were cultivated on a medium with casein 2.5 g/L. Lipolytic enzymes were analysed on nutrient agar supplemented with $CaCl_2$ 1% and Tween 80 1%, and amylases on a medium with starch. The colony growth rate was determined on 3 culture media, namely: PDA, MEA and CZA.

Culture media were sterilized by autoclaving for 15 minutes at 121 ˚C.

Methods

Average hourly growth rate estimation

The fungal species were grown in tubes and kept at 4˚C in the refrigerator until use.

The average hourly growth rate was determined on the previously mentioned culture media, at temperatures of 20˚C and 30˚C, using the formula:

$$V = \frac{Colony\ diameter\ (mm)}{time\ (hours)}$$

Qualitative method for enzymatic activity

To test the production of enzymes, the diameters of the modified zones around the fungal colonies cultivated in Petri dishes on the media mentioned above were measured.

The enzyme indices were calculated according to the formula:

$$I_E = \frac{Zone\ diameter}{Colony\ diameter}$$

Submerged cultivation

The submerged cultivation of fungi was carried out in liquid medium with molasses, in Erlenmeyer flasks (200 mL/flask), on a rotary incubator, at 150 rpm and 20˚C, for 7 days. Dry biomass, pH and dry matter (in the filtrate) were measured in the collected samples. The obtained biomass was separated by filtering and washing with distilled water, dry matter was analyzed with a thermobalance at a temperature of 103˚C.

Guaiacol assay method for laccase activity

The method is based on the oxidation reaction of guaiacol in the presence of laccase, with the formation of a reddish brown coloration.

Guaiacol can be used as a hydrogen donor (substrate) in the assay of laccase. Upon oxidation, it forms tetraguaiacol with a molar extinction coefficient $\varepsilon = 26.6\ mM^{-1}cm^{-1}$.

A reaction mixture consisting of 1) Guaiacol (2 mM) 1 ml; 2) Sodium acetate buffer (10 mM) 3 ml; 3) Enzyme 1 ml was prepared. A control is prepared, which contains distilled water instead of the enzyme. The reaction mixture was incubated at 30˚C for 15 minutes and then the absorbance was read at 450 nm in a UV-Vis spectrophotometer. 1 enzymatic unit (U) was defined as the amount of enzyme required to oxidize 1 µmol of guaiacol per minute. Enzyme activity was calculated according to the formula (Isik et al., 2023):

$$\text{Laccase activity (LA)} = \frac{Abs \times Vol \times DF}{\varepsilon \times t \times v}$$

Where:

LA = Laccase activity

A = Absorbance 450 nm

V =Total mixture volume (ml)

v = enzyme volume (ml)

t = incubation time

ε = Guaiacol [12100 M^{-1} cm^{-1}] molar absorption coefficient.

DF=dilution factor.

RESULTS AND DISCUSSIONS

Mushroom cultures developed in tubes or Petri dishes on the mentioned media showed white color, fluffy appearance and characteristic flavor (Figure 1).

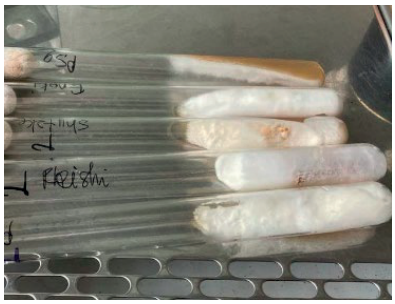


Figure 1. Appearance of cultures in tubes on PDA medium

The average hourly growth rates on PDA, MEA and CZA culture media at temperatures of 20˚C and 30˚C are shown in Figures 2 and 3.

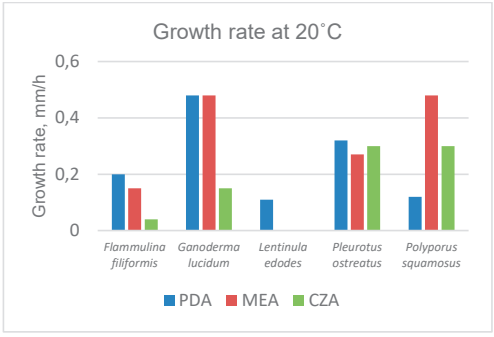


Figure 2. Average hourly growth rates of colonies on PDA, MEA and CZA at 20˚C

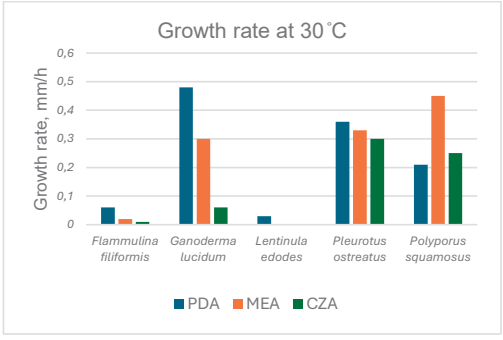


Figure 3. Average hourly growth rates of colonies on PDA, MEA and CZA at 30˚C

Colony growth of the 5 species of basidiomycetes was strongly influenced by the composition of the culture medium. In general, the highest growth rates were recorded for the PDA and MEA media, while for the CZA medium the growth was minimal for the species of *Flammulina filiformis* and *Lentinula edodes*. For the following studies this was important for choosing the optimal parameters for biomass accumulation and enzymes production. The temperature had a less important influence on the growth of the studied mushroom colonies. *Flammulina filiformis, Ganoderma lucidum* and *Lentinula edodes* species seem to grow better at 20˚C, while *Pleurotus ostreatus* and *Polyporus squamosus* have comparable growth rates at 20˚C and 30˚C.

The results of determining the enzyme indices according to the mentioned formula are shown in Table 1.

Table 1. Enzyme index values of fungal colonies

Enzymatic indices					
Species	ILc	IC	IA	IP	ILi
Flammulina filiformis	1,4	0,25	0	6	0
Ganoderma lucidum	2,7	2	0	2	5
Lentinula edodes	2,2	2	0	0	3
Pleurotus ostreatus	4,6	1,1	0	3	4,6
Polyporus squamosus	1	1,5	0	1,1	0

Note: ILc=laccase index; IC=cellulase index; IA=amylase index; IP=protease index; ILi=lipase index.

The aspect of the modified areas around the producing colonies is shown in the Figures 4-7. Laccase was highlighted in the case of *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes* species, known for their growth on lignocellulosic materials. *Polyporus squamosus* had the lowest laccase production, the reverse side of the colony being slightly coloured in reddish brown. Laccase biosynthesis started after the first 3 days of colony development.

Cellulolytic enzymes could be visualized on the medium with CMC also after the first 3-5 days of development. By staining the culture medium with 1% Congo red solution and washing with a 1M NaCl solution, the colorless hydrolysis zone could be visualized around the colonies of *Ganoderma lucidum, Lentinula edodes, Polyporus squamosus* and *Pleurotus ostreatus*.

Some species of Basidiomycetes have synthesized proteases and lipases. *Flammulina filiformis* and *Pleurotus ostreatus* had the highest proteolytic indices, of 6 and 3, respectively. *Ganoderma lucidum, Lentinula edodes* and *Pleurotus ostreatus* synthesized lipases, highlighted by the appearance of an opaque area around the colonies.
No fungal colony synthesized amylolytic enzymes.

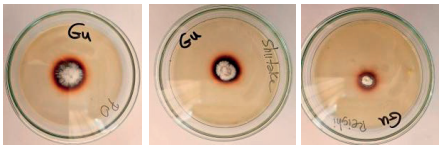


Figure 4. Appearance of basidiomycetes colonies on the medium for laccases: *Pleurotus ostreatus, Lentinula edodes, Ganoderma lucidum*

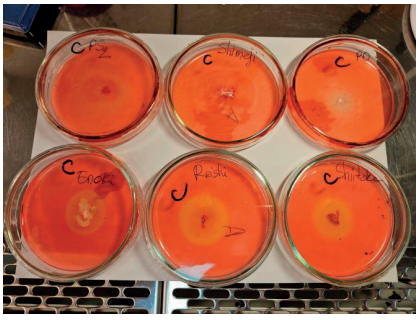


Figure 5. Appearance of basidiomycete cultures on the medium for *cellulolytic* enzymes

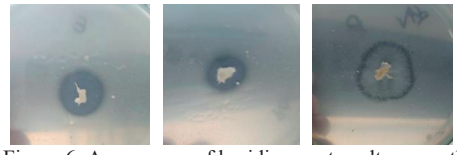


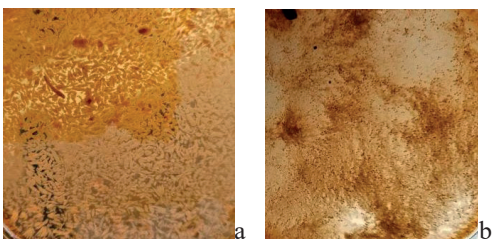
Figure 6. Appearance of basidiomycete cultures on the medium for proteolytic enzymes: *Flammulina filiformis, Pleurotus ostreatus, Polyporus squamous*

Figure 7. Appearance of basidiomycete cultures on the medium for lipolytic enzymes: *Pleurotus ostreatus, Ganoderma lucidum, Lentinula edodes*

The accumulation of biomass in liquid media
The mushroom species were cultivated in the liquid medium with sugar cane molasses and mineral salts, on the rotary incubator, at 150 rpm, for 7 days. Dry biomass, pH and dry matter% values in the filtrate were analysed. The results presented in the Table 2 demonstrated that the largest amount of biomass was obtained in the cultures of *Pleurotus ostreatus* and *Lentinula edodes*, of 3.49 and 3.24 g/L, respectively. The mycelium developed in the form of filaments, sometimes forming spheres with a diameter of several millimetres (Figure 8 and Figure 9). The accumulation of biomass was correlated with the decrease of dry matter in the filtrates, and the pH values in the culture liquid varied between 4.7 and 7.1 units, depending on the species.

Table 2. The results of biomass accumulation in the culture medium with molasses

Species	Dry biomass, g/L	pH in filtrate	Dry matter in filtrate, %	Laccase activity, U/mL
Control	-	5,7	9,8	0
Flammulina filiformis	2,0	6,54	9,2	0
Ganoderma lucidum	6,8	4,92	8,1	6
Lentinula edodes	16,2	4,73	3,7	4
Pleurotus ostreatus	17,5	5,77	3,5	10
Polyporus squamosus	13,1	7,16	2,7	0



a b

Figure 8. The *mycelium* of a) *Pleurotus ostreatus*, b) Polyporus squamosus in liquid medium

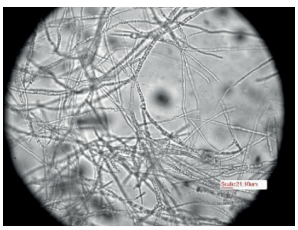


Figure 9. Hyphae of *Polyporus squamosus* on microscope camera

After 7 days of thermostating in the rotary incubator, the quantitative analysis of the

laccase activity by the guaiacol method showed that only three strains synthesized the enzyme in the culture medium: *Pleurotus ostreatus, Ganoderma lucidum*, and *Lentinula edodes*.
Comparable results were obtained for the same reaction conditions by other authors, namely by Suleman et al., 2020, who determined a maximum activity between 10 and 26 U/mL for different fungal isolates; Kumar et al., 2016. found that the laccase activity for an *Aspergillus flavus* strain was 26 U/mL, and Isik et al., 2023, obtained an enzyme activity of approximately 300 U/mg from *Trametes versicolor*. Using other substrates such as ABTS, syringaldazine, or 2,6-dimethoxyphenol, in different test conditions, the values of laccase enzymatic activities are different.
Similar studies were carried out using different species of basidiomycetes, cultivated in culture media with diverse composition (Krupodorova et al., 2021). *Agaricus bisporus* (Ma et al., 2014; Salmones et al., 2018), *Ganoderma adspersum* (Badalyan et al., 2019), *Lentinula edodes* (Krupodorova et al., 2020) *Pleurotus* spp. (Phadke et al., 2020) were cultivated in different conditions.
Although mushrooms contain valuable nutrients for food, a relatively small number of publications is dedicated to the study of the influence of cultivation parameters on their growth. The presence and nature of carbon and nitrogen sources, mineral salts and growth factors greatly influence both the yield of biomass production, as well as the growth time and the duration of the lag phase.
The various researches on the growth of the fungal mycelium have shown that there is no single commonly accepted standard media for the optimal fungal growth, because the influence of the type of nutrients on the mycelial growth can vary depending on fungi species and their strains. Media such as PDA, CZA and MEA were found to be the best for the growth of many basidiomycetes.
In addition, the various agro-industrial by-products, such as molasses, have a complex nature and variable composition over time, making it difficult to determine some physiological characteristics such as the rate of substrate uptake.

CONCLUSIONS

Five species of basidiomycetes (*Pleurotus ostreatus, Flammulina filiformis*, *Ganoderma lucidum*, *Lentinula edodes*, and *Polyporus squamosus)* were tested in terms of growth speed on different agar media, enzyme production and biomass production in liquid media.
Mushrooms are becoming more and more important for the human diet due to their nutritional, organoleptic and pharmacological characteristics. Considering the optimal concentration of minerals, the low value of lipids and the caloric content, the nutritional value of mushrooms was re-evaluated. In addition, it seems that some mushrooms can prevent some diseases such as forms of cancer, hypercholesterolemia and hypertension.
Colony growth of the 5 species of basidiomycetes on PDA, MEA and CZA was strongly influenced by the composition of the culture medium, while the temperature had a less significant influence.
The best producers of laccase and cellulase were *Pleurotus ostreatus* and *Ganoderma lucidum*, which had values of 3 and 4 respectively for laccase enzyme indices.
The maximum biomass values in the culture medium with molasses were recorded for the *Pleurotus ostreatus, Lentinula edodes*, and *Polyporus squamosus* species, being 17.5, 16.2, and 13.1 g/L respectively.
Only three species of basidiomycetes, namely *Pleurotus ostreatus, Ganoderma lucidum* and *Lentinula edodes*, synthesized laccase in the liquid medium used in this study.

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NUTRITIONAL COMPOSITION OF BEE PRODUCTS AND CURRENT TRENDS ON THEIR PROCESSING

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Abstract

In present times, natural bioactive substances are of great interest, especially appreciated by consumers whose preferences tend towards functional local foods. Among the most well-known natural resources are beehive products, recognized for their beneficial effects on the human body. This article aims to highlight current studies on the nutritional composition of various bee products, including honey, beeswax, propolis, bee pollen, royal jelly, and bee venom. In the same time, current trends in exploiting beehive products indicate a continuous search for ways to enhance their utilization. Through innovative methods such as extractions, fermentations, and ingredient combinations, new horizons are being explored regarding the nutritional benefits and applications of these products. The present work outlines the review of processing methods applied for enhancing utilization of bee products emphasizing the effects of their consumption on health and their potential applications in the food and pharmaceutical industries.

Key words*: bee products, nutritional composition, processing.*

INTRODUCTION

Apis mellifera is known as the bee and is one of the most beneficial insects in the whole world, because it plays an essential role for the environment, pollinating flowers and plants (Azam et al., 2019). Since ancient times, bee products have been used as natural remedies against numerous diseases, due to the diversity of their composition and chemical properties (Al-Hatamleh1 et al., 2022). The medicinal therapeutic use of beehive products is called Apitherapy (Abdela et al., 2016). Nowadays, the research of new safer and more active molecules from functional foods is a main trend in green chemistry. This trend is reinforced by scientific data demonstrating the importance of functional nutrients in the prevention and treatment of many diseases. In recent years, consumers have increasingly demanded more food options, especially without chemical additives and rich in bioactive constituents (Laaroussi et al., 2023). Natural bioactive substances are of great interest and especially appreciated by consumers whose preferences tend towards functional natural foods. Among natural products containing bioactive ingredients, honey and other bee products are very popular as healthy alternatives to synthetic supplements (Liu et al., 2023). Natural extracts from bee products are recognized worldwide as a remarkable source of bioactive compounds with diverse functionalities, such as antioxidant, antimicrobial and anticancer agents. Some of these compounds are used in the pharmaceutical field but also for the development of new functional foods with the aim of improving the bioactivity of current food products, their properties and replacing other synthetic components (Fuente-Ballesteros et al., 2023). Bee products are naturally secreted by bees through glands (e.g., venom, beeswax, and royal jelly) or produced by collection and processing by bees (e.g., nectar, bee pollen, and wax) (Liu et al., 2023). Bee products exhibit a wide range of desirable characteristics, including antimicrobial and antioxidant properties, and the most popular products of the hive are honey, pollen and their extracts, propolis, royal jelly and bee venom (Bartkiene et al., 2020).

MATERIALS AND METHODS

The objective of this review was to collect the latest information on the composition of bee products and to highlight their effects on health as well as their potential for food, medical, cosmetic and biotechnological applications. For

this study we used international platforms ScienceDirect, MDPI and the search tool Google Scholar and ResearchGate to analyze and synthesize the information. Following the keywords used, honey, pollen, bee bread, bee venom, beeswax, propolis, royal jelly, composition, processing, bee products, honey varieties, we checked 500 articles, and according to the keywords of interest, the number of articles decreased to 150, of which 71 can be found in the bibliography.

1. BEE PRODUCTS COMPOSITION

1.1. Bee honey

Bee honey is a sweet natural substance produced by bees (Kerkich et al., 2023) and is consumed worldwide by humans. Bees produce this concentrated aqueous mixture by converting sugar sap from pollen collected from plants by means of hypopharyngeal gland enzymes (e.g. amylase, glucosidase and glucose oxidase). Bee honey is the most widely consumed and well-known bee product (Sawicki et al., 2023) and although it is speculated that it has been used since ancient times as a natural remedy (Nikhat et al., 2021) there is a lack of knowledge regarding its interaction with the body. Lately honey is gaining popularity over sugar for its countless health benefits because it has a low glycemic index (Nikhat et al., 2021) The chemical and medicinal properties of honey are different depending on the types of honey and their origin (Young et al., 2023). There is also a difference in the chemical composition between the types of honey that come from different regions depending on the geographical and botanical variability, thus generating distinct therapeutic activities. High quality honey, differs in appearance, taste, color and aroma. These characteristics of honey are influenced by the floral source from where the bees collect the nectar (Gündoğdu et al., 2019). The specific composition of honey includes carbohydrates, enzymes, vitamins, proteins, phenols, flavonoids, volatile compounds, organic acids and minerals, but it must be emphasized the fact that this composition can be influenced by nectar, season, honey storage as well as the geographical region where the honey is collected (Wang et al., 2023). Honey in general consists of approximately 200 components (Trisha et al., 2023) mainly 80–85% carbohydrates (46–72% glucose, 7–61% fructose and 1–11% sucrose), 15–17 % water, 0.3% protein (including arginine, histidine, isoleucine, lysine, methionine, threonine, tryptophan, valine and other amines) and 0.2% ash. . Bee honey also contains vitamins B1, B6 and niacin, making it a healthy natural product with excellent antioxidant and antibacterial properties (Liu et al., 2023). It has been used in both traditional and modern medicine, as it exhibits a wide range of therapeutic properties, such as anti-inflammatory, anti-diabetic, anti-mutagenic, anti-cancer, beneficial properties for respiratory, gastrointestinal, cardiovascular and system protection nervous **(**Bartkiene et al., 2020). In recent years, special attention has been directed towards Manuka honey, which contains unique compounds given by the nectar of *Leptospermum scoparium* flowers (Wang et al., 2024).

1.2. Bee wax

One of the lesser known and less studied bee product is beeswax. This bee product is actually a natural substance secreted by special wax glands present in the abdominal segments of worker bees. The wax is used to build hexagonal honeycomb cells to store honey and protect larvae and pupae. In food technology, beeswax is mainly used as a food additive and glazing agent **(**Sawicki et al., 2023). Beeswax can be used in the food processing industry, in the cosmetic industry, in art as well as in medicine. The latest studies show that wax has therapeutic properties in the healing of bruises, burns and inflammations (Fratini et al., 2016). The color of the wax is given by the presence of propolis and pollen, and the predominant characteristic of the wax is its smell. According to Kurek-Górecka et al. (2020), beeswax is considered as source of vitamin A, represented by the carotenes content, which accelerates skin regeneration.

1.3. Bee propolis

Propolis is another bee product that has important health benefits. This product contains a large number of compounds with anti-inflammatory, antioxidant, antiviral and antimicrobial properties. Bees prepare and use propolis as a sealing material to protect against the penetration of microorganisms (fungi and

bacteria) into the hive and to create the most sterile environment known in nature (Anjum et al., 2018). The most recent studies have focused on the chemical compounds of propolis depending on the plant source and geographical area, as well as on propolis collected by non-stinging bees. The stingless bees are originally from tropical and subtropical countries (dos Santos et al., 2021). Propolis has antioxidant, anti-inflammatory and antimicrobial actions. It contains 50% resin, 30% resinous vegetable oils and beeswax, 10% essential oils, 5% pollen and 5% other components. Various active compounds have been identified in propolis, including polyphenols, phenolic aldehydes and ketones. Propolis is usually viscous at room temperature and can vary in color from plant to plant (including green, black, dark brown, and yellow) and exhibit different characteristic aromatic odors (Liu et al., 2023). The main pharmacological activities of propolis are related to flavonoids and phenolic compounds - the major bioactive constituents of this product. The properties of propolis flavonoids to reduce the formation or eliminate free radicals allow efficient regeneration of damaged tissue, and the antimicrobial properties of propolis prevent wound infection. It is used in traditional medicine as an antimicrobial, anti-inflammatory, anesthetic, antiseptic, antimutagenic and anti-cancer agent and also exhibits wound healing, antioxidant and cardioprotective properties (Liu et al., 2023). Propolis is very popular and is used in a variety of commercial preparations, including pharmaceuticals, because it is a natural product with no side effects (Abdelrazeg et al. 2020).

1.4. Bee pollen

The bees are the ones who create the pollen grains, mixing their secretions with the pollen collected from the flowers, resulting in grains between 1.4 mm and 4 mm in size (Giampieri et al., 2022). It is considered a nutritional treasure due to its active components, which possess significant properties in terms of health and medicinal benefits. The essential elements of bee pollen contribute to the improvement of various body functions and provide protection against a wide spectrum of diseases (Khalifa et al., 2021). Bee pollen, a nutritious product, consists of 15–25% protein, 45–60% carbohydrate, 5–10% fat, and 10–19% fiber. However, chemical compounds can vary due to both plants source and geographical origin (Spulber et al., 2020). Bee pollen is a treasure trove of active natural metabolites, it is the main source of nutrients for bees, such as minerals (calcium, magnesium, iron, zinc, copper), proteins, carbohydrates, vitamins (A, C, E, niacin, thiamin, biotin and folic acid), fiber and lipids, including fatty acids, Omega-3 and Omega-6, phenols (flavonoids and phenolic acids), carotenoids and phytosterols (Lu et al., 2022; Sokmen et al., 2022 Aylanc et al., 2023; Liu et al., 2023). In addition to its nutritional values, bee pollen presents an inexhaustible source of powerful antioxidant compounds such as resveratrol, quercetin, kaempferol, cinnamic and caffeic acids (Laaroussi et al., 2023). Due to its nutritional characteristics, the rich content of minerals, vitamins, fibers and bioactive compounds, but also the fact that it contains all the essential amino acids needed by humans, pollen is defined as "the best food product in the world" (Sokmen et al., 2022; Liu et al., 2023;).
Pollen is a popular beehive product widely used in traditional medicine for the prevention and self-treatment of various pathologies and has attracted the interest of many researchers around the world. Several human health-promoting effects have been reported in the analysis of bee pollen extracts, including cardioprotective, anti-inflammatory, anticarcinogenic, and hepatoprotective effects. Furthermore, new research has shown that bee pollen offers promising benefits for Parkinson's disease, depression and polycystic ovarian syndrome. Due to its well-known nutritional and medicinal benefits, bee pollen is commonly used as a natural food supplement (Alshallash et al., 2023; Laaroussi et al., 2023).
The main bioactive components in bee pollen are phenolic acid derivatives and polyphenolic compounds. Flavonoids are a class of secondary plant compounds that exhibit various essential physiological and pharmacological activities. Studies have shown that bee pollen exhibits strong biological functions, including antibacterial, antioxidant, anticancer, anti-inflammatory, hepatoprotective, anti-atherosclerotic and immunomodulatory activities (Liu et al., 2023), as well as an essential role in mitigating cardiovascular

diseases risks (Dinu et al., 2023). Recently, considerable interest has been shown in the use of bee pollen in food systems as a functional ingredient to enrich product quality characteristics. In this context, bee pollen has been used in the enrichment of yogurt, cheese, bread and fermented beverages (such as kombucha, white wines, malt and fermented milk beverages) as a (bio)techno-functional ingredient with strong antioxidant and antimicrobial activities, improving the nutritional and functional characteristics of the final products (Laaroussi et al., 2023). Another area of interest regarding the pollen is that with its fermentation at a temperature higher than 25°C a pollen similar to pasture is produced **(**Miłek et al., 2023). Recent research demonstrates that bee products, but especially pollen, is a sustainable product and can be used as a functional product for biomaterials (Sanyal et al., 2023).

1.5. Bee bread (bee pasture)

Forage is a valuable product of the hive, made from bee pollen, honey and secretions from the bees' salivary glands (Ghosh & Jung, 2022). The resulting mixture is stored in brood cells, which undergo lactic fermentation. This fermentation takes place during storage, which increases the bioavailability of pollen compounds, as cell walls are partially destroyed during fermentation. The lactic acid bacteria in the hive of bee pollen represent the protein base of the food in the hive, hence the name bee bread. (Ćirić et al., 2022). Natural fermentation in the hive increases the bioavailability of bee pollen and also keep in the form of pasture, which can be kept for a long time without losing its nutritional value (Kaškonienė et al., 2020). Like other products of the beehive, the biological properties of bee bread depend on the climatic conditions, the honey plants sources, the geographical area as well as of season. From a nutritional point of view, bee bread is more valuable compared to bee pollen, due to its better digestibility properties (Ćirić et al., 2022; Aylanc et al., 2023).

Like bee pollen, grass contains the same main chemical components, i.e. proteins, carbohydrates and lipid substances (saturated and unsaturated fatty acids). It also contains other nutrients such as minerals, carotenoids, phytohormones and vitamins (Miłek et al., 2023). Pasture contains peptides and free amino acids and is an excellent product that could supplement the population's nutrient deficiencies to achieve a balanced diet. Pasture contains antioxidant compounds (e.g. carotenoids) and natural preservatives (e.g. lactic acid). However, it should be noted that the presence of free amino acids in bee products can lead to the formation of biogenic amines, which are ultimately undesirable compounds in food products (Bartkiene et al., 2020). The latest research shows that extracts from bee bread can have antifungal, antitumor, antioxidant and antibacterial effects (Poyraz et al., 2023).

1.6. Royal jelly

Royal jelly is a milky white secretion produced by the mandibular and hypopharyngeal glands of worker bees and is used to feed bee larvae and adult queens (Yu et al., 2023). The chemical composition of royal jelly includes 3–6% fat, 15% carbohydrates, 18% protein, 50–60% water, 1% vitamins and 1.5% minerals. Royal Jelly Major Protein (MRJP) accounts for 82-90% of total royal jelly protein. The molecular weights of the MRJP family (MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5) range between 49 and 87 kDa, with MRJP1 showing the highest protein content (31%), followed by MRJP3 (26%), MRJP2 (16%), and MRJP5 (9%).

The highest vitamin content in royal jelly is represented by pantothenic acid (52.8 mg/100 g), followed by niacin (42.42 mg/100 g). Royal jelly also contains a considerable number of bioactive substances, including proteins with antimicrobial properties, fatty acids and peptides, the composition and content of which may vary depending on the plant and postharvest conditions. Studies have shown that royal jelly exhibits antioxidant, anti-inflammatory, anti-aging, neuroprotective, antibacterial, anti-allergic, and antitumor pharmacological properties and is widely used in the food, cosmetic, and pharmaceutical industries (Liu et al., 2023).

1.7. Bee venom

It is the action of the bee to defend itself when it feels in danger. In the abdomen of the bee, there is a gland where venom is produced, with multiple uses in apitherapy. This venom is a

natural substance, a dense, colorless liquid, with a specific, pungent smell, it is soluble in water and insoluble in alcohol (Abdela et al., 2016). Bee venom is used in traditional medicine to treat various diseases. The latest research refers to the use of bee venom as an alternative treatment for breast cancer, which indicates that bee venom has an anti-cancer effect (Kwon et al., 2022), it can have an antibacterial effect for resistant pathogens (Gökmen et al., 2023) and can be used in various skin diseases, being used in preparations as cosmetic products or can be used as a dressing in the treatment of various skin wounds (Abd El-Wahed et al., 2021). Research shows that Lyme disease, HIV, Parkinson's and Alzheimer's diseases are among the most well-known diseases in the treatment of which bee venom is used (Al-Ameri et al., 2022).

To summarize the presented information regarding bee products composition, Table 1 presents a synthesis with the main results of the study.

Table 1. Chemical and nutritional composition of bee products

Region	Composition	Reference
	Propolis	
Romania	Water content: 0.35-0.66 % Ash: 0.94-0.98 % Na (103.29-110.33 mg), Ca (587.05-840.40 mg), K (97.34-128.79 mg), Mg (144.24-148.99 mg), P (132.00-152.63 mg), Fe (41.97-54.58 mg), Mn (4.91-5.67 mg), Cu (2.32-3.13 mg), Zn (15.85-42.73 mg), Cd (0.24-0.55 mg)	Moraru et al., 2024
Spain	Moisture: 16.26% Ash: 0.85% Ca (856.56 mg/kg), Cu (1.51 µg/kg), Fe (107.82 mg/kg), Zn (73.72 mg/kg), K (1690.15 mg/kg), Mg (217.79 mg/kg), Mn (14.22 mg/kg), Pb (4056.98 µg/kg), Hg (5.59 µg/kg) Total polyphenols: 42.72 Pinocembrin–Galangin Eq./100g Total flavonoids content: 1.64-4.95 QE g/100g Antioxidant activity: 1114.28 µM (TEAC), 2535.40-3918.18 µM (CEAC)	Rendueles et al., 2023
Portugal	Water content: 2.42-6.56 % Ash: 0.79-1 % Total polyphenols content: 107.96-226.73 mg GAE/g extract Total flavonoid content: 31.9-40.94 mg QE/g extract Total ortho-diphenol content: 272.3-332.27 mg GAE/g extract Antioxidant activity: 10.81-21.49 EC_{50} µg/mL (DPPH), 8.38-10.53 EC_{50} µg/mL (ABTS)	Caetano et al., 2023
Italy	Dry matter: 3.23-3.82 % Ash: 0.7-2.06% Total phenols: 222.44-442.26 mg GAE/g Total flavonoids: 64.35-115.62 mgQE/g Antioxidant activity: 4.97-5.66 mMTE/g (ABTS), 1.2-1.76 mMTE/g (FRAP)	Grassi et al., 2023
Lithuania	Brix: 11.1 Total phenolic content: 87.2 mg RUE/10 g Total flavonoid content: 8.4 mg RUE/10 g Radical scavenging activity: 27.0 mg RUE/10 g	Adaškeviciut et al., 2019
Egypt (extracts - 10 g of sample in 100 ml methanol)	Gallic acid 3.4 ± 0.12 mg/mL Vanillic acid 2.45 ± 0.25 mg/mL Synringic acid 4.24 ± 0.35 mg/mL p-Coumaric acid 24.5 ± 0.05 mg/mL Ferulic acid 26.5 ± 0.16 mg/mL Caffeic acid 11.4 ± 0.04 mg/mL Quercitin 2.24 ± 0.02 mg/mL Rutin 6.4 ± 0.11 mg/mL Catechin 2.1 ± 0.13 mg/mL Epicatechin nd α-Catechin nd Kaempferol 0.59 ± 0.19 mg/mL Apigenin 3.57 ± 0.21 mg/mL 3,4-Dimethoxycinnamic acid nd Naringenin 2.56 ± 0.28 mg/mL Luteolin 1.3 ± 0.24 mg/mL	Mohdaly et al., 2015

Malaysia	Total phenolic content: 10.017-17.043 mgGAE/g Total flavonoid content: 0.204-0.830 mgQE/g Antioxidant activity DPPH (IC_{50}, mg/g): 30.77 (20% ethanol crude extract) and 96.28 (100% water crude extract); ORAC (mg Trolox Equivalent/g Propolis): 3.821 (20% ethanol crude extract) and 0.623 (100% water crude extract)	Lim et al., 2023
Brazil	Total phenolics: 192.03-336.91 mg GAE/g Flavonoids: 16.70-34.38 mg QE/g	Dutra et al., 2023
Brazil	Total phenolic compounds: 2741.71 mg GAE/100 g (Green propolis), 1191.55 mg GAE/100 g (Brown propolis), 901.79 mg GAE/100 g (Dark propolis) Antioxidant capacity: Green propolis: 293.90 Trolox µM/g (ABTS), 422.83 µM Ferrous Sulfate/g (FRAP), 491.68 (EC_{50} Expressed in g of Sample/g of DPPH) Brown propolis: 109.29 Trolox µM/g (ABTS), 179.54 µM Ferrous Sulfate/g (FRAP), 1054.38 (EC_{50} Expressed in g of Sample/g of DPPH) Dark propolis: 162.57 Trolox µM/g (ABTS), 161.29 µM Ferrous Sulfate/g (FRAP), 1090.72 (EC_{50} Expressed in g of Sample/g of DPPH)	Vieira et al., 2023
Bee pollen		
Portugal	Ash: 3.3±0.5 g.100 g^{-1} Water: 5.3±0.5 g.100 g^{-1} Lipid: 5.2±3.7 g.100 g^{-1} Protein: 19.3±3.6 g.100 g^{-1} Fiber: 5.4±0.5 g.100 g^{-1} Carbohydrate: 67.1±1.3 g.100 g^{-1}	Aylanc et al., 2023
Portugal	Antioxidant activity (DPPH): 0.20 mg/mL Reducing power activity: 5.0 mg GAE/g	Ertosun et al., 2023
Spain	Total phenolic content: 1612.6 mg/100 g Total flavonoid content: 256.8 mg/100 g Antioxidant activity: 65.7% (DPPH), 57.4% (ABTS)	Rojo et al., 2023
Italy	Polyphenols: 27.37 mg GAE/g FW Flavonoids: 25.37 mg QE/g FW Flavonols: 13.50 mg QE/g FW Anthocyanins: 58.16 mg C3GE/L Carotenoids: 11.78 µg/g FW Antioxidant activity: 5.31 TEAC (ABTS), 0.15 EC_{50} mg/mL (DPPH), 587.64 µmol TE/g FW (ORAC), 15,054.81 Fe^{2+} µM (FRAP), 1.03 mg EDTAE/g FW (Fe^{2+} Chelation)	Chelucci et al., 2023
Lithuania	Brix: 27.2 Total phenolic content: 47.2 mg RUE/10 g Total flavonoid content: 32 mg RUE/10 g Radical scavenging activity: 26.7 mg RUE/10 g	Adaškeviciut et al., 2019
Poland	Total phenolic content: 10.36 mg GAE/g Total Carotenoid content: 24.96 µg/g Antioxidant activity: 26.61 µmol TE/g (FRAP), 17.61 µmol TE/g (DPPH)	Miłek et al., 2023
Egypt (extracts - 10 g of sample in 100 ml methanol)	Gallic acid 5.9 ± 0.05 mg/mL Vanillic acid 0.35 ± 0.15 mg/mL Synringic acid 0.59 ± 0.08 mg/mL p-Coumaric acid 2.48 ± 0.25 mg/mL Ferulic acid 4.2 ± 0.18 mg/mL Caffeic acid 4.21 ± 0.22 mg/mL Quercitin 6.4 ± 0.30 mg/mL Rutin 3.46 ± 0.14 mg/mL Catechin 4.8 ± 0.18 mg/mL Epicatechin 2.1 ± 0.08 mg/mL α-Catechin 0.58 ± 0.05 mg/mL Kaempferol 1.65 ± 0.24 mg/mL Apigenin 2.4 ± 0.25 mg/mL 3,4-Dimethoxycinnamic acid 45.8 ± 0.16 mg/mL Naringenin 3.34 ± 0.12 mg/mL Luteolin 2.8 ± 0.10 mg/mL	Mohdaly et al., 2015
United States	Pollen collected from industrial hemp Moisture: 76.8-81.6 % Ash: 1.55-1.87 %	Dingha and Jackai, 2023

	Crude fiber: 1.50-2.67 % Protein: 6.05-6.89 %		
-	Carbohydrates/100g: 31.69 Lipids/100 g: 20.44 Proteins/100 g: 23.6 Total phenols content: 22.62 mg GAE/g dw Total flavonoids content: 85.75 mg QE/g dw Antioxidant activity DPPH: 413.85 mmol TE/g FRAP: 36.56 µmol TE/g TEAC: 86.06 µmol TE/g CUPRAC: 155.18 µmol TE/g		Tirla et al., 2023
Bee bread			
Portugal	Ash: 3.5±0.2 g.100 g^{-1} Water: 7.6±0.2 g.100 g^{-1} Lipid: 3.6±1.0 g.100 g^{-1} Protein: 22.6±0.8 g.100 g^{-1} Fiber: 4.4±0.6 g.100 g^{-1} Carbohydrate: 65.6±1.5 g.100 g^{-1}		Aylanc et al., 2023
Poland	Total phenolic content: 9.53 mg GAE/g Total Carotenoid content: 33.14 µg/g Antioxidant activity: 26.98 µmol TE/g (FRAP), 15.83 µmol TE/g (DPPH)		Miłek et al., 2023
Greece	Total phenolic content: 6.49-14.64 mg GAE/g Total flavonoids content: 2.34-5.49 mg QE/g Antioxidant activity: 0.18-1.80 IC_{50} (DPPH), 0.38-1.80 IC_{50} (ABTS)		Didaras et al., 2021
Lithuania	Brix: 19.0 Total phenolic content: 19.63 mg RUE/10 g Total flavonoid content: 7.88 mg RUE/10 g Radical scavenging activity: 17.23 mg RUE/10 g		Adaškeviciut et al., 2019
Honey			
Lithuania	Brix: 17.40 Total phenolic content: 5.6 mg RUE/10 g Total flavonoid content: 3.6 mg RUE/10 g Radical scavenging activity: 4.06 mg RUE/10 g		Adaškeviciut et al., 2019
Republic of Serbia	Sunflower honey Moisture: 17.01 % Ash: 0.13% HMF (hydroxymethylfurfural): 2.08 mg/kg Sucrose: 0.332 % Glucose: 37.07 % Fructose: 40.24 %		Živkov Baloš et al., 2023
Romania	Rape honey Moisture: 17.30-19.12% HMF: 7.40-48.60 mg/kg Total phenolic content: 12.50-31.04 mg GAE/100 g Total flavonoids content: 7.66-16.23 mg QE/100 g Antioxidant activity: 49.06-55.80 (DPPH) Fructose: 34.87-37.61% Glucose: 28.25-36.09% Sucrose: 0-0.62% Turanose: 0.26-4.16% Maltose: 0.89-5.97% Trehalose: 0.91-11.34% Melesitose: 0.60-2.95% Raffinose: 0-0.36%		Pauliuc & Oroian, 2020
Brazil	From *Melipona fasciculata* Moisture: 27 % Brix: 71 Apparent sucrose: 1% Total phenols: 122 mg kg^{-1} Total flavonoids: 87 mg kg^{-1} Ascorbic acid: 51.8 mg kg^{-1}	From *Melipona subnitida* Moisture: 27.2 % Brix: 72.1 Apparent sucrose: 1.74% Total phenols: 202 mg kg^{-1} Total flavonoids: 121.8 mg kg^{-1} Ascorbic acid: 75 mg kg^{-1}	da S. Sant'ana et al., 2020

	Acetic acid: 536 mg kg $^{-1}$ Maleic acid: 0.6 mg kg $^{-1}$ Succinic acid: 0.2 mg kg $^{-1}$ Fumaric acid: 0.6 mg kg $^{-1}$ Tartaric acid: 745 mg kg $^{-1}$	Acetic acid: 3584 mg kg $^{-1}$ Maleic acid: 0.0 mg kg $^{-1}$ Succinic acid: 0.45 mg kg $^{-1}$ Fumaric acid: 0.8 mg kg $^{-1}$ Tartaric acid: 9865 mg kg $^{-1}$	
United States	Buckwheat honey Antioxidant activity: 1.01 µmol TE/g (ORAC), 78.1 ED_{50} 10 min mg eq./ml (DPPH) Total phenolic content: 0.32 GAE mg/g	Wild plants honey Antioxidant activity: 0.39 µmol TE/g (ORAC), 89.5 ED_{50} 10 min mg eq./ml (DPPH) Total phenolic content: 0.17 GAE mg/g	Corey et al., 2022
United States	Water content:16.4-18.2% HMF: 5.5-12.1 mg/kg Total phenolic content: 81.6-105.7 mg GAE/100 g		Nyarko et al., 2023
Iraq	Sucrose: 2.2-2.9% Moisture: 13.53-16.07 g/100 g Melanoidin: 0.25-0.44 Antioxidant activity: 14.26-22.15 mg AAE/g, 7.87-95.62 IC50 mg/mL (DPPH) Total phenol: 55.33-120.33 mg GAE/100 g Coumaric acid: 0.00-2.34 µg/mL Catechin: 0.00-2.68 µg/mL Quercetin: 0.00-0.30 µg/mL		Hameed et al., 2024
Egypt	Sidr honey Moisture: 19.03 % HMF: 11.33 mg/kg Glucose: 26.62 g/100 g Fructose: 35.28 g/100 g Sucrose: 8.87 g/100 g Maltose: 8.13 g/100 g		El-Wahed et al., 2023
Saudi Arabia	Sidr honey Moisture: 18.03 % HMF: 20.92 mg/kg Glucose: 22.51 g/100 g Fructose: 40.33 g/100 g Sucrose: 8.94 g/100 g Maltose: 8.22 g/100 g		El-Wahed et al., 2023
Saudi Arabia	Moisture: 14.9 % HMF: 3.8 mg/kg Glucose: 31.5 % Fructose: 39.7 % Sucrose: 2.8 %		Raweh et al., 2023
Royal jelly			
Romania	Water content: 64.75-65.56 % Protein: 36.13-41.75 mg/g Ash: 0.93-1.46 % Na: 182.67-187.53 mg, Ca: 200.79-382.12 mg, K: 1182.42 – 1659.43 mg, Mg: 365-77-423.32 mg, P: 1125.75 -1648.38 mg Fe: 17.87-19.43 mg, Mn: 4.20-5.32 mg, Cu: 6.26-7.12 mg, Zn: 19.71-21.95 mg, Cr: 1.03-2.95 mg Cd: 0.70-1.51 mg		Moraru et al., 2024
Poland	Water content: 68.4 % Total protein: 14.12 % Antioxidant activity: 10.52 % (DPPH), 0.19 µmol TE/100 g (FRAP) Total phenolic content: 189.72 mg GAE/100 g Total flavonoids content: 11.3 mg/100 g		Sidor et al., 2021
Lithuania	Brix: 13.9 Total phenolic content: 20.8 mg RUE/10 g Total flavonoid content: 14.2 mg RUE/10 g Radical scavenging activity: 6.7 mg RUE/10 g		Adaškeviciut et al., 2019

2. PROCESSING OF BEE PRODUCTS

2.1. Bee honey

Bee honey at the time of extraction contains pollen, beeswax, bee remains, practically many other unwanted materials. These impurities must be removed, for a better quality of honey and for a longer shelf life. A major problem for tropical countries is that honey can ferment. For this to happen, different conditions of temperature and time are needed to inactivation of yeasts and moulds, these being the only microorganisms that develop in honey (Kebede et al., 2024). The pasteurization of honey can be done by heating it for a few seconds at a temperature of 70-75 °C to eliminate the yeast, but then it is rapidly cooled, to not affect the quality of the honey (Eshete Y. & Eshete T., 2019).

2.2. Bee wax

Bee wax is a semi-solid product from inside the hive, mixed with honey and resins. In order for this to be a product utilized to its maximum capacity, it must go through different treatments. The classic method consists in soaking the wax for 24 hours in clean water, so that the honey and resins are removed. Then it is melted in boiling water, after which it is strained and squeezed by hand. Thus, the freshly obtained wax is clean and of superior quality.

2.3. Bee propolis

Bee propolis is found in two forms, in the form of an aqueous extract and in the form of an ethanolic extract. Most uses of propolis are found in the form of ethanolic extract, where ethanol of at least 70% is used to extract propolis (Kebede et al., 2024), and the method used to concentrate the propolis extract is done under reduced pressure. The duration of extraction varies from 10 days and can reach up to 30 days, because the propolis weight/extract volume ratio is usually made. There are also extracts in the form of powder, where the raw material after harvesting is crushed, sieved to keep pure propolis and then extracted using different solvents (water, glycol, ethyl alcohol or ethanol) (Galeotti et al., 2018).

2.4. Bee pollen

Bee pollen is most often found in the form of aqueous, lipid and freeze-dried extracts. Different ingredients are used for these extracts, especially for the extracts used in the cosmetic industry (water, oil, glycerine, propylene glycol) (Kebede et al., 2024). Pollen grains can also be used as such in the cosmetic industry, after which have previously been dehydrated. This beehive product is less used as such in the food industry, due to its low digestibility as well as the allergenic nature it can have (Yin et al., 2022). Being a product with a potential maximally as a functional food, the use of inoculated fermentation was considered.

The latest researches emphasize that this fermentation destroys the cell wall thus increasing digestibility and the accumulation of nutrients and active compounds found in pollen (Di Cagno et al., 2019). Thanks to these improvement research, fermented pollen is used in various food applications (Cheng et al., 2024). Pollen is found in various fermented food products (wine, yogurt, cheese), both for its unique flavour and for its properties. Spontaneous pollen fermentation is carried out with the help of bacteria (*Pseudomonas* spp. *and Lactobacilli* spp.) and yeasts (*Saccharomyces* spp.) which are present in bee saliva. This naturally fermented product inside the hive is called bee pasture. Regarding the laboratory fermentation of bee pollen, this fermentation is carried out with microorganisms (*Lactobacillus rhamnosus*, *Lactobacillus kunkeei*, and *Hanseniaspora uvarum*) to obtain artificial bee bread. The fermentation process improves the nutritional properties and bioavailability of pollen through a number of mechanisms. First, fermentation facilitates the breakdown of complex molecules such as proteins, polysaccharides and lipids, transforming them into smaller and more digestible forms, which makes them more accessible to the body (Aylanc et al., 2023).

Second, the presence of microbial enzymes during fermentation improves the activity of digestive enzymes, contributing to the process of digestion and absorption of nutrients (Rul et al., 2022). Third, the probiotics present in fermented pollen support gut health and optimize digestive function, thus supporting nutrient absorption and improving their bioaccessibility (Yan et al., 2021). These combined effects contribute to improving the digestion process and nutrient bioavailability in fermented pollen.

2.5. Bee bread

Bee bread is quite difficult to extract from inside the hive. This separation of the bread from the bees inside the hive is done in four stages. The first stage is the drying of the bee bread, for 8-10 hours at 40°C. The second stage is the maceration of the pasture. After drying, the bee bread is cooled and then ground, up to 4.9-5 mm (Kebede et al., 2024). The third stage represents the filtering of the matter, and for this stage a special machine with an air flow is used, so that all the wax particles disappear. And the fourth stage represents the disinfection of bee bread, with the help of methylene and ethylene oxide, under the action of gamma rays.

2.6. Bee venom

Regarding the processing of bee venom for its use in the cosmetic industry, there are some essential steps, starting from the extraction of the venom with a venom collector, then purified. The clean product is diluted with water, centrifuged, lyophilized and refrigerated before use (Kebede et al., 2024).

CONCLUSIONS

As a general conclusion regarding bee products, they can be divided into two categories, bee collection products (honey, propolis, pollen, pasture) and products made with the help of bee's secretions (wax, royal jelly, venom). Beehive products are essential components in the manufacture of medicinal products, cosmetic products, or as functional foods. Each of these products mentioned in the article is differentiated by the presence of different active chemical substances, therefore extraction, isolation and purification techniques are necessary for almost all beehive products.

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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY EVALUATION OF OLIVE MILL POMACE EXTRACT

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Abstract

Olive oil production generates different by-products such as olive mill wastewater and olive pomace OP, considered low-cost sources of bioactive compounds including polyphenols that show remarkable antioxidant properties. The objective of this work study was to conduct an assessment of the total polyphenol content TPC and antioxidant activity of different OP extracts recovered through liquid-liquid solvent extraction. OP samples were obtained from both two and three-phase extraction processes of olive oil production. Several solvents (water, methanol, ethanol, and n-hexane) have been used to extract phenolic compounds with ultrasound-assisted techniques (UAE). Folin-Ciocalteu assay was used to determine the TPC in olive oil pomace samples. The total antioxidant activity of phenolic extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and phosphomolybdate assay. The highest extraction efficiency of phenolic compounds was obtained using a combination of ethanol:water (80:20). Three-phase decanter system showed higher values of TPC (0.7-0.8 g gallic acid equivalent/L extract), antioxidant activity (80-105% antiradical activity), and total antioxidant capacity (90-109% TAC) compared with the two-phase. Based on the results, OP has antioxidant qualities and may be useful in food formulation.

Key words*: total phenolic content, antioxidant activity, olive pomace, food formulation.*

INTRODUCTION

Olive oil production is an essential global agro-industry, especially in Mediterranean countries, and is considered a key element associated with several health-beneficial effects (Takaç 2009; Alkhalidi 2023).

One of the most iconic and representative ingredients in the Mediterranean diet are the oils and fats of *Olea europaea* L. as a good source of unsaturated fatty acids (around 72% monounsaturated fatty acids primarily oleic acids, and 14% polyunsaturated fatty acids (Barjol 2013; Wani et al., 2018;).

Various studies have reported that olive oil consumption is associated with several health benefits, including reduction of risk factors of coronary heart disease and prevention of several chronic diseases (such as atherosclerosis), cancer, chronic inflammation, strokes, and other degenerative diseases (Skaltsounis et al., 2015; Pavez et al., 2019).

These beneficial health effects have been attributed to the bioactive and phenolic compounds which are considered major contributors to their antioxidant and health-promoting effects by analytical and technical approaches of modern medicine as well as food science and chemistry (Zhao et al., 2022).

Olive oil production involves several mechanical extraction processes, such as i) press olive oil extraction, ii) three-phase centrifugal olive oil extraction, and; iii) two-phase centrifugal olive oil extraction (Ochando-Pulidon et al., 2020).

The olive oil industry generates a large amount of by-product, solid wastes, and liquid effluents such as olive pomace (OP), olive mill waste (OMW), and olive mill wastewater (OMWW) depending on the extraction techniques used for olive oil manufacture (Del Mar Contreras et al. 2020; Abbattista et al., 2021). Industrial olive processing byproducts and wastes have been recognized as one of the most critical problems in terms of environmental pollution due to their high content of organic matter and phenolic compounds (Roig et al., 2006). Discharges of processing biomass, particularly liquid effluent,

cause toxicity, contamination, pollution, and economic damage to the respective manufacturers (Safarzadeh Markhali, 2021). They cause diverse environmental impacts in terms of resource depletion, land degradation, air emissions, and waste generation (Pampuri et al., 2021). However, they also contain valuable nutritional substances (Quero et al., 2022). Nowadays these discharges are valorized by several pharmaceutical and food industries, mainly due to their high phenolic content (Rathi et al., 2018).

Olive pomace (OP) produced from a two-phase centrifugation system contains around 65% up to 70% moisture while the pomace generated from a three-phase decanter contains a lower amount of moisture quantity (45%). (Skaltsounis et al., 2015).

This by-product contains mainly water, seed, and pulp and is considered harmful to the environment, due to the phytotoxic and antimicrobial properties, low pH, relatively high salinity and organic load, and the phenolic and lipid constituents (Piotrowska et al., 2011).

Olive pomace as the main residue derived from olive oil extraction, presents a typical composition of lignocellulosic biomass which contains lignin (30.0–41.6%), cell wall polysaccharides (35.3–49.0%) as cellulose, pectic polymers and hemicellulose (xylans, glucoroxylans, xyloglucans and, manans), oil (7.5–14%) and minerals (4.4–6%) (Rodrigues et al., 2015; Miranda et al., 2019;).

Olive pomace is also a valuable food by-product containing natural phenols and polyphenols with health benefits related to their antioxidant activities (Zhao et al., 2022).

It contains large amounts of bioactive compounds with a wide range of physiological properties, such as anti-atherogenic, anti-allergenic, anti-cancer, anti-inflammatory, antioxidant, anti-microbial, anti-thrombotic, cardioprotective, and vasodilatory effects (Lozano-Sánchez et al., 2014).

Olive pomace is an excellent source of high-added value compounds such phenolic compounds, carbohydrates, and proteins (Bermúdez-Oria et al., 2019). It is characterized by containing squalene and has been reported as the source of several phytochemicals such as tocopherols, peptides, and quercitin (Rodrigues et al., 2015).

OP and OMWW could be considered also a low-cost and renewable natural source of antioxidants due to its high content of phenolic compounds with potential influence on human health, such as hydroxytyrosol, tyrosol, and luteolin (Caporaso et al., 2018; Speroni, 2020).

Since there are many benefits that phenols of OP bring, several authors have reported its use as a raw material for obtaining multiple valuable compounds with health-promoting properties (Niknam et al., 2021). Thus, thanks to its low cost and large availability, several extraction techniques for recovery of olive pomace antioxidant components have been developed to re-valorize the olive oil by-product and minimize the environmental impact associated with its disposal (Cepo et al., 2018; Gulon et al., 2020;).

In Albania, olive oil production is one of the major agricultural sector, with 15.500 tons produced in the 2022 crop year according to the International Olive Council (IOC) (Dawson et al., 2022).

The main goal of the current study is to evaluate the total phenolic content (TPC) and antioxidant activity of different OP extracts recovered through liquid-liquid solvent extraction. To this aim, it is necessary to consider the extraction techniques and solvents used to recover antioxidant compounds of OP samples obtained from both two and three-phase decanter of olive oil production systems.

Samples of OP1 and OP3 were obtained by a 3-phase centrifugal extraction process, while the samples of OP2 were obtained from the processing of olives using a Pieralisi Leopard Decanter with multi-phase decanter technology. Samples of OP1 were obtained by the production of extra virgin olive oil from the processing of organic Kalinjoti cultivar using a three-phase extraction system. According to genetic studies, Kalinjoti is recognized as the

MATERIALS AND METHODS

Sample collection

The olive pomace was obtained directly from three olive oil industries located in the southern and central parts of Albania. Olive pomace samples labeled with a specific code (OP1, OP2, and OP3) were collected during the crop year 2022-2023.

most common native cultivar growing mostly in the Vlora region of Southern Albania (Thomaj and Panajoti, 2003).
Samples of OP2 and OP3 were collected from the processing of various olive varieties after the centrifugation step of two and three-phase extraction systems, respectively.
Samples of OP were dried at 45°C to 50°C for 48h in a tray dryer to prevent the degradation of phenolic compounds (Pikuli et al., 2023). To obtain smaller particle sizes (diameter 1 mm), dried OP samples were milled for 7 s, with a flour mill.
The moisture content of olive pomace samples was determined gravimetrically by drying 10 g of olive pomace for 48h till constant weight in a force air drying oven LBX OVF series, at 105±10ºC (Moya et al., 2020).

Extraction of phenolic compounds
The recovery of phenolic compounds from olive pomace has been performed by solid-liquid extraction methods using different solvents, including water, methanol, ethanol, and hexane (Gullon 2020).
Pikuli et al. (2023) optimized the extraction of phenolic compounds from olive pomace using ethanol:water (80:20) at temperature of 30-40ºC and an extraction times of 60 to 180 min. Ultrasound-assisted solid-liquid extraction of bioactive compounds from OP was performed at 30ºC with ultrasound pulses every 5 s using an ultrasonic bath, model Cole-Parmer 8893 (47 kHz, 230 W). UAE was carried out using 10 g of OP and 50 mL of each extraction solvent (water, methanol:water (80:20), ethanol:water (50:50), ethanol:water (80:20), and n-hexane) (Pampuri et al., 2021).
The supernatants of phenolic compounds were filtered using 0.45 µm Millipore syringe filters and stored at - 20ºC for further analysis.

Evaluation of the Total Phenolic Content (TPC)
The total phenolic content was carried out using the Folin-Ciocalteu method with some modifications described previously by Spinelli et al. (2015). Total phenolic compounds were quantified by a calibration curve previously built (0-5 mg GA/mL; R^2 = 0.9997; y = 2.6276x - 0.0168) using a standard solution of gallic acid (Sigma-Aldrich), and the total phenolic content was expressed as gram gallic acid equivalents per liter (g GAE/L extract) (Quero et al., 2022). All analytical tests were carried out in triplicate. The experimental procedure is described below. Initially, 20µl of phenolic extract (diluted in water), 2500µl of water, and 400µl of Folin-Ciocalteu phenol were added to each test tube.
The test tubes were vigorously mixed using a vortex, and placed in the dark for 8 minutes. 500 µl of sodium carbonate Na_2CO_3 (7%) was added to each tube and mixed again. All test tubes were placed in a water bath previously preheated to 40°C to react for 30 minutes. After that, the test tubes were removed from the water bath and allowed to reach room temperature. The absorbance of each solution was measured in a UV-vis spectrophotometer at a wavelength of λ=750nm.

Total Antioxidant Activity (TAA) of Phenolic Extract
The total antioxidant activity of phenolic extracts was carried out using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay according to Ballesteros et al. (2014). A calibration curve was previously built using 3mg DPPH dissolved in 100 ml of methanol to prepare the 1, 1-diphenyl-2-picrylhydrazyl solution. The experiment was conducted on microtiter plates. The following information is a brief description of the experimental procedure. The phenolic extract was obtained from the extraction of olive pomace. A quantity of 10mg of phenolic extract was dissolved in 1 ml of water. After that 20 µl of dissolved phenolic extract (dissolved in water), 980 µl of methanol, and 1000 µl of DPPH solution were added to each test tube. The test tubes were mixed in a vortex and were incubated in the dark for 30 minutes. DPPH is a stable radical that appears in purple and absorbs at a wavelength of 517 nm. The absorbance of each test tube's material was measured using a UV-vis spectrophotometer at the same wavelength. Total antioxidant activity was expressed as % RAC (anti-radical activity). To calculate the DPPH scavenging activity of the different fractions, the following formula was used:
Percentage inhibition (%) = [(Abs of control - Abs of the sample) / (Abs of control)] x 100.

Total Antioxidant Capacity (TAC) of Phenolic Extract

Total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by Jan et al. (2013), with some modifications.

Briefly, 20 µl aliquot of OP extracts was added into test tubes. A pre-prepared buffer solution (phosphomolybdate reagents) of 3 ml was added. The tubes were well-mixed and incubated in a pre-heated water bath at 95°C for 90 minutes. After cooling down the samples to room temperature, the absorbance of each mixture was measured in a UV-vis spectrophotometer at a wavelength of λ=695 nm. Ascorbic acid was used as a standard to calculate the total antioxidant capacity expressed as a percentage (%TAC). Total antioxidant capacity was calculated using ascorbic acid as a standard according to the following formula:

%TCA = [(Abs. of control − Abs. of sample) / (Abs. of control)] × 100.

All reagents utilized were of analytical grade and purchased from Sigma Aldrich.

Statistical analysis

All analyses were performed in triplicate, and the results were expressed as mean values ± standard deviation. The statistical analyses were evaluated by one-way ANOVA Tukey's test with a significance level α set at 0.05, using SPSS ver. 22 software.

RESULTS AND DISCUSSIONS

The olive pomace (OP) chemical composition is influenced by the olive cultivar, growing conditions, and the extraction process used (Poti et al., 2022). Samples of OP had a brown dark color and pH values ranged from 4.6 to 5.4. The moisture content of olive pomace samples is displayed in Figure 1.

The moisture content of sample OP2, collected from the two-phase centrifugation process, ranged from 38% to 49%. On the other hand, the olive pomace samples obtained by the three-phase extraction systems (OP1 and OP3) contained a larger amount of water, with moisture levels ranging from 60% to 72%.

The recovery of phenolic compounds from OP has been carried out using various solvents in solid-liquid ultrasound-assisted extraction, including water, methanol:water (80:20), ethanol:water (50:50), ethanol:water (80:20), and n-hexane. The extraction yield was determined by using gravimetric analysis. Briefly 1 mL of each extract was weighed before and after being placed at 100 ± 1°C overnight. Figure 2 displays the amount of extract (in grams) obtained from 10 grams of dry OP (%w/w).

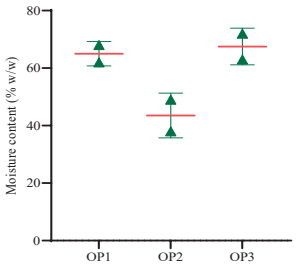

Figure 1. Moisture content of olive pomace samples

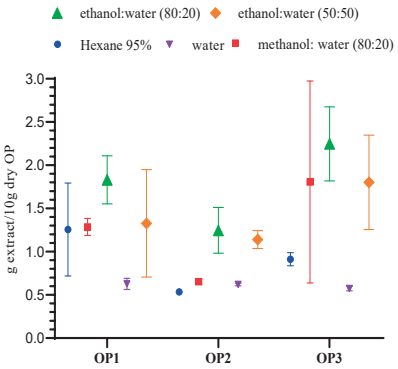

Figure 2. Yield extract of olive pomace provided by ultrasound assisted extraction (UAE)

Data regarding total phenolic content, total antioxidant activity, and capacity of olive pomace samples are presented in Table 1. TPC of OP samples was quantified by Folin-Ciocalteu assay which depends on the reduction of Folin-Ciocalteu reagent by phenolic compounds under alkaline condition.

The highest amount of TPC, TAA, and TAC compounds was recorded in the samples of olive pomace OP1 and OP3, obtained from three-phase production systems. The highest TPC values in olive pomace samples were obtained by using methanol:water (80:20) and

ethanol:water (80:20) as extraction solvents, 0.7505±0.0105 mg GAE/L extract and 0.7832±0.0460 mg GAE/L extract (Tukey's test with $\alpha = 0.05$), respectively.

Studies conducted in Spain (Gómez et al., 2020) and Italy (Aliakbarian et al., 2018) have reported similar range of TPC in olive pomace, obtained through various combinations of water-solvent extraction. Generally, solvents have been found to extract a higher TPC than water and acidified water (Zhao et al., 2022). However, this is the first report in Albania on the extractable TPC range (0.7505-0.7832 mg GAE/L extract) from olive pomace. In this study, the combination of ethanol:water (80:20) was the most common solvent used for phenolic compounds extraction, considered a green, nontoxic, and environmentally friendly medium compared to other organic solvents (Rodriguez-Rojo et al., 2012).

Table 1. Total phenolic content (TPC), total antioxidant activity (TAA), and total antioxidant capacity (TAC) of olive pomace samples

TPC (g GAE/L extract)			
Solvents-UAE	**OP1**	**OP2**	**OP3**
water	0.5521 ± 0.0145	0.2446 ±0.0013	0.5245 ±0.0788
methanol:water (80:20)	0.7505 ±0.0105	0.3341 ±0.0066	0.5113 ±0.0657
ethanol:water (80:20)	**0.7832 ±0.0460**	0.3841 ±0.0250	0.5457 ±0.0039
ethanol:water (50:50)	0.7652 ±0.0512	0.3681 ±0.0560	0.5842 ±0.01707
TAA (%RAC)			
	OP1	**OP2**	**OP3**
water	67.2911 ±5.2556	60.2112 ±1.8586	70.5365 ±2.1262
methanol:water (80:20)	79.1557 ±8.2821	85.6641 ±0.8125	101.6614 ±0.5534
ethanol:water (80:20)	**104.242 ±5.5341**	84.6522 ±4.9167	91.1491 ±7.8173
ethanol:water (50:50)	90.3727 ±2.2457	83.2298 ±1.8750	88.8198 ±2.2998
%TAC			
	OP1	**OP2**	**OP3**
water	67.165 ±3.3524	41.6651 ±5.6148	70.9350 ±1.2689
methanol:water (80:20)	79.3032 ±6.3572	43.7053 ±1.6522	47.6354 ±8.7512
ethanol:water (80:20)	**109.604 ±0.9864**	52.2223 ±2.6422	89.9117 ±4.8113
ethanol:water (50:50)	99.7032 ±3.4680	37.942 1±5.6443	73.7440 ±6.1254

According to the data presented in Table 1, olive pomace samples obtained from the Kalinjoti cultivar (OP1) showed higher levels of TPC, TAA, and TTAC compared with OP samples obtained from other cultivars. Hence, it can be concluded that the OP obtained from the Kalinjoti cultivar is a good source of bioactive compounds and may be useful in food formulation.

The content of bioactive polyphenols in olive pomace is variable depending on numerous factors: olive cultivar, the olive oil extraction process, and the type of pomace that remains as the by-product (traditional extraction, two-phase process or three-phase process, etc.) (Dermeche, 2013).

The influence of the extraction solvent (water, methanol, or ethanol) and the olive oil production processes (2 or 3-phase centrifugation systems) on the yield extract of phenolic compounds, Total Phenolic Content, Total Antioxidant Activity by DPPH (% Anti-Radical Activity), and Total Antioxidant Capacity by phosphomolybdate test (%TAC) of olive pomace samples are shown in Figure 3.

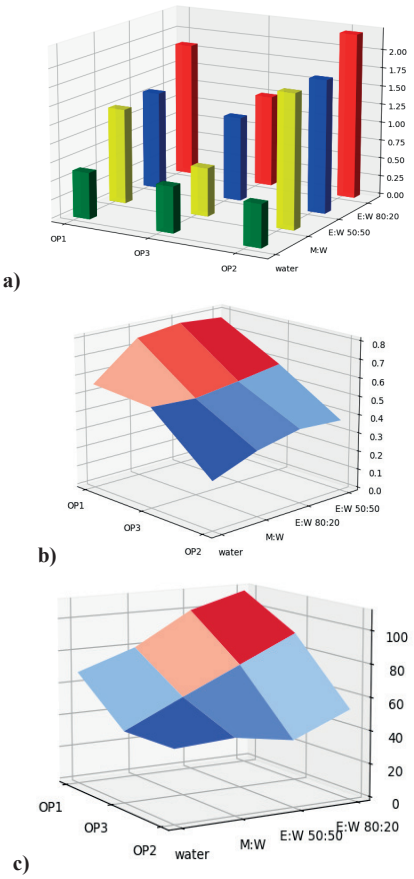

Figure 3. Three-dimensional plots showing the effect of extraction solvent and olive oil production processes on extraction yield (a); on TPC (b); and on TAC (c) of olive pomace

The optimum extraction efficiency of antioxidant compounds was obtained using a combination ethanol:water (80:20) as extraction solvent (Figure 3a). The highest levels of TPC and TAA were obtained from olive pomace samples of 3-phase centrifugation systems using ethanol:water (80:20) (OP1 and OP3) as extraction solvent (Figure 3b,c). The influence of different olive oil production processes (2 and 3-phase extraction systems) on the recovery rate of TPC, TAA, and TAC is presented in Figure 4.

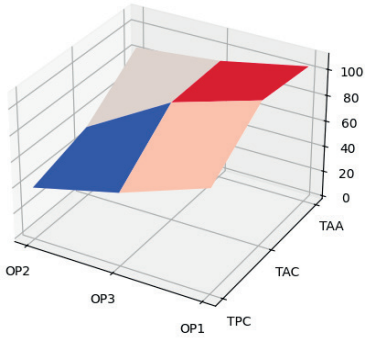

Figure 4. Three-dimensional plots showing the influence of olive oil production processes on the recovery rate of TPC, TAA, and TAC from olive pomace using ethanol:water (80:20) as extraction solvent

The results showed that significantly higher levels of TPC, TAA, and TAC in an aqueous solution of ethanol (80:20) were obtained from olive pomace generated by 3-phase centrifugation processes (OP1 and OP3) in comparison to 2-phase centrifugation systems (OP2).

CONCLUSIONS

In the present study, we focused mainly on the evaluation of the total phenolic content and antioxidant activity of olive pomace samples generated from the manufacturing of olive oil using two and three-phase centrifugation systems. Among different food-grade solvents, ethanol:water (80:20) was selected as the most appropriate solvent for phenolic compounds recovery from OP. The obtained results showed that olive pomace extracts contained a significant amount of phenolic compounds and antioxidant activity. However, they significantly depended on some factors such as olive cultivars, olive oil production processes, and the polarity of the extraction solvent. Satisfactory phenolic and antioxidant yields proved that OP was a low-cost, renewable, and abundant source of bioactive compounds. Simple solvent extraction using food-grade solvents could be successfully applied. The study highlights the usage of olive pomace generated from the olive oil industry as a valuable source of bioactive compounds in food formulations.

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QUALITY EVALUATION OF SOME COMMERCIAL HONEY SAMPLES

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Abstract

Honey is one of the most valuable natural product used for thousands of years in human nutrition. The quality of honey must be controlled analytically with the aim of preserving the consumer from commercial speculation. The values of the physicochemical parameters for ten commercial honey samples attest that all samples appeared to conform to the European Legislation. However, food products such as honey are easily adulterated both in domestic and foreign trade. An alternate analytical technique used to detect adulterations and authenticity of honey implies both FTIR spectrum screening and multivariate analysis of samples. The spectral data of ten honey samples were acquired in the range of 4000–400 cm^{-1}. Out of them, there were two adulterant materials. A spectral range of 1800 to 650 cm^{-1} was selected in order to achieve a satisfactory cluster discrimination. Based on spectral variations, cluster analysis may also be used to categorize and differentiate between pure and contaminated honey.

Key words*: 5-hydroxymethylfurfural, ATR-FTIR spectroscopy, cluster analysis, multivariate analysis, polyphenols.*

INTRODUCTION

Honey is the most important primary product of apiculture, from a qualitative point of view, as well as from an economic perspective. It was also the first apiarian product used by mankind from ancient times. Throughout time, in almost all the cultures there is evidence of honey being used as a food source with therapeutic benefits and as a symbol in magical and religious ceremonies (Eteraf-Oskouei & Najafi, 2013; Al-Ghamdi & Ansari, 2021).

According to the Explanatory Dictionary of the Romanian Language, honey is “a yellowish semi-liquid substance with a sweet taste and a pleasant aroma, very rich in sugars, vitamins, and enzymes, gathered and produced by bees from the nectar of the flowers” (Coteanu et al., 2016). The nectar that the bees suck from the flowers turns into honey through a kind of "kiss" between the bees that participate in the honey maturation process, through which small drops of nectar are transferred from one bee to another, moment when the nectar is enriched with the enzymes that ennoble it, then the excess water is removed. The production of honey by bees begins with the collection of nectar or manna and ends with the filling of the cells of the honeycomb, in which the honey is stored. The requirement to convert complex carbohydrates (sucrose, maltose, etc.) into simple, assimilable sugars (glucose and fructose) so that the bees may completely digest them throughout the winter months gives rise to enzymatic processing. Afterwards, the nectar or manna is diluted with saliva and by reducing the water content, the enzymatic processes are blocked and the storage space is decreased (Giosanu et al., 2022; Vîjan et al., 2023; Albu et al., 2024).

Honey has water, carbohydrates, pollen, minerals, enzymes, vitamins, pigments, aromatic compounds and acids in its composition. Carbohydrates include fructose (38%), glucose (31%), sucrose (1%), maltose and other disaccharides (7%), melezitose (characteristics of manna honey). Minerals in honey, such as: potassium, sodium, calcium, magnesium, chlorine, sulphates, phosphates, silicon, have a low content in flower honey, but they have a high content in manna honey. These minerals are responsible for the extraordinary qualities of honey. Thiamine (vitamin B_1), riboflavin (vitamin B_2), nicotinic acid (vitamin B_3), vitamin K, folic acid (vitamin M), biotin (vitamin H), and pyridoxine are among the vitamins found in honey. Honey contains trace levels of vitamins, amino acids, and proteins due to pollen (5%) in the honey. The enzymes in honey are: glucose oxidase,

catalase, phosphatase, diastase (which transforms starch into dextrins) and invertase (which turns sucrose into fructose and glucose). In flower honey, the enzymes have a double origin: vegetable (the enzymes in the nectar) and animal (the enzymes in the bee's saliva). The composition and therapeutic effects of different types of honey are determined by the various plants visited by bees, respectively by their active principles (Hills et al., 2019; Giosanu et al., 2022; Vîjan et al., 2023; Albu et al., 2024).

The source of the nectar determines the honey's scent, which can range from subtle (acacia) or bitter (chestnut honey) to the distinct perfume of linden, raspberry, etc. The colour of honey can vary from almost colourless (acacia honey) to dark brown (manna honey).

A good honey (16–18% moisture) is viscous at normal temperature (20°C). If the humidity is higher than 21%, the honey flows like water, which means that it was obtained and/or stored inadequately, and it will degrade quickly due to its poor quality.

Honey acidity is maximum 4 mEq/kg for flower honey and 5 mEq/kg for manna honey. Normal pH values for honey are between 3.4 and 6.1.

One of the factors used to confirm the authenticity of honey is electrical conductivity. In flower honey, the electrical conductivity is very different according to the type of honey. The pollen spectrum is the basic criterion in order to be able to correctly assess the type of honey, due to the fact that the morphology of the pollen grains is characteristic for each individual plant species (Pérez et al., 2007; Castro-Vázquez et al., 2008; Bogdanov, 2009; Alvarez-Suarez et al., 2010; Bodó et al., 2021; Giosanu et al., 2022; Vîjan et al., 2023; Albu et al., 2024).

Since the physicochemical parameters of honey types are generally similar, it is difficult to distinguish between different types of honey based on the physicochemical analysis of this product (Giosanu et al., 2022; Vîjan et al., 2023).

The food sector finds FTIR (Fourier transform infrared) spectroscopy to be an alluring technique since it offers quick, easy, and non-destructive evaluations of chemical and physical components. The development of multivariate data analysis techniques and advancements in FTIR instruments make this technology perfect for large-scale, quick screening and for the description of minor food components down to parts per billion (ppb) levels.

In order to verify the quality of the tested honey samples, one analysed ten honey samples as regards several physicochemical parameters, such as: moisture, ash, pH, free acidity (FA), electrical conductivity (EC), total sugar content (TSC), 5-hydroxymethylfurfural (5-HMF), and several biochemical parameters, such as: total polyphenol content (TPC), total flavonoid content (TFC), total tannin content (TTC), and the antioxidant activity. Additionally, multivariate analysis and ATR-FTIR spectroscopy were combined to identify and assess honey adulteration.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals and reagents were acquired from Merck in Darmstadt, Germany.

Honey samples

Ten honey samples, eight from primary honey producers and two commercial honey samples (Greek and Manna honey) were analyzed. The eight samples of honey from primary producers from Romania are: two sunflower (S) from Argeș - Costesti (AG-C) and Argeș - Gliganu (AG-G), three acacia samples (A) from Argeș - Costesti (AG-C), Argeș - Mosoaia (AG-MO) and Argeș - Vedea (AG-V) and three multifloral samples (M) from Tulcea - Casimcea (TL-C), Arges - Mozaceni (AG-MZ) and Giurgiu-Bolintin (GR-B). Greek and Manna honey were purchased from the supermarket.

Physicochemical determinations

By means of a C-561 Consort multimeter, one determined the electrical conductivity (EC) and the pH according to the methodology suggested by Vîjan et al. (2023). The EC results were expressed as microsiemens per centimeters (μS/cm).

Free acidity (FA) was determined by titrating aqueous solutions of honey using 0.1 M sodium hydroxide solution to pH 8.30 according to the

methodology suggested by Vîjan et al. (2023). The FA results were expressed in milliequivalents of acids per kilogram of honey (mEq/kg).
Moisture and ash (mineral content) were determined gravimetrically by oven drying at 105–110°C, and by the calcination of dry residue at 550–600°C, respectively, until the samples were brought to a constant mass. The moisture and ash results were expressed as percent (%).
By colorimetric analysis, according to the methodology suggested by Dubois et al. (1956) one determined the total sugar content (TSC) expressed as g glucose/100 g. Similarly, by colorimetric analysis, according to the methodology suggested by Vîjan et al. (2023), one determined 5-hydroxymethylfurfural (5-HMF) content expressed in milligrams 5-HMF per kg (mg 5-HMF/kg).

Bioactive compounds determinations
By colorimetric analysis and in agreement with the methodology suggested by Vîjan et al. (2023), one determined the total polyphenol content (TPC), the total flavonoid content (TFC), and the total tannin content (TTC). The results were expressed as mg gallic acid equivalent (GAE)/100 g for TPC and TTC, and mg catechin equivalent (CE)/100 g for TFC, respectively.
The antioxidant activity, expressed as a percent of inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH I%), was calculated according to the approach proposed by Vîjan et al. (2023).

UV-Vis and ATR-FTIR analysis
The spectral measurements were made with a UV-Vis Perkin-Elmer Lambda25 spectrometer and an FTIR Jasco 6300 spectrometer. Pike Technologies' ATR attachment with a diamond crystal enables direct sample FTIR spectra collection without any special further processing. The FTIR spectra were recorded in the region of 4000-400 cm^{-1}, detector TGS, and apodization Cosine. JASCO Spectra Manager II was used to process the spectrum data. Between measurements, the ATR crystal was washed with pure acetone and then dried with soft tissue (Topală & Tătaru, 2019; Topală et al., 2020). All measurements were made at room temperature (T= 23°C). For every sample, three replicate spectra were obtained, and the average spectrum was calculated.
Infrared Spectra were exported from Spectra Manager, in ASCII (dx) format, into the Unscrambler Software (Edition X 10.4, Camo Oslo Norway) for chemometric analysis. Spectra were pre-processed using the second-derivative transformation, the Savitzky-Golay derivation (Topală et al., 2020; Vîjan et al., 2023). The principal component analysis (PCA) model was developed using cross-validation. PCA was performed on the entire spectral range (4000 to 400 cm^{-1}), Validation: Cross Validation, Algorithm: Singular Value Decomposition (SDV). The spectral range 1800–650 cm^{-1} was chosen for Hierarchical Cluster Analysis (HCA).

Statistical Analysis
At least three replications' worth of data were presented as mean ± standard deviation.

RESULTS AND DISCUSSIONS

The parameters of honey samples, presented in Tables 1 and 2 (i.e., electrical conductivity (EC), pH, free acidity (FA), moisture, ash, total sugar content (TSC), 5-hydroxymethylfurfural (5-HMF), total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and antioxidant activity (DPPH I%) showed strong oscillations depending on the honey harvest zone.
EC values ranged from 206.83 to 519.17 µS/cm, which suggested that the analyzed samples came from nectar honey (certified with EC values lower than 800 µS/cm, and ash values below 0.6%), according to Directive 2014/63/EU.
The pH values recorded for the analyzed honey samples range within the standard limits (pH 3.40–6.10) that ensure the freshness of the honey.
The free acidity values that ranged from 6.83 to 24.13 mEq/kg (below the allowed maximum of 50 mEq/kg, according to Bogdanov, 2009) attest the freshness of all the samples.
All honey samples presented a safe moisture content for storage and consumption (12.66–18.74%), below the maximum value (20%) established by the Codex Alimentarius (2001) standard.

The 5-HMF content of all the tested samples was below the maximum level of 40 mg/kg required by the International Honey Commission Standard, which attests the fact that no heat treatment had been applied to the honey (Bogdanov, 2009). The honey from Manna and Greece had the greatest 5-HMF levels (33.94 and 36.07 mg/kg, respectively).

Table 1. Honey electrical conductivity (EC), pH, free acidity (FA), moisture, ash, total sugar content (TSC) and 5-hydroxymethylfurfural (5-HMF) based on the geographical origin (GO) of the honey

Honey	GO	EC (µS/cm)	pH	FA (mEq/kg)	Moisture (%)	Ash (%)	TSC (g glucose/100 g)	5-HMF (mg/kg)
S	AG-C	448.67±1.15 a	3.93±0.01 a	15.50±0.19 a	14.92±0.13 a	0.18±0.01 a	59.99±0.03 a	19.08±0.09 a
	AG-G	519.17±1.15 a	3.97±0.01 a	17.83±0.19 a	16.14±0.13 a	0.23±0.01 a	61.65±0.03 a	19.65±0.09 a
	P	*<0.001*	*<0.001*	*0.002*	*0.001*	*0.177*	*<0.001*	*<0.001*
A	AG-C	283.83±1.15 a	4.45±0.01 a	8.83±0.19 a	12.90±0.13 c	0.11±0.01 b	62.55±0.03 b	16.22±0.09 a
	AG-MO	239.00±1.15 a	4.33±0.01 a	6.83±0.19 a	14.32±0.13 b	0.17±0.01 a	68.46±0.03 a	16.17±0.09 a
	AG-V	206.83±1.15 a	4.45±0.01 a	6.83±0.19 a	15.85±0.13 a	0.13±0.01 ab	60.35±0.03 b	15.82±0.09 a
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
M	AG-MZ	385.33±1.15 a	4.12±0.01 b	11.17±0.19 a	13.65±0.13 a	0.16±0.01 a	78.36± 0.03 a	24.97±0.09 a
	GR-B	377.38 ±1.15 a	4.42±0.01 a	9.43±0.19 a	13.22±0.13 a	0.15±0.01 a	62.88± 0.03 b	25.02±0.09 a
	TL-C	363.17±1.15 a	4.55±0.01 a	6.92±0.19 b	12.66±0.13 b	0.12±0.01 a	60.92± 0.03 b	25.92±0.09 a
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.034*	*<0.001*	*<0.001*
Greek honey		437.00±2.28	4.75±0.05	24.13±0.25	14.62±0,28	0.16±0.02	58.05±0.18	33.94±0.49
Manna honey		440.67±1.51	4.56±0.06	23.62±0.12	14.10±0.17	0.17±0.08	59.26±0.07	36.07±0.33

Means with the same letter in each column are not significantly different at a 5% level, according to Duncan`s Multiple Range Test.
P values for GO influence significance were presented according to the One-Way Analysis of Variance (at a significance level of α=.05).
S = sunflower honey, A = acacia honey, M = multifloral honey. AG-C = Arges-Costesti, AG-G = Arges-Gliganu, AG-MO = Arges-Mosoaia, AG-MZ= Arges-Mozaceni, AG-V=Arges-Vedea, GR-B = Giurgiu-Bolintin, TL-C=Tulcea-Casimcea

Table 2. Total phenolic (TPC), flavonoid (TFC) and tannin content (TTC), and antioxidant activity (DPPH I%) depending on the geographical origin (GO) of the honey

Honey	GO	TPC (mg GAE/100 g)	TTC (mg GAE/100 g)	TFC (mg EC/100 g)	DPPH (I%)
S	AG-C	186.71±0.01 a	73.99±0.01 a	17.70±0.05 a	27.71±0.02 a
	AG-G	198.47±0.01 a	84.11±0.01 a	20.30±0.07 a	28.62±0.02 a
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*
A	AG-C	118.69±0.01 a	77.62±0.01 a	12.16±0.05 a	13.36±0.02 c
	AG-MO	81.41±0.01 b	62.13±0.01 a	12.16±0.07 a	19.21±0.02 b
	AG-V	70.45±0.01 b	48.45±0.01 a	11.18±0.07 a	23.13±0.02 a
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*
M	AG-MZ	140.51±0.01 a	50.05±0.01 a	20.05±0.06 a	26.49±0.02 a
	GR-B	134.05±0.01 a	43.65±0.01 a	19.55±0.05 a	25.48±0.02 a
	TL-C	130.15±0.01 b	40.25±0.01 a	14.73±0.04 b	23.41±0.02 a
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Greek honey		102.39±1.05	28.72±1.03	12.93±0.24	18.14±0.71
Manna honey		116.49±0.02	37.41±0.02	12.34±0.32	21.62±0.04

The antioxidant properties of honey are mostly caused by the phenolic compounds produced under abiotic and biotic stress conditions. The results from Table 2 showed that sunflower honey had the highest phenolic, tannin, and flavonoid content, whereas acacia, Greek and Manna honey presented the lowest phenolic, and flavonoid content. Our results showed that all analysed honey samples had corresponding values of the quality indicators, in accordance to the EU legislation and the Codex Alimentarius standard (Codex Alimentarius, 2001; Council Directive 2001/110/EC; Directive 2014/63/EU).

The FTIR Spectroscopy is used in combination with multivariate analysis to detect adulterated honey and assess honey quality. The honey samples spectra were analyzed in the present

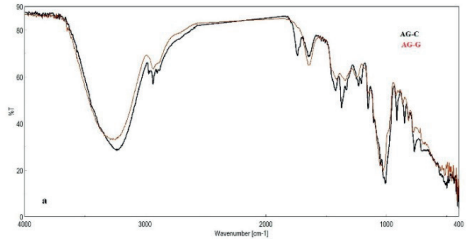
study in two regions: 1800–650 cm^{-1} and 3000–2800 cm^{-1}. According to Sivakesava and Irudayaraj (2001), the ideal spectral range for distinguishing between pure honey and honey contaminated with simple sugars (glucose, fructose, and sucrose) is between 1800 and 750 cm^{-1}. The fingerprint spectral is region of 1500–800 cm^{-1} and is mainly due to the absorptions of monosaccharides and disaccharides present in the honey (Limm et al., 2003; Damto et al., 2023; Vîjan et al., 2023).

The bands at 1474–1199 cm^{-1} are responsible for bending of O–C–H, C–C–H and C–O–H (Tewari & Irudayaraj, 2004). According to Gok et al., 2015, the anomeric region was a peak between 950 and 750 cm^{-1} and was often preferred for the spectral analysis of carbohydrates. The area that stands out in the assessment and description of honey is the band from 890 to 810 cm^{-1} typical for the C-H deformation or vibration anomeric region of carbohydrates (Tul'chinsky et al., 1976; Damto et al., 2023).

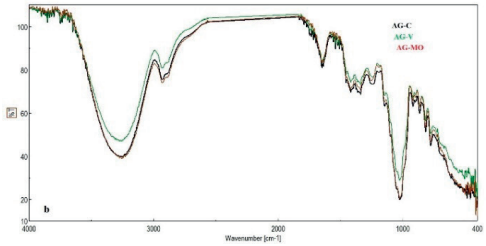
Figure 1 and Table 3 show the ATR-FTIR spectra of the tested honey with major high bands. Generally, one noted some differences in the FTIR spectrum analysis of the honey samples.

Table 3. ATR-FTIR Assignments for honey samples

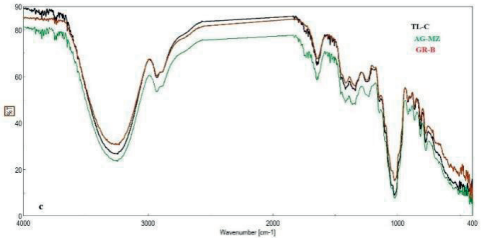
Honey	Sunflower (S)		Acacia (A)			Multifloral (M)			Greek	Manna
Harvest zone	AG-C	AG-G	AG-C	AG-MO	AG-V	TL-C	AG-MZ	GR-B	from trade	
ν(C-H) tretching of carboxylic acids + ν(NH_3) of free aminoacids	2935	2933	2930	2930	2935	2930	2933	2930	2931	2931
CH_3 sym stretch	2881	2887	2888	2892	2892	2883	2879	2883	2884	2883
C=O stretch in unconjugated ketones, carbonyls in ester groups (frequently of carbohydrate origin)	1736	1744	1743	1732	1747	1733	1743	1741		1743
δ(O-H) from H_2O	1646	1644	1646	1646	1646	1646	1646	1645	1645	1645
δ(O-H) in C-OH group + δ(C-H) in the alkenes	1420	1415	1418	1418	1418	1418	1416	1417	1417	1417
Stretching C-O, deformation C-H, deformation N-H	1373	1373	1357	1339	1360	1373	1362	1340	1339	1341
ν(C–H) + ν(C–O) in carbohydrates	1231 1152	1244 1148	1243 1145	1252 1145	1242 1145	1243 1146	1232 1146	1244 1146	1266 1105	1254 1100
ν(C-O) in C-OH group + ν(C-C) in carbohydrates	1046 1008	1049 1028	1051 1024	1048 1024	1049 1025	1046 1024	1048 1024	1044 1024	1046 988	1048 1023
δ(C–H)	915	917	917	917	916	917	916	917	923	918
Ring vibrations (mainly from carbohydrates)	850	862	863	864	863	863	862	863	859	863
-C–H bending (mainly from carbohydrates)	814	816	816	816	815	816	816	816	831	817
Anomeric region of carbohydrates	768	776	776	775	776	775	774	776	767	776



(a)



(b)



(c)

Figure 1. ATR-FTIR spectra of honey samples with different botanical origins: sunflower (a), acacia (b), and multifloral (c)

The spectra of natural honey and commercial honey have similar characteristics and spectral overlaps but differ in the wavelength of the characteristic peaks (Figure 2 and Table 3). However, the Greek honey sample's spectra showed a shift to a longer wavelength and a

broadening of the absorption bands at the range 950–810 cm^{-1}, typical for the C-H deformation or vibration anomeric region of carbohydrates (Figure 2).

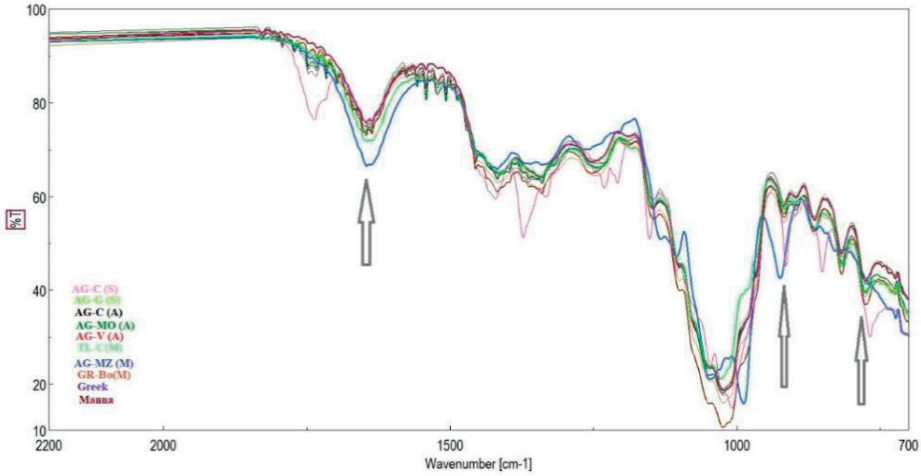
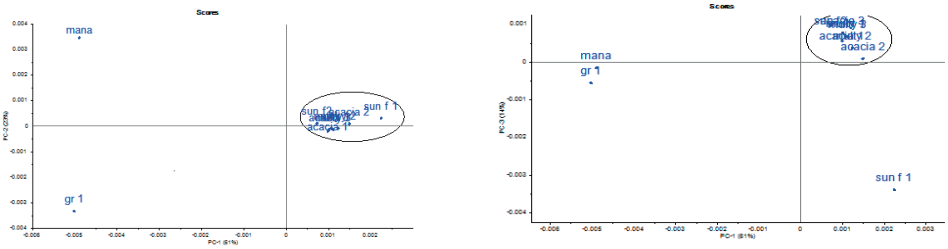


Figure 2. FT-IR spectra of honey samples with the indication of spectral changes for commercial honey (Greek and Manna honey)

From these results it is possible to differentiate two groups of honey, commercial (probably adulterated) and nonadulterated honey. To make these results clearer, PCA was applied to FTIR spectra of all groups, obtaining evident discrimination.

A clear splitting of the data can be observed as depicted in Figure 3 by the first two principal components in the 4000–650 cm^{-1} region. The first three principal components (PCs) for the honey samples under consideration account for 98% of the total variance (PC1=61%, PC2=23%, and PC3=14%). This suggests that the three components were sufficient to provide a good separation between the groups. Commercial honey (Greek and Manna honey) is separated from sunflower, acacia, and multifloral honey obtained from primary honey producers.



(a) (b)

Figure 3. 2-D scores obtained from PCA of FTIR spectra of honey for the first two PCs (a), and PC3 versus PC1 (b)

Hierarchical cluster analysis (HCA) involves a measurement of the similarity between objects about to be clustered and samples with the maximum similarities were clustered preferentially (Yi et al., 2013). Thus, it was possible to separate two groups, adulterated and nonadulterated honey. The spectral area from 1800–650 cm^{-1} was selected for successful discrimination of clusters (Damto et al., 2023). The results obtained are represented in Figure 4 in the form of dendrogram. The clusters determination for the analysed honey samples indicates a clear separation in two distinct categories, a category of commercial and other non-adulterated honey.

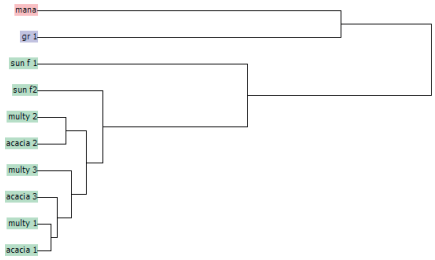


Figure 4. Hierarchical clustering of all samples in the 1800–650 cm^{-1} (fingerprint) spectral region

CONCLUSIONS

The physicochemical parameters values of the ten analyzed honey samples indicate that all samples are conform to the European Legislation. However, the levels of physico-chemical and biochemical parameters of honey samples fluctuated significantly depending on the geographical origin of honey. Thus, sunflower honey was noted for its high content of phenolic compounds, flavonoids and tannins, whereas acacia, Greek and Manna honey presented the lowest phenolic, and flavonoid content.

The adulterated honey was identified by the use of multivariate analysis from FTIR spectrum screening. The Greek and Manna honey samples spectra showed a shift to a longer wavelength and a broadening of the absorption bands as the concentration of sample increases. The bands in the spectral region 1800–650 cm^{-1} were selected for successful discrimination of clusters. Our results suggest that the two commercial honey (Greek and Manna honey) were probably adulterated.

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JUJUBE, NUTRITIOUS FRUIT ON CHINESE TRADITIONAL DIET

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Abstract

Jujube (Ziziphus jujuba), the deciduous tree species belonging to the Rhamnus family, Ziziphus genus, is priced for its sweet and nutritious fruit. Jujube is an important fruit tree in China and the largest dried fruit tree species. Origin from China, the jujube has a cultivation and utilization history of more than 7000 years. Despite act as a common fruit on Chinese diet, jujube also play very important role as herb in Chinese traditional medicine. Furthermore, jujube also act as a culture symbol in traditional Chinese culture with rich meanings. This paper gives a brief review on Chinese jujube cultivation and utilization, and give prospect of the jujube industry.

Key words*: jujube, cultivation, nutrition, production.*

INTRODUCTION

Jujube (*Ziziphus jujub*a), also called Zao in Mandarin Chinese, or Chinese date, red date, is the most important *Ziziphus* species for fruit in the Rhamnaceae family (Gao, Wu, & Wang, 2013). It is a medium-sized tree, that grows up to 7 – 10 meters high and has shiny deciduous foliage. The stone fruit varies in size depending on the cultivar, and has thin, dark red skin surrounding sweet, white flesh. The nutritious jujube fruit is rich for sugar, vitamin, and mineral contents.

Jujube is one of the oldest cultivated fruit trees in the world (M. Liu et al., 2020). It originated in the middle and lower reaches of the Yellow River, in northern China. The cultivated jujube is domesticated from wild jujube (*Ziziphus jujuba* var. *spinosa*), also called sour jujube. History of jujube cultivation and utilization in China could be traced back to more than 7000 years ago. Jujube was introduced to Chinese neighbour countries such as Japan and Korea 2000 years ago. Now days, jujube has spread to more than 40 countries, including the United States, Australia, and European countries such as Romania, Italy, and Spain (Crawford, Shan, & McCarthy, 2011; Stănică, 2019; Yao, 2013).

For its delicious and nutritious fruit, easy management, low cultivation cost and high economic benefits, ecological friendly characteristics as well, jujube meet the need of consumers, growers, marketers, governments and society. Thus, the jujube is considered a super fruit for the future (M. Liu et al., 2020).

This paper presents a brief review on Chinese jujube cultivation and utilization, and give prospect of the jujube industry.

JUJUBE CULTIVATION IN THE WORLD

Jujube cultivation in China

Comparative analysis on chloroplast genomes of 326 jujube, including 133 cultivated, and 193 wild jujube genotypes revealed that initial domestication and cultivation of jujube took place within the middle and lower reaches of the Yellow River. The divergence time of cultivated jujube and wild jujube is 1.82 million years ago (Yang et al., 2024). A large-scale genome resequencing analysis of 672 accessions including 359 cultivated, 291 wild and 22 semi-wild jujube also revealed that wild jujube originated from the lower reaches of Yellow River, and domesticated at the Shanxi-Shannxi Gorge (Y. Li et al., 2024). A carbonized jujube

kernel found in the Peiligang, a Neolithic age historical site, in Xinzheng city, Henan province, China, revealed that jujube utilization and cultivation history could be traced back to 7000 years ago (M. Liu et al., 2020). Morphology analysis on jujube stones and AMS (Accelerator Mass Spectrometry) ^{14}C dating on millet seeds in other three Neolithic sites in Northern China suggest that jujube was cultivated as early as 6 200 years ago (K. Li et al., 2024).

Despite for its long cultivation history, jujube is the most planted dry fruits in China. According to the China Forestry and Grassland Statistical Yearbook 2021, the total yield of dry jujube is 4 404 820 tons, ranked the 1st in all the dry fruits (National Forestry and Grassland Administration, 2022).

The traditional jujube cultivation areas are middle and lower reaches of the Yellow River, which is the Henan, Hebei, Shanxi, Shannxi and Shandong provinces. In the new century, jujube cultivation in China developed rapidly and changed a lot, the cultivation area greatly increased, and the main production area has transferred to Xinjiang, northwest of China. In the year 2005, the total dry jujube yield in these 5 provinces is 2 330 336 tons, more 90% of the total yield (2 463 087 tons) in China. In the year 2020, the total yield in China has increased to 4 404 820 tons, almost doubled than 2005, and the production in Xinjiang has increased to 2 465 811 tons, 55.98% of the total production of whole country. Of the top five provinces, the production in Shanxi and Shannxi still increased, while the Henan, Hebei and Shandong provinces decreased.

The main reason of jujube cultivation area shift is economic factors. In the new century, China has experienced rapid economic development. The urbanizing in east provinces is very fast, while the huge population require sufficient crop field to stabilize food supply. With the expanding of cities, jujube orchards in the suburbs are the first replaced by buildings and shrink quickly. At the same time, the rapid increase in land and labor prices has reduced the profits of jujube cultivation. Meanwhile, in the west province Xinjiang, which has a land area of 1 664 897 km^2, almost four times of California, the land price is very cheap, and the environment are very suitable for high quality jujube production (Xiao, Na, & Rong, 2021).

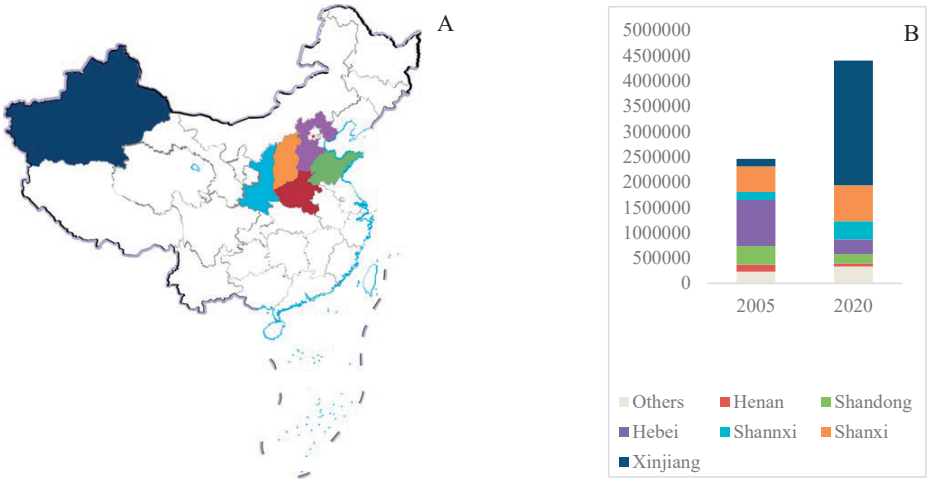

Figure 1. Jujube production in China. A. the main jujube cultivation provinces in China, B. jujube dry fruit yield in China, year 2005 and 2020

China has abundant jujube germplasm resources. According to The Chinese Fruit Tree Records: Jujube, there are more than 700 jujube cultivars recorded in China. The National Jujube Repository, constructed by Pomology Institute of Shanxi Academy of Agricultural Science, in Taigu, Shanxi province has preserved ~1000 jujube genotypes (D. Li, Niu, & Tian, 2013).

The most two cultivated dry jujube cultivars in China are 'Huizao' and 'Junzao'. In Chinese, 'Hui' means grey, illustrating the greyish ripen fruit colour and the dark green tree vigour. 'Jun'

means the best horse, implying this is the best jujube cultivar. The origin of 'Huizao' is Xinzheng, Henan province, while 'Junzao' is from Jiaocheng, Shanxi province. These two cultivars were introduced to Xinjiang in 1990s and performance well.

The most cultivated fresh jujube cultivar in China is 'Dongzao'. In Chinese 'Dong' means winter. Since the fruit ripen time of this cultivar is early October, early winter in Northern China. This cultivar is from Zhanhua, Shandong province, and Huanghua, Hebei province. Both are coastal areas of the Bohai Sea. This cultivar was introduced to many jujube cultivation areas and performance well. The first jujube whole genome sequence is from the 'Dongzao' cultivar (M.-J. Liu et al., 2014).

Jujube cultivation in other Countries

Besides China, Korea, Japan and Iran have the longest jujube cultivation history. The introduction of jujube to Korea and Japan are believed to be more than 2000 years (M. Liu et al., 2020). In Iran, records on palace roof and columns of ruins of Persepolis indicates utilization and cultivation of jujube in Iran could be traced back to 2 600 years ago. In 2021, the jujube cultivation area in Iran is ~5,000 ha, with a production of 7,500 ton. About 33 genotypes can be identified. Two of them, 'Siojan' and 'Majan' were registered as cultivars (Ghouth, 2021).

According to Historia Naturalis, written by Gaius Plinius Secundus (AD 23-79), jujube was introduced to Italy by Octavian Augustus, a Counsellor of the Roman Emperor. Then it spread to other Mediterranean countries. (STĂNICĂ, 2019).

In northern Italy, a small town Arqua Petraca, jujube tree is cultivated almost every garden. Local people use jujube fruits for liquor, jam, snacks, and celebrate jujube festival in first week of October. Researchers introduced and tested jujube cultivars suitable to be grown in Italy and get promising results. Two fresh-eating, without thorns cultivars, 'Dongzao' and 'Meimizao', with big tasty fruit are proposed for intensive planting (Cossio & Bassi, 2011).

In Romania, most ancient jujube populations exist in semi-spontaneous status in Dobrogea area, located between the Danube River and the Black Sea. This site is close to the antique Greek, Roman and Byzantine colonist ruins. Implies jujube may be introduced from the Mediterranean basin (Stănică, 2019). The University of Agronomic Science and Veterinary Medicine of Bucharest (USAMV) had carried out complex jujube research and extension for more than twenty years. The China-Romania Joint Jujube Key Research Laboratory has been established with the collaboration of USAMV with Chinese Hebei Agricultural University. Study on jujube germplasm collection, introduction, elevation and food production development have been performed systematically.

Jujube was first brought to the United States in 1837 by Robert Chisholm, and plant in Beaufort, North Carolina. In 1876, G. P. Rixford introduce jujube from southern France to Sonoma Valley, California. In 1908, the USDA Agricultural Explorer Frank N. Meyer imported first group of jujube cultivars from China to the U.S., and planted them at the USDA Plant Introduction Station at Chico, CA. Now days, jujube are widely planted across the U.S., primarily as a dooryard fruit tree. Few small-hectarage commercial planting primarily serve ethnic niche market in California. The New Mexico State University carry out complex jujube research and extension programs (Yao, 2013). A recent study on genetic diversity of jujube cultivars in the United States with single nucleotide polymorphism (SNP) markers identified 23 synonymous cultivar groups (Sapkota et al., 2024).

In Australia, the jujube was introduced from the US by Gidgegannup grower Jim Dawson in 1994 (Crawford et al., 2011). Jujube are mainly grown in the Perth Hills, the northern Rangelands, the South West and Great Southern regions of Western Australia. The counter-seasonal production to the northern hemisphere provides an opportunity for Australia jujube for the increasing demand of target markets including China, Singapore, and Malaysia, especially during the lunar new year festivals.

Despite for some genotypes introduced abroad China long time ago, the most cultivated jujube cultivars in the foreign countries outside China is Li, Lang, Sugarcane, Honey jar, and so on.

JUJUBE FRUIT UTILIZATION

Jujube is a multi-purpose plant. The jujube tree produces strong wood, yield honey, in addition to delicious fruit. The present research paper is an in-depth review in the utilization of jujube fruit.

Use as food

When directly consumed as fruit, the jujube can be eaten both fresh or dried (dehydrated). Basically, the jujube varieties could be divided as fresh or dry. Most of the dry jujube varieties (cultivars) could be eat directly as fresh jujube when they are fully ripened and not dehydrated, perhaps not so crisp as those fresh varieties (cultivars). For the fresh varieties, when they are dehydrated, there won't be any dry materials left (Qu et al., 1993).

The fresh jujube fruits have high sugar content, vitamins, edible cellulose, minerals, cyclic adenosine monophosphate, and cyclic guanosine monophosphate. Fresh jujubes are known for their high vitamin C content, mostly ranging from 300-600 milligrams per 100 grams and even higher, surpassing kiwifruit, which is known for its high vitamin C (Gao et al., 2013). Most western people think the taste of fresh jujube is similar to apple when they first try it.

Traditionally the jujube fruit will be picked after fully ripen and dried by air, sun or heat. Now days in Xinjiang, since the dry climate, the fruits are dehydrated on the tree before pick. The skin of dry jujube is fully red, that's the name 'red jujube' comes.

The dry jujube, or red jujube, could be eat directly, but for most times, jujube will be cooked together with other foods. In China, rice and wheat flour are people's stable foods, and red jujube are usually used as adjuncts for them. In the past times, only on big days such as festivals, weddings or funerals, people could eat jujube. Now days, jujube has become common food on the diet.

The most usual use is to boil them with rice and beans as soup dish. According to Chinese lunar calendar, on the eighth day of the last month, housewives will clean the kitchen, use all kinds of grains, nut, and fruits in the cabinet to cook Laba Congee, one kind of special soup. From then on, the kitchen will be prepared to celebrate the spring festival, the most important festival in China and east Asia countries. Jujube is the necessary component of Laba Congee, which means jujube is also a daily fruit in the kitchen.

Other than the spring festival, another two most important festival on Chinese lunar calendar is the Dragon Boat Festival, on fifth day of the fifth month, and the Mid-Autumn Festival, on the fifteenth day of the eighth month. On the Dragon Boat Festival, people will eat Zongzi, cooked rice in bamboo leaves. On the Mid-Autumn Festival, mooncake is necessary for celebration. Jujube is also an important component in zongzi or mooncake.

Another traditional food in feast banquets is Babaofan, type of steamed rice, with jujube, and other six kinds of beans or nuts. The name Babaofan implies 'eight treasures rice' in Chinese.

Mantou, type of white coloured steamed bread by yeasty wheat flour, is the daily food in northern China. On festival days, jujube will be decorated on top of Mantou for good looking.

Smashed together with beans, then make vegetarian fillings of Baozi, type of Chinese buns, is another common way people use red jujube.

The jujube fruits could also be processed as preserved fruits, jams, candies, vinegar, liquors, beverages, and so on. Since dehydrated, the red jujube could be stored under normal temperature for long times.

Use as tradition medicine

Jujube has been commonly consumed in traditional Chinese medicine for thousands of years. The classic ancient Chinese medical text Huangdi Neijing (475 – 221 BC), recorded jujube as one of extremely valuable fruits. In Shennong Bencao Jing (300 BC – 200 AD), jujube was regarded as one of the top-grade medicinal herbs, that could extend one's life expectancy by nourishing blood, increasing sleep quality and improving digestive system. Many other medical books reported use of jujube as herb in tradition Chinese medicine (Shahrajabian, Khoshkharam, Zandi, Sun, & Cheng, 2019).

In China, people also believe the concept 'You are what you eat'. Since the jujube skin colour is red, same colour with blood, people believe it is good for blood, especially for women. Clinical practice and pharmacological research have confirmed that jujube has potential hemato-poietic functions, effective to blood deficiency

treatment. Flavonoid, polysaccharide and triterpenoid within red jujube could serve as the potential active ingredients accounting for the aforementioned health benefits.

Other than blood deficiency, jujube also has other health benefits such as anti-inflammatory, anti-cancer, gastrointestinal protective, anti-oxidant, anti-insomnia, neuroprotective, and so on (Table 1).

In many classic traditional Chinese medicine prescriptions, jujube is an important component and play essential role (Chen et al., 2017).

Table 1. Pharmacological Properties of Jujube Fruit

No.	pharmacological property	Subject	Observation	References
1	Anticancer activity	MCF-7 and SKBR3 breast cancer cells	ZE1, ZE2, and ZE4 exerted significant antiproliferative effects on estrogen receptor MCF-7 (IC50 values of 14.42, 7.64, 1.69 μg/mL) and SKBR3 (IC50 values of14.06, 6.21, 3.70 μg/mL)	(Plastina et al., 2012)
		melanoma cells	50% inhibitory concentration of DPP at 3.99 mg/mL after 24 h of treatment, decreased significantly to 3.36 mg/mL after 48 h	(Hung, Hsu, Chang, & Chen, 2012)
		Colitis-associated colon carcinogenesis in AOM/DSS-treated	Dietary jujube increased colon length and suppressed the activation ofNF-κB/IL-6/ JAK1/STAT3 signaling pathway.	(Periasamy, Wu, Chien, Liu, & Liu, 2020)
2	Anti-inflammatory activity	male ICR mice (20−22 g);	inhibitory effects on the inflammatory cells activated by Euphorbia kansui and prostratin, a phorbol ester isolated from Euphorbia fischeriana fraction F to be the most active part	(Yu et al., 2012)
		Wistar albino rats (180−240 g)	significantly attenuated the effect of carrageenan in rat paw and extended to 2 and 3 h at 100, 200, and 400 mg/kg; inhibited granuloma formation at 200 and 400 mg/kg; markedly decreased serum nitrite/nitrate at 200 and 400 mg/kg	(Goyal, Sharma, & Singh, 2011)
3	Antiobesity activity	mouse embryo 3T3-L1 cells	CHCl3-F efficiently suppresses adipogenesis in 3T3-L1 preadipocytes	(Kubota et al., 2009)
4	Immunostimulating activity	3-month-old Kunming mice (males and females,20 ± 2g)	CZSP dramatically increased thymus and spleen indices, and peritoneal macrophages CZSP, ZSP3, ZSP3c, ZSP4, and ZSP4b induced the proliferation of spleen lymphocyte	(J. Li, Shan, Liu, Fan, & Ai, 2011)
5	Antioxidant activity	DPPH, superoxide anion, hydroxyl radical-scavenging activity, and reducing power	ZSP3c and ZSP4b containing more uronic acid had the stronger free radical scavenging activities	(J. Li, Liu, Fan, Ai, & Shan, 2011)
		DNA damage protective activity, DPPH, FRAP, reducing power, inhibition of lipid peroxidation, bleaching ability of β-carotene in linoleic acid system,	jujube fruits from Ningxia, Gansu, and Shaanbei grown in the semiarid regions ofloess plateaus showed fairly higher antioxidant activities	(Sun, Liang, Shan, Viernstein, & Unger, 2011)
		Fruit flies	Jujube fruit powder supplementation increased flies' ability to resist starvation stress and ROS stress.	(Ghimire & Kim, 2017)
6	Hepatoprotective activity	Kunming male mice (18-22 g)	reduced activities of CCl4-elevated ALT, AST, and LDH in serum, and hepatic MDAlevel at 400 mg/kg; better profile of HI, normal GSH-Px, and SOD activities in liver	(D. Wang et al., 2012)
		male ICR mice (25-28 g)	decreased ALT and AST, attenuated histopathology of hepatic injury, and ameliorated the oxidative stress in hepatic tissue at 200 mg/kg	(Shen et al., 2009)
7	Gastrointestinal protective activity	32 rabbits	reduced intestine MDA level and increased antioxidant enzyme activities in rabbits with ischemia/reperfusion (I/R) of the small intestine	(B. Wang, 2011)
8	Anti-hyperglycemic activity	Mice fed 60% high-fat and 10% fructose diet	Dried jujube and chokeberry reduced the HFFD mice body weight, attenuated blood glucose and triglyceride concentrations.	(Jeong & Kim, 2019)
		Rat L6 myotube	These polycyclic triterpenoids induced glucose uptake in a glucose transporter-4-dependent manner, and finally promoted glucose uptake in rat L6 myotubes.	(Kawabata et al., 2017)

One important concept in traditional Chinese medicine is 'to treat disease before they occur'. To keep health by dietary supplementation is one way to achieve this concept.

Jujube has both functions as food or as medicine, it can prevent and treat disease by strengthening peoples' body through daily diet.

CONCLUSION AND FUTURE DIRECTION

Jujubes are considered the "super fruit" of the future. Jujube fruit is a dietary supplement with high contents of bioactive compounds such as dietary fibers, mineral, and natural antioxidant

compounds. Nevertheless, fresh jujube has a short shelf-life. Thus, converting fresh jujube to processed products is the best way for preserving it for a long-time.

Based on available studies, jujube pro-ducts can possess good antioxidant, antiobesity, and antitumor activity. As a result, these products can be consumed as functional food.

As a perennial tree species, the jujube cultivation could last many years once it is established. The tolerance to drought, salt, and deficient soil, compose the adaptability to many environmental conditions, especially in arid and semi-arid areas where other crop species could not achieve satisfactory yield. The jujube could provide significant nutrition source and solution of food supply in these areas. Especially under the context of climate change and global warming.

Deep processing could increase the economic benefits and market of jujube. The nutritional and pharmacological benefits of jujube have been recognized by more and more people. For the jujube industry, the current issue is to develop more deep processed products, and fill the 'culture gap' to make more people accept jujube. Jujube fruit is suitable for processing, could be easily processed to many kinds of food products. Yet to expand the jujube industry, we need to develop more jujube products, especially leisure foods such as snacks, chips, pops, strips, and so on. These products better meet the consumption needs of the young generation. Therefore, the comprehensive and multi-level development of deep processed red dates and health products with specific functions, in order to effectively utilize red dates and fully play their role in food and medical health, is a serious challenge. Deep processing of red dates will gradually showcase their obvious product advantages and broad market prospects, accelerating the healthy development of the red date industry.

There are some cultural influences accompany with jujube consumption habits. For example, the Chinese name of jujube, Zao, is the same pronounce with the character 'early'. This good meaning drives Chinese people to plant jujube trees in yards, eat jujube on daily diets, to encourage people more diligent and achieve success early. This custom is quite common in East Asia countries. In traditional Chinese culture, newlyweds are given the blessing of having a precious son at an early age. Therefore, on the day of marriage, people will place jujube, peanuts, longans, and lotus seeds on the new couple's bed, which is the best blessing.

In western countries, there are also some cultural phenomena in jujube consumption. In Arqua Petraca, small town with jujube cultivation in Italy, a local beloved jujube liquor, *brodo di giuggiole,* or jujube broth, give birth to an idiom, *andare in brodo di giuggiole* (to go in jujube broth), meaning to live in a state of bliss.

Yet these cultural influences are not too familiar with the world, especially between the east and western people. It is necessary to fill up these cultural gaps, make jujube familiar to more people, and became components on more diets.

Another obstacle barrier in jujube production development that needs to be overcome is the cultivar assay and selection. Jujube has abundant germplasms, yet the characteristics of each genotype need to be determined by systematic assay. Furthermore, the most adaptable and suitable cultivar for different region with different climate and soil conditions need to be selected. In general, good taste, high and stable yield, and thorn-less cultivar is most well-come in the market. Resistance to pests and diseases are also factors need to be considered.

Fast and simple propagation techniques for cloned seedlings are also need to be developed. Especially propagate seedlings close to the new cultivation areas, to avoid long-distance transport of the seedlings. Local seedlings have stronger adaptability and less risk of disease and pest transmission, more suitable for jujube cultivation development.

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THE POTENTIAL OF *Candida lipolytica* ICCF 214 (ATCC 16618) TO PRODUCE BIOSURFACTANS

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Abstract

Biotechnological processes, particularly those involved in biosurfactant production, can pose significant financial challenges. The viability of biosurfactant production hinges on the creation of processes that make use of cost-effective raw materials. This study focuses on exploring the potential of Candida lipolytica ICCF 214 (ATCC 16618) reference strain for biosurfactant production using a mixture of vegetable oil and other chemical compounds used for fermentation medium (1% glutamic acid, urea, or glucose) as carbon sources. Based on the obtained results, we can conclude that all three fermentation media stimulated Candida lipolytica ICCF 214 strain to produce biosurfactants, but further analysis of surface tension and antimicrobial activity of the obtained biosurfactants is required. Additionally, we observed that media with a volume of 100 ml produced higher quantities of biosurfactants, due to the good aeration of the fermentation medium. However, even the ones with 150 ml showed decent performance during the bioprocess.

Key words: *bioemulsifiers, biosurfactants, microbial fermentation, Candida lipolytica, yeasts.*

INTRODUCTION

Surfactants are molecules with both hydrophilic and hydrophobic properties, enabling them to accumulate at interfaces, reduce interfacial tensions, and form structures like micelles. These attributes make surfactants and emulsifiers essential in various industries including industrial, agricultural, food, cosmetic, and pharmaceutical sectors. Originally sourced from renewable origins like fats and oils, a majority of surfactants nowadays are derived from petrochemicals (Ambaye et al., 2021). Nonetheless, a subset known as biosurfactants is biologically synthesized by yeasts or bacteria, categorized as glycolipids, lipopeptides, fatty acids, polymeric, or particulate compounds. Biosurfactants present advantages over synthetic counterparts due to their biodegradability, lower toxicity, effectiveness in extreme conditions, and environmentally friendliness (Ambaye et al., 2021). Despite these benefits, biosurfactants are not yet economically competitive with synthetics, necessitating cost-effective production strategies to facilitate their widespread adoption. They can be produced from various substrates, predominantly renewable resources like vegetable oils and industrial wastes (Amalesh et al, 2012). Selecting low-cost substrates is essential for the economic feasibility of biosurfactant production. Key factors for successful biosurfactant production encompass the development of cost-effective processes, utilization of low-cost materials, attainment of high yields, and customization of biosurfactants for specific applications.

In the past few years, there has been a surge in interest in isolating and identifying new microbial polysaccharides and surfactants that could potentially be used in enhanced oil recovery processes. The prospect of discovering a new microbial gum, such as xanthan or gellan, or a distinctive bioemulsifier like emulsan which possesses unique properties enabling its use as a gelling agent, emulsifier, stabilizer, flocculant, lubricant, or dispersing agent, has fueled this interest.

This study endeavors to explore the potential of the microorganism *Candida lipolytica* to produce biosurfactants from a mixture of vegetable oil and other chemical compounds employed in fermentation media (1% glutamic acid, urea, or glucose) as carbon sources. The kinetics, characterization, properties, and toxicity of the resulting biosurfactant are assessed for potential environmental applications.

MATERIALS AND METHODS

Microorganism

The microorganism utilized within this study belongs to the Collection of Microorganisms of Industrial Importance (CMII-ICCF-WFCC 232), namely *Candida lipolytica* ICCF 214 (ATCC 16618), from the Institute of Chemical-Pharmaceutical Research and Development in Bucharest (ICCF BUCHAREST).

Cultural conditions and biosurfactant production

Initially, the strain of *C. lipolytica* was inoculated into test tubes containing a suitable medium for yeast, specifically YMPG (Yeast Malt Peptone Glucose), freshly prepared. The transfer of the microorganism of interest was carried out under sterile conditions, with each test tube containing about 10 ml of YMPG medium. *C. lipolytica* was transferred onto the fresh medium, and after 48 hours, under controlled temperature conditions (28°C), robust development of the microorganism was observed. In total, five test tubes were utilized for this procedure. Two cultures steps were needed to initiate the experiments, a preinoculum and inoculum phase.

- **PREINOCULUM**: was represented by the strain of *C. lipolytica* on solid (or liquid) medium.
- **INOCULUM** Phase: was prepared in liquid YMPG medium.

The YMPG (DSMZ 186 medium) universal medium for yeast contained Glucose: 10g, Yeast extract: 3 g, Malt extract: 3 g, Peptone: 5 g, Agar: 17 g, Distilled water: 1000 ml pH: 6.5-7, Sterilization in autoclave at 115°C for 30 minutes. A total of 200 ml of YMPG medium was prepared for inoculation, without agar: Glucose: 2 g; Yeast extract: 0.6 g; Malt extract: 0.6 g; Peptone: 1 g. The volume was adjusted to 200 ml with distilled water. The pH of the prepared medium was determined to be 6.36. It was then adjusted to pH 7 using a 30% NaOH solution.

In the Inoculation Phase, Erlenmeyer flasks with a volume of 500 ml were utilized, each containing 100 ml of the liquid YMPG medium. Fermentation was initiated from the inoculum. In the literature, the inoculum volume varies between 2-10% (Albuquerque et al., 2006). In a first experiment we used a 5% inoculum.

The inoculation media were agitated in an orbital shaker at 220 rpm at 28°C for 48 hours. At 24-hour intervals from the start of inoculation, we monitored the evolution of the following parameters: pH and optical density (OD), read using a UV-VIS spectrophotometer at 540 nm, dilution 1:50; in the case of large inoculum volumes, we determined the biomass uplift (%).

The bioprocess

Three different fermentation media were used for this experiment.

M1 was the first fermentation medium tested for obtaining biosurfactants from the *C. lipolytica* strain. A batch of 500 ml medium was prepared, distributed as follows: two Erlenmeyer flasks with 100 ml medium each and two flasks with 150 ml medium each. The M1 medium contains: 0.1% NH_4NO_3; 0.02% KH_2PO_4; 0.02% MgSO4x7H_2O; 6% corn oil; 1% glutamic acid. After homogenizing all components, the pH was adjusted to 5.73.

M2 was the second fermentation medium tested for obtaining biosurfactants from the *C. lipolytica* strain. A batch of 500 ml medium was prepared, distributed as follows: 2 Erlenmeyer flasks with 100 ml medium each and 2 flasks with 150 ml medium each. The M2 medium contains: 0.25% $(NH_2)_2CO$ (urea), 1.36% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 7.5% corn oil. The final pH was adjusted to 5.32.

M3 was the third fermentation medium used for obtaining biosurfactants from the *C. lipolytica* strain. The M3 medium contains: 0.01% NH_4NO_3; 0.02% KH_2PO_4; 0.02% $MgSO_4$ x7H2O; 0.2% yeast extract; 10% glucose, 10% rapeseed oil (canola oil). The final pH was 5.67. The fermentation process was performed for 96 hours duration. During the fermentation process, the pH and optical density (OD) were monitored. The OD measured with a UV-VIS spectrophotometer, at 540 nm, in 1:50; 1:100 dilutions. The dry biomass parameter was determined at the end of fermentation. The quantity of the biosurfactant obtained, and the Emulsification index were also determined.

The Shoorl method, also known as the phenol-sulfuric method, is a technique used to determine the concentration of reducing sugars, such as glucose and fructose, in a solution. This method is based on the reaction between the

reducing sugars and phenol and sulfuric acid, which forms an intense color that can be measured spectrofotometrically.
The procedure involves first preparing a solution of reference sugars to create a calibration curve. Then, test samples are mixed with phenol and sulfuric acid, triggering the color formation reaction. The intensity of the formed color is then measured with a spectrophotometer at a specific wavelength, and the concentration of sugars is determined based on the light absorption.
The Shoorl method is widely used in biochemistry and microbiology laboratories for the rapid and accurate determination of sugar concentrations in a variety of biological samples and solutions.

Biomass determination

For biomass determination, 5 ml samples were mixed in pre-weighed tubes with chilled distilled water and centrifuged at 5000 rpm for 20 min. Following two washing cycles, the resulting cell pellet was dried in an oven at 90°C for 24 h. All the assays were carried out in triplicate and did not vary more than 5%.
Further, in order to separate biomass from the medium and preserve biosurfactants / substances of interest in the supernatant, with pH values ranging between 2 and 4, the pH was adjusted using a 30% NaOH solution to reach a pH of 7-7.5. Subsequently, the fermentation media was centrifuged at 7500 rpm for 20 minutes at 4°C. After centrifugation, biomass was separated from the supernatant, and the percentage of dry biomass was determined. The biomass was transferred from the centrifuge tubes to initially weighed Petri dishes and dried at 37°C for 24 – 48 hours until constant weight. The supernatant was refrigerated to facilitate the separation of the vegetable oil (corn and rapeseed oil used in preparing fermentation media). Prior to refrigeration, a simple filtration was performed. The volumes of supernatant obtained after centrifugation were measured.

Isolation of the Biosurfactant

The biosurfactant was extracted using different solvent system methods. Following the protocol, the cell-free broth was acidified with 6 M HCl to reach pH 2.0 and then precipitated with two volumes of methanol (96 %Purity). After 24 h at 4 °C, samples were centrifuged at 5000 g for 30 min, washed twice with cold methanol and dried at 37 °C for 24–48 h. The yield of isolated biosurfactant was expressed in g/L. In the second method, the biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. (2005). Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 1C. The resulting precipitate was collected by centrifugation and then evaporated to dryness to remove any residual acetone. Subsequently, the precipitate was re-dissolved in sterile water. The third method was that described by Amézcua-Veja et al. (2007). A sample of the cell broth was placed in a separatory funnel and an equal volume of ethyl acetate was added. The broth and ethyl acetate mixture formed two phases. The upper phase was transferred to a round-bottom flask. The lower phase was extracted with an equal volume of ethyl acetate three times for complete recovery of the biosurfactant. The round-bottom flask was placed on a roto-evaporator and the ethyl acetate was evaporated under vacuum at 80 °C. The residue obtained was washed twice with hexane and dried in the oven until a constant a constant weight was achieved.
From the three extraction methods, experiments were conducted with the first two methods. Consequently, 10 ml of supernatant from samples 1, 3, and 5 were precipitated with two volumes of methanol (96% purity, reagent from Merck), and another set of 10 ml samples were precipitated with three volumes of acetone (97% purity, reagent for analysis, Merck). After 24 hours, it was found that the method with methanol yielded a greater amount of precipitate compared to the samples where acetone was used as the solvent.
For the seven samples, precipitation was performed with 100 ml of cold methanol, brought to pH = 2 with 10% HCl.
For each sample, 100 ml of supernatant with a pH of 2 and 200 ml of cold methanol were added. They were left for 24 hours at 4°C, then centrifuged at 5000 rpm for 20 minutes at 4°C. The resulting precipitate was washed twice with methanol and centrifuged again. The resulting precipitate was dried for 24-48 hours at 37°C, or by drying in an oven at 105°C for 4 hours, until a constant weight was reached.

The emulsification index

Four milliliters of supernatant were taken from each of the seven aforementioned samples, and the emulsification activity was monitored using different substrates (sunflower oil, corn oil, and motor oil). Six milliliters of each oil were added to each sample for emulsification index analysis. After 24 hours, the stability of the formed emulsion was observed by agitating the samples using a vortex mixer for two minutes.

The emulsification index was calculated by dividing the height of the emulsified layer by the total height, multiplied by 100.

RESULTS AND DISCUSSIONS

The pH evolution in two of the *C. lipolytica* inoculum cultures was evaluated (Figure 1).

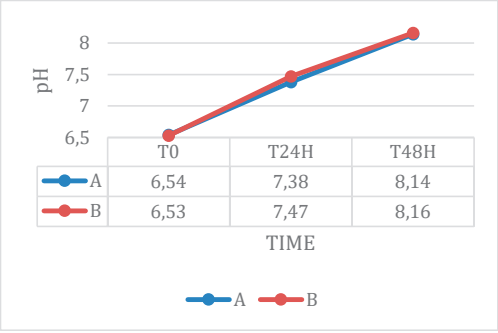

Figure 1. The pH evolution for the inoculum A and B

The pH levels can influence enzyme activity, nutrient availability, and product formation, among other factors. Maintaining optimal pH conditions is essential for maximizing the efficiency and yield of the fermentation process. Tracking pH evolution allows for adjustments to be made as needed to ensure the desired outcomes are achieved.

In the context of a fermentation process, optical density is an important tool in monitoring and controlling biotechnological processes.

The OD evolution in two of the *C. lipolytica* inoculum cultures is presented in Figure 2.

It was observed that in the first 24 h from inoculation both cultures were in exponential growth phase, while after 48 hours of inoculation, the culture was fully grown.

Considering the pH and optical density values, inoculum B demonstrated superior growth compared to A, therefore it was chosen to start the BIOPROCESS (Figure 3).

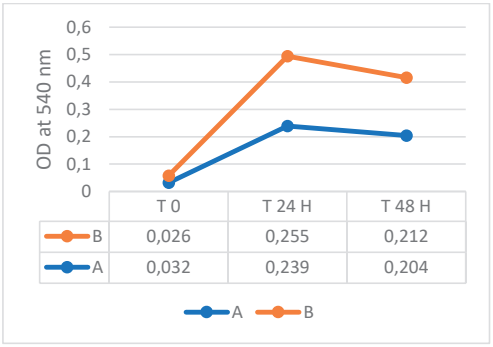

Figure 2. The Optical density (OD) read at 540 nm for the inoculum A and B

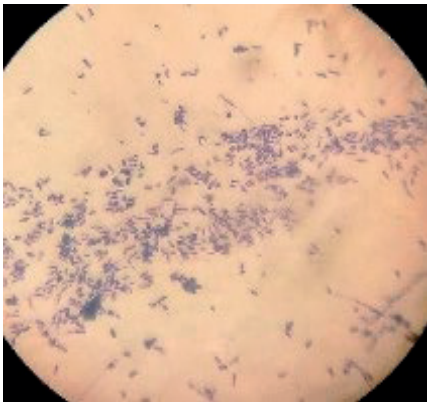

Figure 3. Microscopic aspects of *C. lipolytica* culture stained with methylene blue

The inoculation of the three fermentation media marked as **M1, M2**, and **M3,** has been carried out using inoculum B, which demonstrated superior growth compared to A, has been performed. An initial sampling was at time T 0, followed by an initial 96 hours fermentation process (the flasks were stirred at 200 rpm, 28°C). Depending on the observed values of the key process parameters, it was decided whether to potentially extend the fermentation process to either 120 hours or 144 hours.

For medium M3, the sugar content was additionally determined using the Shoorl method after the inoculation phase.

It was noted that M3 originally had a sugar content of 7.85 g%. Medium M3 was initially supplemented with 10% glucose monohydrate.

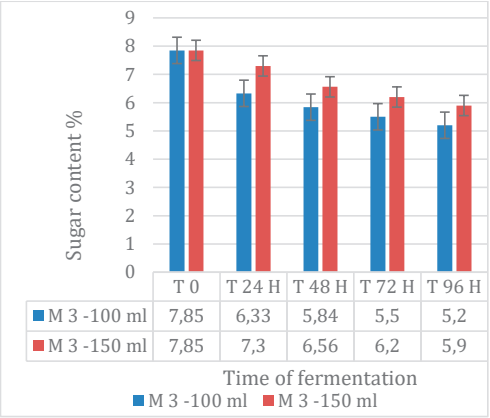

Figure 4. Sugar content (%) for medium M3

Observations indicated that the culture in medium M3 metabolized approximately 0.3 % glucose after 48 hours of fermentation, indicating the possibility of prolonging the bioprocess for an extended period of time, such as 144 hours.

The sugar content refers to the concentration or amount of sugars present in a solution or medium. In the context of fermentation processes, sugar content is often monitored as it serves as a crucial substrate for microbial growth and metabolism. Yeasts and other microorganisms typically utilize sugars as a source of energy for fermentation, converting them into various products such as alcohol, organic acids, or gases.

Measuring the sugar content helps to understand the progress of fermentation, as the consumption of sugars and the production of fermentation by-products can be monitored over time. This information is vital for optimizing fermentation conditions, controlling product quality, and maximizing yields. Various methods, such as spectrophotometry, chromatography, or enzymatic assays, can be employed to determine the sugar content accurately.

In figure 5 is presented the pH evolution from T0 until the completation of fermentation for the bioprocess (96 h). In Figure 6 is presented the evolution of optical density, read at 540 nm, using a UV-VIS spectrophotometer.

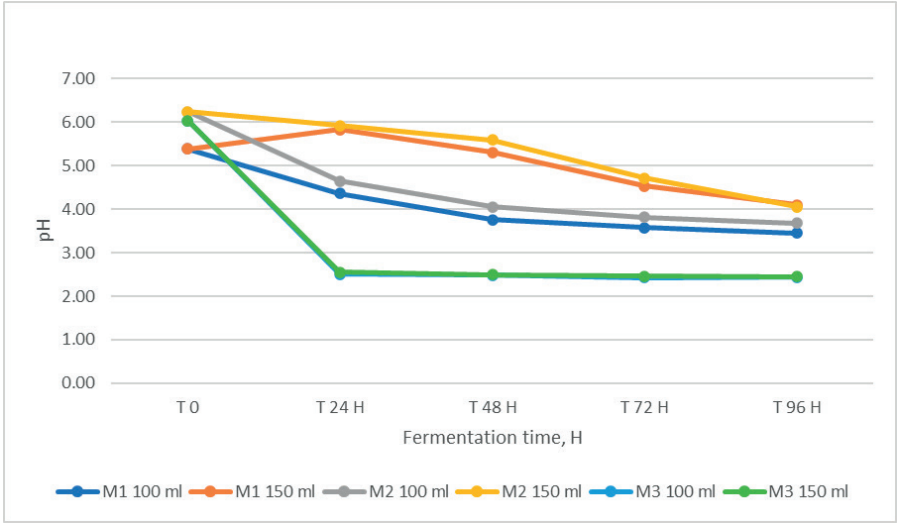

Figure 5. The pH evolution from T 0 until the completation of fermentation for the bioprocess (96 h).

Figure 6. The evolution of optical density, read at 540 nm, using a UV-VIS spectrophotometer

At the end of fermentation, pH and OD values were determined from the duplicate Erlenmeyer flasks, in which no samples were taken during the bioprocess. These measurements were compared with values determined both during the fermentation process and at its end. This procedure was carried out to identify any significant differences in process parameters.

After analyzing the parameters measured at the end of fermentation, we grouped together the flasks where there were no significant differences. Thus, from the 12 flasks with three different fermentation media, the following resulted after grouping: seven samples suitable to further investigations. From those with 100 ml of medium, they were combined with those made in duplicate for M1, M2, and M3. From those with 150 ml, only M1 and M2 were combined, and for M3, separate ones were made for both samples with 150 ml (the one in duplicate from which no samples were taken and the one from which samples were taken for analysis during the fermentation process).

This resulted in a total volume of medium after combining the flasks.

In Table 1 are presented the values for dry biomass (Figure 7), expressed in g/L, for each of the experimental substrate and fermentation volume.

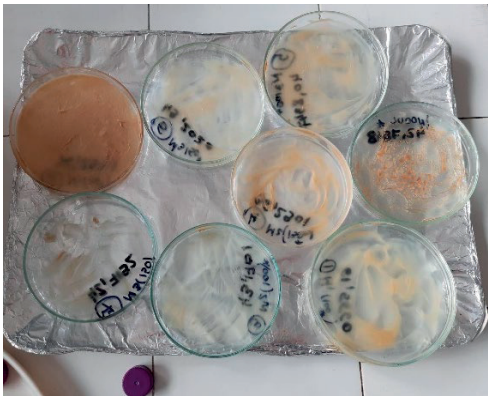

Figure 7. Dry biomass

Table 1. Calculation of dry biomass, g/L

Reunited medium	Dry biomass (g/L)
M 1 100 ml	8.07
M 1 150 ml	12.28
M 2 100 ml	2.32
M 2 150 ml	3.00
M 3 100 ml	4.65
M 3 150 ml	4.31
M 3 150 ml *	1.32
INOCULUM A	4.73

Biosurfactant yield

Candida strains have been shown to produce biosurfactants with different yields, which are dependent of the medium and culture conditions.

Crude biosurfactant of 13.99 g/L was extracted from the 96h culture of *C. lipolytica ICCF 214* showing a growth-associate production. Similarly, a biosurfactant yield of 4.5 g/L was extracted from the 144h culture of *C. sphaerica* (Rufino et al., 2013). On the other hand, the yield of another biosurfactant produced by *C. sphaerica* was 9 g/L after 144 h of experiment, which is in accordance with the values previously reported in the literature (Sarubbo et al 2007; Rufino et al., 2013). reported a yield of 8 g/L for a biosurfactant produced by *C. lipolytica* using canola oil and glucose as substrates. In Table 2 is presented the values for biosurfactant content, expressed in g/L.

Table 2. Biosurfactant content obtained with precipitation of methanol, g/L, 96 h

Reunited medium	Sample notation	Biosurfactant content (g/L)
M 1 100 ml	1	2.12
M 1 150 ml	2	0.43
M 2 100 ml	3	3.12
M 2 150 ml	4	4.47
M 3 100 ml	5	4.00
M 3 150 ml	6	2.13
M 3 150 ml *	7	1.89

In Table 3 are presented the values for biosurfactant content, expressed in g/L.

Table 3. Biosurfactant content obtained with precipitation of acetone, g/L 96 h

Reunited medium	Sample notation	Biosurfactant content (g/L)
M 1 100 ml	1	12.45
M 1 150 ml	2	4.45
M 2 100 ml	3	9.45
M 2 150 ml	4	13.99
M 3 100 ml	5	5.74
M 3 150 ml	6	-
M 3 150 ml *	7	2.82

In Table 2 and Table 3 are presented the values for biosurfactant content (g/L), the purification has been with methanol and acetone. Can observe that the precipitation with acetone produced for M2 the largest quantity of biosurfactant content.

Emulsification index

Biosurfactant production is often assessed by measuring emulsification. Although a direct correlation has been found between surface activity and emulsification activity and the emulsion index has been used as a screening method, the ability of a molecule to form a stable emulsion is not always associated with lowered surface tension activity. Sunflower oil, corn oil and motor oil were the substrates. The water–oil emulsions were compact and remained stable for more than six months at room temperature, suggesting that the addition of the biosurfactant to a remediation process may enhance the availability of the recalcitrant hydrocarbon. Different oil-degrading microorganisms produce surface active substances and some make a stable oil-in-water emulsion. These microorganisms can be categorized into two groups - those that produce a low-molecular-weight surfactants, which typically but do not form stable emulsions, and others that produce polymers that primarily act as emulsion stabilisers but have minimal impact on surface tension. A few bacteria and yeasts have both types of properties. The present data suggest that *C. lipolytica* ICCF 214 could be included in the latter category of microorganisms, as this strain was able to produce a biosurfactant that could reduce the surface tension and make stable emulsions with motor oil, as will be shown later. In figure 8 is presented the emulosification index. Table 4 presents the hydrophobic substrates tested for emulsification by the cell-free broth containing the biosurfactant from *C. lipolytica* ICCF 214.

Figure 8. Emulsification index

Table 4. Emulsification index (EI) %

Reunited medium	Sample notation	EI -24 H %- substrat sunflower oil	EI -24 H %- substrat corn oil	EI -24 H %- substrat motor oil
M 1 100 ml	1	15	60	65
M 1 150 ml	2	30	30	65
M 2 100 ml	3	No emulsion formed	No emulsion formed	15
M 2 150 ml	4	No emulsion formed	No emulsion formed	7
M 3 100 ml	5	42	86.3	43
M 3 150 ml	6	47	77.7	50
M 3 150 ml *	7	58	55	60

In the Table 4 we can observe that the best result for emulsification index (%) was for medium M3, using the corn oil substrate. Albuquerque et al (2006) obtained the similar index emulsification.

CONCLUSIONS

Based on the obtained results, we can conclude that all three fermentation media produced biosurfactants using the Candida lipolytica ICCF214 strain, but further analysis of surface tension and antimicrobial activity of the obtained biosurfactants is required. Additionally, we observed that media with a volume of 100 ml produced higher quantities of biosurfactants, indicating good aeration of the fermentation medium. However, even the ones with 150 ml showed decent performance during the bioprocess

- The biosurfactant isolation method using two volumes of methanol will be employed, and other solvent extraction methods (such as chloroform) will be explored.
- Regarding the emulsification index (E24), medium M3, with a volume of 100 ml, exhibited the largest emulsified layer, prompting further analysis of the chemical composition of the obtained product.
- Other new fermentation media or microorganisms will be attempted to optimize the bioprocess for biosurfactant production.

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CORRELATION OF THE CHEMICAL PARAMETERS WITH THE SENSORIAL PROPERTIES OF WINE

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Abstract

Wine quality is afforded by chemical properties but also tightly connected to perception of appearance, olfactory and taste properties. In order to assess the most important chemical parameters of wine that are priority for wine consumers, 21 wine samples were studied. This means 3 varieties of bottled wines from 2018 harvest, vinified in dry, medium dry, medium sweet and sweet, from 4 different wine regions and 6 different vineyards from Romania. For assessing the chemical parameters, 9 laboratory analyses were performed (sulphites, total acidity, volatile acidity, pH, alcohol content, anthocyanins, residual sugar, total polyphenols, tannins). Organoleptic analyses were performed according to BLIC test by 26 tasters panel. Using specific attributes, they evaluated the visual aspect, the olfactory properties and the taste and finally a quality overall mark was delivered. The statistical analyses of the correlation between chemical parameters and sensorial characteristics showed the importance of some laboratory determinations on the perception of wine quality by a common consumer. But the results revealed that very good values of the chemical parameters are not a guarantee of high acceptability of the consumers.

Key words*: wine, sensorial characteristics, chemical parameters, statistical analyses, organoleptic analyses.*

INTRODUCTION

Experience and needs could influence consumer preferences. Gender and age influence wine choices, label information have also a positive effect in choices, but knowledge is the most important factor in preferences and consumption of wine (Troiano S. et al., 2020). Highest level of wine expertise could be attained when consumers are able to recognize fine wine properties, like complexity, harmony or persistence, in association with socio-cultural aspects, like origin, winemaking, and performed aesthetic assessments independently from wine enjoyment (Malfeito-Ferreira, 2021). Price and quality differences perceived are not exclusively influenced by features objective of the product. Most of consumers, and even experts, seem not to be able to make difference by sensory characteristics and cannot rank wines according to their price. Consumers tend to consider that a higher price absolutely means a higher quality (Troiano S. et al., 2020).

The first important intrinsic sensory of consumers expectations according to flavor and taste of beverages is the color. (Vinha et al., 2018). Singularity of olfaction (ortho- and retronasal pathways) means to transfer the stimulus from nose receptor cells to orbitofrontal cortex (OFC) where conscious processing takes place without passing through thalamus. Accordingly, these senses contribute to the perception of aroma (Malfeito-Ferreira, 2021). Tongue is not essential for taste perception, by Mahood K et al. (2017) opinion, which means that taste perception could be influenced by nutrition or health implications. For all these variations of perception, some specialists have created different reasoning or quality equation. Visalli et al. (2023) performed a Free-Comment Attack-Evolution-Finish (FC-AEF) which describe temporal aspects of tasting to the free description of wine. They pretend that a predefined list of descriptors can be measured using the Attack-Evolution-Finish (AEF) method described prior also by Mathieu et al. in 2020. AEF was adapted to replace the list of attributes by FC using the so-called Free-Comment AEF method (FC-AEF). FC-AEF provided additional information compared to AEF. FC-AEF can be used both with consumer and expert panels and was used to collect temporal data about two Bordeaux and two Rioja wines. Also, Visalli et al. said in 2023 that consumers are used to assess the influence of culture and expertise on temporal sensory evaluations of wines. The purpose

of this study is to analyze the physio-chemical parameters and sensory attributes and then to corelate them to see which of them are important for common consumer.

MATERIALS AND METHODS

Materials

For this study there were collected 21 wine samples from local stores.

There were 3 varieties of authentic Romanian wines (Fetească Neagră - FN, Fetească Albă - FA and Busuioacă de Bohotin - BB) from 2018 harvest year, vinified as dry, medium dry, medium sweet and sweet, from 4 different wine Regions and 6 different vineyards. All the wine samples were bottled in glass (750 ml volume) and had cork closure (Table 1).

Table 1. Wine samples used for experiments

no. of sample	sample code	color	taste	region	vineyard	quality
1	FN1	red	dry	Transylvanian Plateau	Lechința	PDO
2	FN2	red	dry	Muntenia and Oltenia's Hills	Dealu Mare	PDO
3	FN3	red	dry	Muntenia and Oltenia's Hills	Pietroasele	PGI
4	FN4	red	dry	Muntenia and Oltenia's Hills	Severinului	PDO
5	FN5	red	dry	Dobroga's Hills	Murfatlar	PDO
6	FN6	red	dry	Moldova's Hills	Cotnari	PDO
7	FN7	red	medium dry	Muntenia and Oltenia's Hills	Dealu Mare	PGI
8	FN8	red	medium dry	Muntenia and Oltenia's Hills	Severinului	V
9	FA1	white	dry	Moldova's Hills	Cotnari	PDO
10	FA2	white	dry	Muntenia and Oltenia's Hills	Dealu Mare	PGI
11	FA3	white	dry	Muntenia and Oltenia's Hills	Pietroasele	PGI
12	FA4	white	dry	Transylvanian Plateau	Lechința	PDO
13	FA5	white	dry	Dobroga's Hills	Murfatlar	PDO
14	FA6	white	medium dry	Moldova's Hills	Cotnari	PDO
15	FA8	white	medium dry	Muntenia and Oltenia's Hills	Dealu Mare	PDO
16	FA8	white	medium dry	Muntenia and Oltenia's Hills	Severinului	V
17	BB1	rose	dry	Muntenia and Oltenia's Hills	Pietroasele	PGI
18	BB2	rose	medium dry	Moldova's Hills	Cotnari	PDO
19	BB3	rose	medium sweet	Moldova's Hills	Cotnari	PDO
20	BB4	rose	medium sweet	Muntenia and Oltenia's Hills	Pietroasele	PGI
21	BB5	rose	sweet	Muntenia and Oltenia's Hills	Pietroasele	PGI

Methods

All the 21 wine samples were analyzed in laboratory for their chemical and physical characteristics. *Determination of total sulfites* is a spectrophotometric method and was done by enzymatic kit EnzytecTM Liquid SO_2 Total (Sulfite UV Method for the determination of sulfurous acid in food stuffs and other materials, Boehringer Manheim/R-Biopharm Enzymatic BioAnalysis/Food Analysis, Roche, Cat.no.10 725 854 035) and UV-VIS Cintra 10e device (SR 6182 – 13/2009).

Total acidity (SR 6182 – 1/2008) and *volatile acidity* (SR 6182 – 2/2008) were performed by potential method using 0.1N NaOH solution, 7 pH buffer solution and a Metrohm 794 Basic Titrino titrator device. The results presented in Table 2 for total acidity and volatile acidity represented the arithmetic average of triplicate.

Determination of alcohol concentration was performed using an alcoholmeter device (STAS 6182/6-70) and for determination of pH weas used a pH meter device (Mettler Toledo) which was calibrated (SR 6182 – 14/2009) with pH solutions (pH 4,01 pH 7,00 and pH 10,01).

The anthocyanins were determined by pH variation method using UV-VIS Cintra 10e device (SR 6182/35 – 75; OIV-MA-AS2-07B).

Determination of reducing carbohydrates (residual sugar) was performed by enzymatic method using an enzymatic kit D-Glucose/D-Fructose (Sucrose/D-Glucose/D-Fructose UV Method for the determination of sucrose, D-glucose and D- fructose in foodstuffs and other materials, Boehringer Mannheim/R-Biopharm Enzymatic BioAnalysis/Food Analysis, R-Biopharm, Roche, Cat.no. 10 716 260 035) and a spectrophotometer UV-VIS Cintra 10e.

Determination of total polyphenols (TPC) was performed by Folin-Ciocâlteau method, using Folin-Ciocâlteau reagent, 80% methanol solution, 20% Na_2CO_3 solution, galic acid 100 ug/ml stock solution and the same spectrophotometer UV-VIS Cintra 10e. (Folin Ciocâltrau method by Singleton and colab. version, 1999).

Determination of tannins was performed by 35% hydrochloric acid, 96% ethyl alcohol and a spectrophotometer UV-VIS Cintra 10e. (SR 6182 – 45/2009)

Organoleptic properties were assessed by a panel of 26 tasters, unauthorized but passionate of wine, being graduates of a basic wine course. There was not followed a high qualified opinion but of common consumer. They evaluated a series of sensorial characteristics of wine samples, characteristics about aspect, smell and taste, like color intensity, color hue, overall smell, aroma intensity, aroma quality, acidity, astringency, alcohol, smoothness, sourness, harmony and overall quality. Finally applied BLIC technique (Balance, Length, Intensity, Complexity) to every wine sample with a grade from 1 to 5, where 1 means a poor wine and 5 means an outstanding wine.

Statistical correlation between the individual components (chemical parameters) and the sensorial characteristics (determined by panel tasters) was calculated using Anova function by Excel Microsoft Office 2021.

RESULTS AND DISCUSSIONS

Laboratory analysis

All samples were analyzed and the results are presented in Table 2.

Table 2. Physio-chemical parameters of wine sample

no. of sample	sample code	sulfites (mg/L)	total acidity* (g tartric acid/L)	volatile acidity* (g acetic acid/L)	pH	tannins (mg/L)	antho cyanins (mg/L)	alcohol (% vol)	residual sugar (g/L)	TPC (g/L)
1	FN1	106.51	4.71	0.54	3.70	3.00	117.38	13.80	0.21	1.33
2	FN2	127.29	6.02	0.87	3.76	5.60	105.95	14.80	0.27	2.01
3	FN3	129.04	6.10	1.68	3.98	4.60	66.31	14.90	0.19	1.82
4	FN4	113.51	6.11	1.05	3.77	8.30	130.71	14.30	0.19	2.61
5	FN5	122.34	6.28	0.72	3.83	7.49	56.40	14.60	0.20	1.98
6	FN6	123.19	5.45	0.88	3.84	5.70	106.65	14.70	0.47	2.00
7	FN7	118.55	5.39	0.54	3.51	4.90	137.96	13.70	0.67	1.80
8	FN8	107.59	4.46	0.96	3.97	5.69	92.99	13.10	1.01	2.35
9	FA1	119.56	6.14	0.21	3.25	0.72	0.00	13.50	2.12	0.35
10	FA2	100.42	5.87	0.18	3.38	0.46	0.00	13.50	0.65	0.26
11	FA3	108.57	6.42	0.27	3.21	1.12	0.00	13.10	1.64	0.40
12	FA4	100.12	6.24	0.20	3.41	0.45	0.00	13.40	3.98	0.27
13	FA5	109.60	6.54	0.27	3.31	1.40	0.00	13.20	2.44	0.39
14	FA6	126.28	5.78	0.33	3.25	0.97	0.00	13.10	4.09	0.30
15	FA7	157.16	5.31	0.45	3.47	0.87	0.00	13.00	5.55	0.29
16	FA8	125.07	4.74	0.71	3.44	0.91	0.00	13.40	7.93	0.30
17	BB1	123.61	4.80	0.42	3.46	2.72	10.29	13.30	3.99	0.50
18	BB2	132.73	4.52	0.39	3.19	1.70	10.67	12.90	4.42	0.41
19	BB3	130.45	5.51	0.48	3.34	5.35	20.20	12.90	12.52	0.43
20	BB4	193.04	6.16	0.66	3.56	8.08	20.96	14.30	12.22	0.56
21	BB5	167.66	5.86	0.84	3.84	7.28	39.63	14.10	19.58	0.59

**values are arithmetic average of triplicate*

Sulfites

According to the obtained data, FA wines ranged between 100.12 mg/L and 157.16 mg/L of total SO_2, FN wines ranged between 106.51 mg/L and 129.04 mg/L of total SO_2 and BB wines ranged between 123.61 mg/L and 193.04 mg/L of total SO_2. These results complied with the values stipulated in Romanian and European legislation. Colibaba C. et al., 2009, obtained in their study regarding BB from 2008, 128.80 mg/L total SO_2. Tartian A.C. et al., (2015) obtained in BB from 2014, ranged between 70.54 and 133.47 mg/L of total SO_2. According to Ivanova Petropulos V. and Mitrev S. (2014) sulfites have an antioxidant role and could be an antimicrobial agent, as well as potential for bleaching the pigments and elimination of unpleasant odors. They obtained in red wines values ranged between 60.16 mg/L and 103.76 mg/L of total SO_2 and also in white wines values ranged between 89.60 mg/L and 131.80 mg/L of total SO_2

Total acidity, Volatile acidity, pH

Total acidity for FA wines was ranged between 4.74 g /L tartaric acid and 6.54 g /L tartaric acid and Volatile acidity for FA was ranged between 0.18 g/L acetic acid and 0.71 g/L acetic acid. Also, pH results were ranged between 3.21 to 3.47 for FA.

Total acidity for FN was ranged between 4.46 g/L tartaric acid and 6.28 g/L tartaric acid and volatile acidity for FN was ranged between 0.54 g/L acetic acid and 1.68 g/L acetic acid. pH determined for FN was ranged between 3.51 and 3.97 values. Total acidity for BB was ranged between 4.80 g/L tartaric acid and 6.16 g/L tartaric acid and volatile acidity for BB was ranged between 0.39 g/L acetic acid and 0.84 g/L acetic acid. pH determined for BB was ranged between 3.19 to 3.84 values. In conclusion, total acidity was in regulated limits for all of 21 samples and volatile acidity was higher for sample FN3 (with the value 1.68 g/L acetic acid). Results also highlighted that 2 samples (FN4 and FN8) had high values to volatile acidity and also high values to pH. This could mean that volatile acidity and pH values are directly influenced. Increasing in pH values could be accompanied by a decreasing of total acidity (Lima S.M. et al., 2015). Also in Syrah, Lima S.M. et al., 2015 obtained total acidity registered between 4.55 g/L tartaric acid and 21.49 g/L tartaric acid and volatile acidity between 0.17 g/L tartaric acid and 0.54 g/L tartaric acid. Antoce and Cojocaru (2018) obtained in their study, total acidity between 4.30 g/L tartaric acid and 6.20 g/L tartaric acid and pH between 3.54 and 3.97 in FN. Balla G. et. al. (2023) determined in FN from 2018, 6.11 g/L tartaric acid for total acidity content. Artem V. et al. in their study published in 2014 identified 7.72 g/L tartaric acid when they determined total acidity in FN from 2013 harvest. Dobrei A. et al. (2018) obtained 6.85 g/L tartaric acid for total acidity characteristic in FN from 2016 and 6.57 g/L tartaric acid for total acidity characteristic for FN from 2017. Bunea C.I. (2014) studied FA and obtained 4.71 g/L tartaric acid for total acidity parameter and 3.80 pH.value. Tartian et al., 2015, registered values between 0.31 g/L acetic acid and 0.39 g/L acetic acid for volatile acidity parameter, values between 5.40 g/L tartaric acid and 6.28 g/L tartaric acid for total acidity parameter and pH value between 3.76 and 3.84 for BB from 2014. Colibaba C. et al., 2009, determined in BB from 2008, 0.50g/L acetic acid for volatile acidity. Tartian et al., 2017, determined total acidity in BB from 2014 and 2015 and obtained 5.70 g/L tartaric acid for 2014 harvest and 5.41 g/L tartaric acid for 2015 harvest. To the same variety, determined pH with values registered between 3.77 and 3.80 for BB from 2014 and pH with values between 3.30 and 3.35 for BB from 2015. Total acidity is generally higher in FA wine samples than FN wine samples and BB wine samples. Volatile acidity is clearly higher in FN wine samples and decreases progressively in BB wine samples and the lowest values are in FA wine samples.

Alcohol content

According to the obtained data alcohol content for all samples was registered between 12.90 % alc. and 14.90% alc. Alcohol concentration for FA was ranged between 12.90% alc. and 14.90% alc. FA wine samples registered homogeneous values regarding to alcohol content, the values varied by half units per cent. Alcohol concentration for FN was ranged between 13.10% alc. and 14.60% alc. The higher alcohol concentrations were ranged in FN samples wine, especially in dry FN. Artem V. et. al. in their study published in 2014 identified 13.55% alcohol content in FN from 2013. Dobrei A. et al. (2018) obtained 12.3% alc. in FN from 2016 and 13.4% alc. in FN from 2017. Balla G. et al. (2022) determined in FN from 2018 a value of 13.64% alcohol content. Alcohol concentration for BB was ranged between 12.9% alc.and 14.80% alc. BB wine samples ranged medium values, between FN and FA. According to Colibaba C. et al., (2009), alcohol content in BB from 2008 was 12.90% alc. The alcohol concentration of BB from 2014 was ranged between 13.91% alc. and 14.41% alc. and BB from 2015 was ranged between 14.55% alc. and 15.24% alc. (Tartian et al., 2017). This means that climate factors influence the alcohol content.

Tannins and Anthocyanins

According to the obtained data tannins were ranged between 0.45 mg/L (in FA) and 1.40 mg/L (in FA), in FN were ranged between 3.00 mg/L and 8.30 mg/L and in BB were ranged

between 1.70 mg/L and 8.08 mg/L. The highest values of tannins content were determined in FN wine samples. These values are higher than the values registered in FA wine samples, while in BB wine samples registered very different values. For two of them was registered comparable values with the values of FN wines samples (BB3, BB4 and BB5).

Anthocyanins values in FA were N/A because are not find in white wines. Anthocyanins values in FN were ranged between 56.40 mg/L and 137.96 mg/L and in BB were ranged between 10.29 mg/L and 39.63 mg/L. Low values of anthocyanins were ranged in BB5. Results are highlighted in Table 2. Lima S. M. et al., 2015, studied Syrah and obtained tannins with values ranged between 0.93 g/L and 2.61 g/L and total anthocyanins values ranged between 55 mg/L and 669 mg/L. Specialists obtained 325.92 mg/L anthocyanins in FN from 2014 (Artem V. et al., 2013), 231.20 mg/L anthocyanins in FN from 2016 and 240 mg/L anthocyanins in FN from 2017 (Dobrei A. et al., 2018).

Residual sugar

According to the obtained data in FN wines were registered values between 0.19 g/L and 1.01 g/L residual sugar, with the highest value to a medium dry wine (FN8). In FA wines the residual sugar was registered between 0.65 g/L and 7.93 g/L, with the highest value to a medium dry wine (FA8). In BB wines the residual sugar determined was between 3.99 g/L and 19.58 g/L, with the highest value to a sweet wine (BB5). Results are centralized in Table 2. The highest values were ranged in BB wine samples, followed by FA wine samples values and then by FN wine samples values. FN samples registered very low values, keeping at low level even FN7 and FN8 (medium dry). FA samples ranged values a little higher than FN samples, this being explained by correlation with the low alcohol content. Higher values were ranged to samples FA6, FA7 and FA8 (medium dry). Similar, the BB samples ranged the highest values in residual sugar, these being correlated with the low alcohol content. Analysis for determination of residual sugar registered values according with the regulated limits but against of regulated results, the registration into a category of taste according to these obtained values and legislative rules is different compared to the taste specified on the label for few samples. Vlassa M. et al., (2010) analyzed 31 samples of bottled wines especially for the purpose of monitoring the quality of commercial wine and observed similar situation about wines that belong to be different as declared on the label.

Ivanova-Petropulos V. Mitrev S. (2014) determined less than 1.5 g/L residual sugar in red wines. Paraschiv et al., (2023), determined 170.7 g/L residual sugar in FA. Colibaba C. et al., 2009, obtained in their study 30.4 g/L residual sugar in BB from 2008. Residual sugar was also determined in BB from 2014 with the value 248.4 g/L and in BB from 2015 with the value 230.0 g/L (Tartian et al., 2017). Paraschiv et al., (2023), concluded in their study that content in sugars of FA wine is influenced by the dose of fertilizers used in soil where the wine variety grew up.

Polyphenols (TPC)

According to the ranged data, polyphenols values in FA were between 0.262 g/L and 0.401 g/L, in FN were between 1.300 g/L and 2.609 g/L and in BB were between 0.409 g/L and 0.585 g/L. FN wine samples showed higher values than FA samples and BB samples due to the contribution of the tannins and anthocyanins (which are not specific to FA samples) to this total content (TPC). The values of FN samples were lower than Antoce and Cojocaru (2018) (between 26.2 g/L and 81.80 g/L) but closer to those assessed by Bărbulescu et al., (2022), (453.77 mg GAE/100 ml in BB from 2022).

Organoleptic analysis

According to the taste panel there were determined three sensorial characteristics with specific attributes to each one: visual aspect (color intensity and color hue), olfactory (overall smell, aroma intensity and aroma quality), taste (acidity, astringency, alcohol, smoothness, sourness and harmony). Finally, overall quality was appreciated. Every specific attribute was evaluated with grades from 1 to 5, where 1 means a poor wine, 2 means an acceptable wine, 3 means a good wine, 4 means a very good wine and 5 means an outstanding wine. Tasters were consisted in 26 testers, actually 15 men and 11 women, from 22 to 57 years old, superior education all of them. They

applied BLIC equation (balance. length. intensity. complexity) for this determination. Finally, there were calculated arithmetic averages for the obtained grades (Table 3).

Table 3 Sensorial characteristics of the wine samples

			visual aspect		olfactory			taste						
no. of sample	sample code	wine type	color intensity	color hue	overall smell	aroma intensity	aroma quality	acidity	astringency	alcohol	smoothness	sourness	harmony	quality overall
1	FN1	dry	4.85	4.65	4.85	5.00	4.96	4.96	4.88	5.00	4.85	4.23	4.42	4.92
2	FN2	dry	4.92	5.00	4.62	4.85	4.88	4.58	4.58	4.92	4.88	4.62	4.77	4.81
3	FN3	dry	4.81	4.92	4.81	4.81	4.77	4.85	4.85	5.00	4.88	4.19	4.42	4.65
4	FN4	dry	4.73	4.54	4.88	5.00	4.00	4.77	4.69	5.00	4.15	4.12	4.23	4.69
5	FN5	dry	4.58	4.92	4.81	5.00	5.00	4.96	4.96	4.77	4.54	4.27	5.00	4.92
6	FN6	dry	5.00	4.92	4.88	4.65	4.23	4.73	4.19	4.31	4.19	4.15	4.27	4.65
7	FN7	medium dry	4.88	4.81	4.54	4.62	4.31	4.62	4.62	5.00	4.73	3.88	4.00	4.38
8	FN8	medium dry	5.00	5.00	4.77	5.00	4.81	4.81	4.65	4.92	4.85	4.31	4.81	4.69
9	FA1	dry	3.96	4.08	4.42	4.15	4.31	4.77	4.81	4.35	4.04	3.58	4.04	4.19
10	FA2	dry	3.58	4.15	4.38	3.92	4.08	4.23	4.46	4.46	4.00	3.81	4.00	4.19
11	FA3	dry	4.08	4.08	4.08	3.88	3.96	4.46	4.15	4.00	3.96	3.96	4.04	4.04
12	FA4	dry	4.04	4.65	5.00	4.19	4.12	4.04	4.00	4.27	4.00	3.88	4.00	4.15
13	FA5	dry	4.00	4.27	4.27	4.12	4.27	4.31	4.31	4.00	4.00	3.81	4.00	4.08
14	FA6	medium dry	4.27	4.38	4.12	3.85	4.00	4.46	4.46	4.46	3.96	3.65	4.04	4.35
15	FA7	medium dry	3.62	4.19	4.23	3.96	4.00	4.46	4.50	4.46	3.92	3.77	4.58	4.46
16	FA8	medium dry	3.96	4.08	4.50	4.46	4.73	4.65	4.62	4.31	4.15	3.77	4.12	4.15
17	BB1	dry	3.62	4.73	4.65	4.54	4.58	3.65	3.65	3.81	4.19	2.77	4.08	4.19
18	BB2	medium dry	3.85	4.50	4.77	4.42	4.46	3.12	3.00	3.69	4.15	2.65	4.04	4.08
19	BB3	medium sweet	3.62	4.23	4.81	4.54	4.58	3.08	2.85	3.69	4.19	2.46	4.08	4.23
20	BB4	medium sweet	3.62	4.69	4.65	4.58	4.69	3.46	3.58	3.77	4.19	3.27	4.12	4.42
21	BB5	sweet	4.38	4.77	4.85	4.85	4.69	3.46	3.50	3.73	4.38	3.12	4.27	4.38

Visual aspect

Color intensity was evaluated with grades from minimum 3.58 to maximum 5.00. Color intensity was the most appreciated in FN wine samples and the least appreciated in BB wine samples.

Color hue was evaluated with grades from 4.08 to 5.00. Color hue was the most appreciated in FN wine samples and then following the BB wine samples and the least appreciated in FA wine samples. FA samples ranged very homogenous values.

The best grades for visual aspect were awarded by FN. Generally, the FN samples with highest anthocyanins content, were more appreciated by color hue. For BB wine samples, the highest value was ranged in BB5, being the most appreciated and also having the highest anthocyanins content and having a superior aspect.

Olfactory

Overall smell was evaluated with grades between 4.08 and 4.88. Excepting FA4, the least appreciated were FA samples. The BB wine samples were appreciated quite good, close to the highest values of FN wines samples.

Aroma intensity was evaluated with grades between 3.85 and 5.00. Obviously, the highest appreciated wine samples were FNs. and the least appreciated wine samples were FAs.

Aroma quality was evaluated with grades between 4.00 and 4.96. Although the highest content of sulfites was in BB and the highest values in volatile acidity was ranged in FN, the FA wine samples were the most depreciated to this characteristic.

Taste

Acidity was evaluated with grades between 3.08 and 4.96. The lowest acidity was appreciated in BB (less than 3.5) and the most appreciated samples by acidity were FN samples. This could be explained by the lowest values of total acidity ranged in BB.

Astringency was evaluated with grades between 2.85 and 4.96. Results highlighting that the astringency wines are preferred by consumers. Although, the tannins content in BB was

significant, these were the least appreciated to the mentioned attribute.

Alcohol was evaluated with grades between 3.69 and 5.00. The alcohol perception as an attribute of taste is correlated with the alcohol content (% alc.) as a chemical parameter. Regarding to the alcohol influence in sensorial properties of wine, it was proved that FN wine samples were the highest appreciated and the BB wine samples were the lowest appreciated, this being explained by alcohol content ranged in tested samples.

Smoothness was evaluated with grades between 3.92 and 4.88.

Sourness was evaluated with grades from 2.46 to 4.62. The highest appreciated wine samples were the FN. BB and FA were medium appreciated, with comparable grades. To FN wines are two exceptions, FN4 and FN6, which were lower appreciated, these being characterized by a high content of anthocyanins and tannins.

Harmony was evaluated with grades between 4.00 and 5.00. The highest appreciated were FN wine samples, excepting FN4 and FN6 which were medium by harmony. The BB wines and FA wines were comparable.

Overall quality

According with panel tasters evaluation, overall quality was between 4.04 and 4.92. Evaluating the attributes, overall quality was appreciated, which proved to be the highest in FN, with the best values in FN1, FN4 and FN8, which had low content in sulfites, higher content in anthocyanins and medium alcohol content. Sensory tests of red wines from 2018 harvest, highlighted those favorite wines was Cadarcă (89 points), Fetească Neagră (85 points), Merlot (84 points) and Cabernet Sauvignon (82 points) (Artem V. et. al. 2014). Perception of women tasters/consumers were more open for medium sweet or sweet wines then for dry wines. Women have also a preference for white and rose wines by color. On the other hand, the men prefer dry and tannic wines by taste and red wines by color. Studies or professional training did not present a tie-breaking criterion.

Fine wines are more appreciate by olfactory characteristics then visual aspect or taste. The arithmetic average of arithmetic averages of each sensorial characteristics of wine samples were calculated and the values are highlighted in Figure 1. Common consumer prefers wines first by olfactory, second by visual aspect and finally by taste.

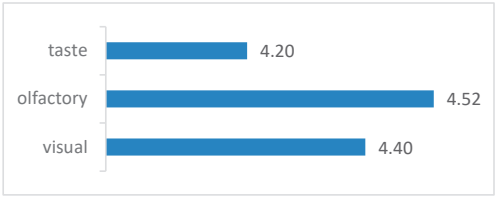

Figure1 Overall sensorial characteristics

Correlation of physio-chemical properties with sensorial characteristics of tasted wines

There were assessed correlations for finding out which of the chemical parameters have higher influence when choosing a wine by a common consumer. There were correlated the all values of parameters with each evaluated attribute (Table 4). The most relevant is the content in phenolic compounds. The pH and TPC had a higher correlation. TPC essentially influence the color intensity, color hue and also aroma intensity and smoothness. The anthocyanins, alcohol content, volatile acidity and tannins content had also a high correlation with color, aroma intensity and astringency. The results suggest that anthocyanins content highly influents the perception of color intensity and also the aroma intensity and smoothness. Although overall quality is highly correlated with the alcohol content, this chemical parameter is reasonable correlated with almost all the sensorial attributes. Volatile acidity is highly correlated with aroma intensity, smoothness, color (intensity and hue) and has a wicked correlation with acidity and astringency. Tannins content is highly correlated with aroma intensity and color hue. Although the residual sugar is lower correlated with overall quality, it could be observed a highly negative correlation with acidity, astringency, alcohol and sourness, the results may suggest that residual sugar content it is not important for common consumer preferences. Sulfites and total acidity expressed an almost non-existent correlation with most of the sensorial atributes. It seems that if the value is within the regulated limits, doesn't influence the consumer perception.

Table 4. Correlation of physio-chemical parameters with the values of sensorial attributes

	color intensity	color hue	overall smell	aroma intensity	aroma quality	acidity	astrin-gency	alcohol	smooth-ness	sour-ness	harmony	overall quality
sulfites (mg/L)	-0,277	0,109	0,026	0,098	0,208	-0,472	-0,406	-0,417	-0,061	-0,396	0,070	0,036
total acidity (g tartric acid/L)	-0,050	-0,112	-0,206	-0,249	-0,305	0,111	0,158	-0,015	-0,253	0,243	-0,100	-0,064
volatile acidity (g acetic acid/L)	0,627	0,623	0,487	0,717	0,305	0,305	0,262	0,459	0,650	0,365	0,519	0,633
pH	0,728	0,792	0,606	0,822	0,549	0,403	0,368	0,546	0,728	0,521	0,718	0,825
alcohol (% vol)	0,587	0,646	0,458	0,607	0,401	0,370	0,363	0,434	0,517	0,510	0,445	0,684
antho-cyanins (mg/L)	0,865	0,679	0,494	0,764	0,309	0,458	0,378	0,692	0,740	0,524	0,433	0,761
Residual sugar (g/L)	-0,449	-0,153	0,107	-0,014	0,145	-0,717	-0,668	-0,688	-0,243	-0,662	-0,228	-0,320
TPC (g/L)	0,867	0,726	0,485	0,777	0,334	0,554	0,470	0,728	0,708	0,622	0,624	0,809
tannins (mg/L)	0,477	0,668	0,578	0,803	0,462	-0,025	0,428	0,177	0,502	0,109	0,464	0,632

CONCLUSIONS

According to the obtained data, almost all the values of *physio-chemical properties* of wine samples were in data range of the Romanian and EU regulation. The FN wine samples registered higher values in alcohol content, volatile acidity, anthocyanins, TPC and tannins. The FA wine samples ranged higher values to total acidity and the BB wine samples ranged higher values to residual sugar and sulfites. The FN wine samples were the highest appreciated by *sensorial characteristics*. The *correlation* highlights that the most important parameters for common consumers are pH, TPC and anthocyanins and the lowest influence have been total acidity and sulfites.

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ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF SMALL PEPTIDES ISOLATED FROM MARINE ALGAE BY GREEN METHODS

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Abstract

This study aimed to investigate new green methods based on combined ultrasound- and enzyme-assisted technologies to isolate bioactive peptides from Cladophora vagabunda green macroalga. Also, their antioxidant and antiproliferative activity was analysed in view of biomedical applications. Algal powder was sonicated in alkaline buffer, pH 8, at 37 °C, for 1 h. The extract was treated with proteinase K and alcalase, respectively, to obtain two protein hydrolysates. Each hydrolysate was fractionated by centrifugal ultrafiltration using filter membranes with molecular weight cut-off (MWCO) at 3 kDa, to separate small peptides. The fractions were analyzed for Trolox Equivalent Antioxidant Capacity (TEAC). The results showed higher capacity of hydrolysates obtained by alcalase treatment to scavenge free radicals. Additionally, the antiproliferative activity was evaluated in HT-29 tumor cells cultivated in the presence of peptides obtained by alcalase treatment and the results showed a decrease of cell viability below 70% after 48 h of cultivation. In conclusion, peptide fractions isolated by green techniques from C. vagabunda alga had significant biological activity and are recommended for further testing as therapeutic agents.

Key words*: algal peptides, Cladophora vagabunda, enzymatic hydrolysis, marine bioactive compounds, sonication.*

INTRODUCTION

High quantities of animal proteins are found in sources such as meat, eggs and milk, while vegetable proteins are found in soy and maize. Lately, the micro- and macroalgal biomass was considered a viable alternative source of proteins (Espinosa-Ramirez et al., 2023). The protein production from marine macrophyte algae (2.5-7.5 tons/ha/year) is higher than that from terrestrial cultures of soy (0.6-1.2 tons/ha/year) (O'Connor et al., 2022). Additionally, proteins from edible seaweeds are nutritionally important, containing high quantities of essential amino acids like lysine, tryptophan, methionine, valine, thus being recommended for human diet (Kazir et al., 2019). The protein extraction yield represented up to 47% when using red algae species (Rhodophyceae) and up to 29% in green (Chlorophyceae) and brown (Phaeophyceae) algae species, the quantity varying according to geographical area and harvesting season (Vieira et al., 2018). However, traditional technologies for algal protein extraction are time consuming and economically unviable (Bleakley & Hayes, 2017).

Currently developed technologies for bioactive compounds extraction from algal biomass are based on green methods of ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and enzyme-assisted extraction (EAE). In case of UAE, the effect of acoustic cavitation could improve seaweed proteins bioavailability by formation and growth of vapor bubbles and facilitating penetration of solvent into the cells to release intracellular compounds (Arya et al., 2023). An ultrasound-assisted treatment in acid or alkaline conditions was efficient for protein extraction from *Ascophyllum nodosum* brown alga (Pan-utai et al., 2023). On the other side, MAE takes place by transfer of microwaves energy to polar water molecules, increasing their vibration, collision, and intracellular heating, and thus leading to an effect of pressurization and algal cell membrane damage (Grosso et al., 2015). EAE using enzymes (cellulases, agarases, xylanase, carragenase) for digestion of cellulose-type polysaccharides (galactans, xylans, fucoidans, laminarin, alginates, carrageenan) present in the

rigid cell wall of algal cells were useful in protein extraction from red alga species *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata* (Fleurence et al., 1995).
Green algae present a chemical composition rich in polysaccharides, phenolic compounds, proteins and amino acids, fatty acids, vitamins and minerals (Lafarga et al., 2020). Extracts of various green algae demonstrated immonomodulatory, antimicrobial, anti-inflammatory and antitumoral activity (Surayot et al., 2016). The genus Cladophora is comprising marine green macrophyte algae with filamentous branched multinucleate cells, widespread from temperate to arctic and tropical waters (Michalak & Messyasz, 2020). *Cladophora vagabunda* is abundantly found in the Black Sea, it has an invasive tendency, but it is still an underexploited biological resource (Marin & Timofte, 2011). Cladophora biomass was reported as valuable raw material for agriculture and cosmetics (Messyasz et al., 2015) and extraction of volatile compounds and fatty acids from *C. vagabunda* indicated applications as bioadditives and biopreservatives (Horincar et al., 2014). In this context, the present paper aimed to investigate new green methods for the isolation of bioactive peptides from *C. vagabunda* macroalga and their antioxidant and antiproliferative activity, in view of valorization as natural ingredients with biomedical applications.

MATERIALS AND METHODS

Biological material and chemical reagents

The biological material consisted of *C. vagabunda* green macrophyte alga collected from the Black Sea between 2 Mai and Vama Veche, in August 2022. The algae were extensively washed in cold tap water to remove the sand, dried in an oven (Memmert, Germany) at 30 °C and ground using an electric grinder until a fine green powder was obtained.
All chemical reagents used in this study were of analytical grade. 2,4,6-trinitrobenzene sulfonic acid (TNBS), 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), proteinase K (E.C. 3.4.21.64), alcalase (E.C. 3.4.21.14), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated. Human HT-29 epithelial cell line derived from colorectal adenocarcinoma, Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS) and penicillin-streptomycin-neomycin antibiotic mixture (PSN) were purchased from Sigma-Aldrich (Germany).

Extraction of algal proteins based on ultrasound-assisted technology

The extraction of proteins from *C. vagabunda* powder was carried out in TES buffer 0.05 M, pH 8 (1:20, m/v) by ultrasonication in a sonication bath (Elma, Germany), at 60 kHz, at a temperature of 37 °C, for 1 h (Figure 1). The temperature was controlled throughout the process not to exceed 37 °C, by sequencing the ultrasonication process in 5 on-off cycles, each lasting 10 min with a break of 2 min. Then, the process continued by magnetic stirring, at 4 °C, for 24 h. The extraction was repeated twice using the residue obtained after centrifugation of the solution at 9000 g, at 4 °C, for 20 min, and all supernatants were pooled together (P1).
The extraction yield was determined on a dry weight (d.w.) basis using the following formula:

$$\text{extraction yield (\%)} = \text{d.w.}_{\text{final}} / \text{d.w.}_{\text{initial}} \times 100$$

The protein content was determined by BCA assay, as previously described (Mihai et al., 2021).

Preparation and purification of algal peptides by enzyme-assisted technology

Enzymatic hydrolysis of protein extract (P1) was carried out using specific neutral proteases of microbial origin under specific conditions of pH and temperature. Thus, the algal extract aliquots were treated with 3% proteinase K in 0.05 M Tris buffer, pH=8, supplemented with 1 mM $CaCl_2$ (PP1) and 3% alcalase in 0.05 M Tris buffer, pH=8 (PA1), respectively (Figure 1). The mixtures were incubated in a shaking water bath (Witeg, Germany), at 55 °C, for 3 h. The pH of the reaction mixture was periodically verified throughout the enzymatic process and maintained at the initial optimal value. At the end of the incubation period, the mixtures were heated at 95 °C for 5 min, to inactivate the enzyme. Then, the solutions were cooled at room temperature and centrifuged at 9000 g, at 4 °C, for 20 min. Each hydrolysate was

subjected to centrifugal ultrafiltration using cellulose membrane filter units with MWCO at 3 kDa (Amicon, Germany) centrifuged at 7500 g in successive cycles of 30 min, according to manufacturer's instructions. Thus, two fractions were separated from each hydrolysate, the permeates (PP1 and PA1 fractions with MW<3 kDa) and the retentates (PP1 and PA1 fractions with MW>3 kDa).

Determination of the degree of hydrolysis

The degree of hydrolysis was determined as free amino groups quantified by TNBS assay, as previously described (Craciunescu et al., 2011). Thus, samples of hydrolysates (PP1, PA1) and the non-hydrolysed protein extract (P1) (0.25 ml) were mixed with 0.05% TNBS in 0.05 M TES buffer, pH=8 (1 ml) and incubated in a shaking water bath (Witeg, Germany) at 50 °C, in the dark, for 1 h. Then, 0.1 N HCl (3 mL) was added and the incubation continued in the water bath, at 50 °C, in the dark, for 10 min. After cooling at room temperature, distilled water (5 mL) was added. The absorbance (Abs) was read in quartz cuvettes at a wavelength of 346 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). Total hydrolyzate was prepared by P1 incubation in 6 N HCl, at 110 °C, 24 h. The degree of hydrolysis was calculated using the following formula:

$$\text{degree of hydrolysis (\%)} = (Abs_{hydrolysate} - Abs_{non\text{-}hydrolysed}) / (Abs_{total\ hydrolysate} - Abs_{non\text{-}hydrolysed}) \times 100$$

Determination of antioxidant capacity

The antioxidant capacity was determined by TEAC assay, as previously described (Gaspar-Pintiliescu et al., 2022). A stock solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1, v/v) and incubation at room temperature, in the dark, for 16 h, to allow free ABTS radicals formation. For the experiment, the stock solution was diluted with distilled water to give an Abs of 0.70 ± 0.02 at a wavelength of 734 nm (blank). The samples (100 µL) were mixed with ABTS reagent (1 mL) and incubated at room temperature, in the dark, for 10 min. The Abs of the reaction mixtures was read at 734 nm using a V650 UV-VIS spectrophotometer (Jasco, Japan). A standard curve was built using Trolox, a synthetic antioxidant, in the range of concentrations of 10-150 µM. The antioxidant capacity was calculated using the following formula:

$$TEAC = (Abs_{blank} - Abs_{sample}) / Abs_{blank} \times 100$$

The results were expressed as mM Trolox equivalents (TE) per g d.w.

Determination of antiproliferative activity

Cell culture and treatment

Human tumor HT-29 epithelial cells were seeded in the wells of a 96-well culture plate at a density of $5x10^4$ cells/mL and cultured in DMEM medium supplemented with 10% FCS, 1% glutamine, and 1% PSN antibiotic mixture. The plates were incubated in standard conditions, at a temperature of 37 °C, in 5% CO_2 atmosphere, for 24 h, to allow cell adhesion. Then, the culture medium was replaced with fresh culture medium supplemented with FCS containing different concentrations of algal peptides in the concentration range of 0.16-2.5 mg/mL. The plates were incubated in standard conditions for 24 and 48 h, respectively. The cells cultivated in MEM medium without treatment, in standard conditions served as negative control.

MTT assay

Cell proliferation was evaluated by MTT assay based on the reduction of yellow tetrazolium salts to purple insoluble formazan by succinate mitochondrial dehydrogenases in metabolically viable cells, a reaction mediated by the reduction NADH to NADPH (Oprita et al., 2008). Thus, at the end of the incubation period, the culture medium in each well was replaced with 0.25 mg/mL MTT solution (100 µL) and the plates were incubated at 37 °C, in 5% CO_2 atmosphere, for 3 h. Then, the MTT solution was replaced with isopropanol (100 µL) to solubilize the formazan crystals by gentle stirring, on a horizontal shaker, for 15 min. The Abs was read at a wavelength of 570 nm using a SpectroStar Nano microplate reader (BMG Labtech, Germany). The Abs values were directly proportional to the number of viable cells. Cell proliferation was calculated using the following formula:

$$\text{cell proliferation (\%)} = Abs_{sample} / Abs_{control} \times 100$$

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n=3). Statistical analysis was performed by two-tailed, two-sample equal variance Student *t*-test on control-sample pairs of interest. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSIONS

In the present study, an experimental protocol based on combined green technologies of UAE and EAE was optimized at laboratory level for sustainable valorization of *C. vagabunda* green macrophyte algal biomass to obtain peptide fractions with bioactivity towards free radicals scavenging and antiproliferative activity in tumor cell culture, in order to be used as natural therapeutic agents (Figure 1).

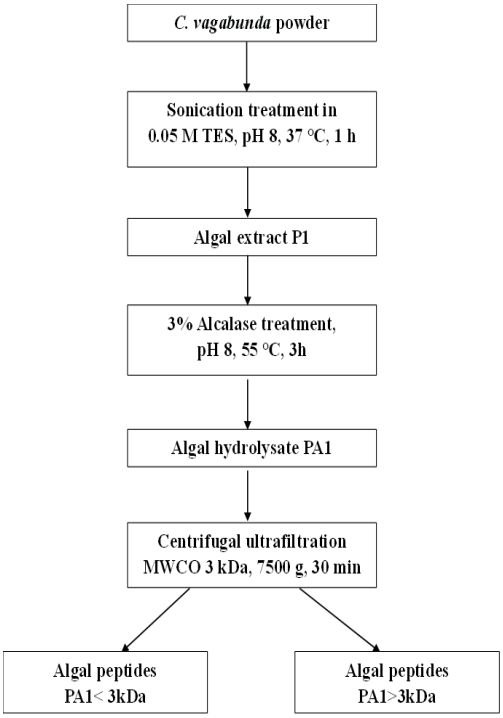

Figure 1. Scheme of combined UAE and EAE technologies to isolate algal bioactive peptides under controlled conditions from *C. vagabunda*

Preparation of algal proteins by UAE

First, an algal protein extract was obtained from *C. vagabunda* green alga powder by ultrasonication with a yield of 23.68%, calculated on a d.w. basis. This value was similar to data reported in previous studies on different green algae from *Ulva* species (7-29%) (Pimentel et al., 2019).

This high extraction rate confirmed the efficacy of the sonication-based optimized technology. It is known that macrophyte algae are characterized by the presence of polysaccharidic cell walls that play an important role in mechanical shear resistance (Shao & Duan, 2022). As a result, in the present study, the extraction of proteins from *C. vagabunda* green macroalga was conducted by ultrasonication treatment at low frequency and high intensity (20-100 kHz), which allowed the destruction of cell walls for improving the bioactive compounds extraction yield. Additionally, the ultrasound-assisted technology applied for a short processing period allowed extraction of thermolabile proteins without damaging their structure and properties, in accordance to previous reports (Ojha et al., 2020; Prandi et al., 2022). The alkaline buffer (pH=8) improved the solubilization of large peptides and proteins from cell cytoplasm and break down of their globular structure, but also facilitated the easy release of small peptides and free amino acids from algal biomass. Similar studies reported that the yield of protein extraction from *Nannochloropsis* spp. was doubled in alkaline solvents (pH=8), compared to water (O'Connor et al., 2022; Parniakov et al., 2015).

Preparation of algal peptides by EAE

Algal peptides were obtained using specific neutral proteases (3% proteinase K and 3% alcalase solutions) with broad action and stability over large pH and temperature domains, to ensure controlled degradation (Figure 1). An indicator of protein degradation and peptides formation is the number of free amino groups, determined in the present study by TNBS assay. The degree of enzymatic hydrolysis of algal proteins reached values of 9.22% under proteinase K treatment (PP1) and 45.87% under alcalase treatment (PA1). These data indicated an efficient hydrolysis of *C. vagabunda* protein extract by alcalase under mild optimal reaction conditions (pH 8, 55 °C, 3 h).

Each hydrolysate was subjected to centrifugal ultrafiltration through membranes with MWCO of 3 kDa, which allowed separation of 2 fractions (permeates with MW<3 kDa and

retentates with MW>3 kDa). The results of protein content analysis in permeates and retentates are given in Table 1.

Table 1. Protein content of protein hydrolysate and peptide fractions from *C. vagabunda*

Protein content (mg/g d.w.)	
Proteinase K hydrolysate	
PP1 (total hydrolysate)	54.52 ± 2.74
PP1 < 3 kDa (permeate)	20.10 ± 1.22
PP1 > 3 kDa (retentate)	106.17 ± 7.91
Alcalase hydrolysate	
PA1 (total hydrolysate)	73.91 ± 3.35
PA1 < 3kDa (permeate)	44.52 ± 2.42
PA1 > 3kDa (retentate)	182.49 ± 7.63

The results represent mean ± SD (n=3).

The results showed that alcalase hydrolysate (PA1) had higher protein content (73.91 mg/g d.w.), compared to proteinase K hydrolysate (PP1) (54.52 mg/g d.w.). Also, peptide fractions isolated from alcalase hydrolysate had ~2-fold higher protein content than those isolated from proteinase K hydrolysate (Table 1). All these data allowed selection of alcalase as optimal protease for preparation of algal peptides from *C. vagabunda* and obtained fractions were further investigated for bioactivity *in vitro*.

Antioxidant capacity of *C. vagabunda* peptide fractions

The high protein content of macrophyte algae and their antioxidant activity is an important reason for separation and valorization in biomedical applications (Surayot et al., 2016).

The antioxidant capacity of peptide fractions isolated from *C. vagabunda* green alga by UAE and alcalase treatment was determined by TEAC assay. The results are presented in Table 2.

Table 2. Antioxidant capacity of alcalase hydrolysate and peptide fractions from *C. vagabunda*

Sample	TEAC (mM TE/g d.w.)
PA1 (total hydrolysate)	163.78 ± 7.34
PA1<3 kDa (permeate)	123.67 ± 5.91
PA1>3 kDa (retentate)	139.43 ± 4.25

The results represent mean ± SD (n=3).

The results showed that all peptide samples presented high potential to scavenge free ABTS radicals. It was observed a decrease of the antioxidant capacity of peptides from permeate fraction, compared to alcalase hydrolysate and retentate. This could be due to smaller size of peptides from permeate containing more amino and carboxyl terminal groups, which decreased the antioxidant activity, as previously reported (Zhao et al., 2020).

The antioxidant activity of peptides could be correlated to their MW, but also to the hydrophobic nature of the amino acids present in their sequences. Thus, in the case of VECYGPRPQF peptide isolated from *Chlorella vulgaris* green macrophyte alga, rich in hydrophobic and aromatic amino acids, it was reported a potent antioxidant activity (Sheih et al., 2010). In the present study, the use of alcalase for algal protein hydrolysis could lead to hydrophobic peptides with increased antioxidant activity due to its known activity of preferential cleavage of the protein before aromatic amino acids, compared to proteinase K hydrolysis that had broader specificity and also cleaved before aliphatic residues and glycine residues.

Previous studies on *Palmaria palmata* macrophyte alga identified mycosporine-like amino acids, such as palythine, porphyra-334, shinorine-330, asterin, palythinol, palythene, usujirene, produced as low MW water-soluble secondary metabolites (Yuan et al., 2009). Their structural characteristics consisting of a cyclohexanone or hexenimine core conjugated to the nitrogen of an amino acid allowed significant antioxidant activity (Bedoux et al., 2014). Similar molecules were isolated from *Porphyra umbilicalis* macrophyte alga and used to obtain Helioguard 365® sunscreen, exhibiting antioxidant activity, capacity to absorb UV radiation and dissipate energy in the form of heat (Pandey et al., 2017), limiting the negative effect of fotons on cellular components (Pimentel et al., 2019).

Antiproliferative activity of *C. vagabunda* peptide fractions

The antiproliferative activity of peptide fractions isolated from *C. vagabunda* green alga was assessed in human tumor HT-29 epithelial cells by MTT assay. The results are presented in Figure 2.

The results showed that alcalase-treated peptide fraction PA1<3 kDa presented similar Abs values at 24 h of cultivation and significantly ($p<0.05$) higher Abs values at 48 h of cultivation, compared to the control, indicating stimulation of cell proliferation. The algal hydrolysate PA1 and peptide fraction PA1>3 kDa could significantly ($p<0.05$) inhibit tumor cell proliferation after 48 h of cultivation, at all tested concentrations, compared to the control. The highest activity was 2-fold inhibition of cell proliferation at 2.5 mg/mL PA1>3 kDa, after 48 h of cultivation. *In vitro* data demonstrated the antiproliferative activity of algal bioactive peptides isolated from *C. vagabunda* in the experimental model based on human HT-29 colon epithelial cells. These *in vitro* results correlated direct proportional to the antioxidant activity of algal peptides and their MW.

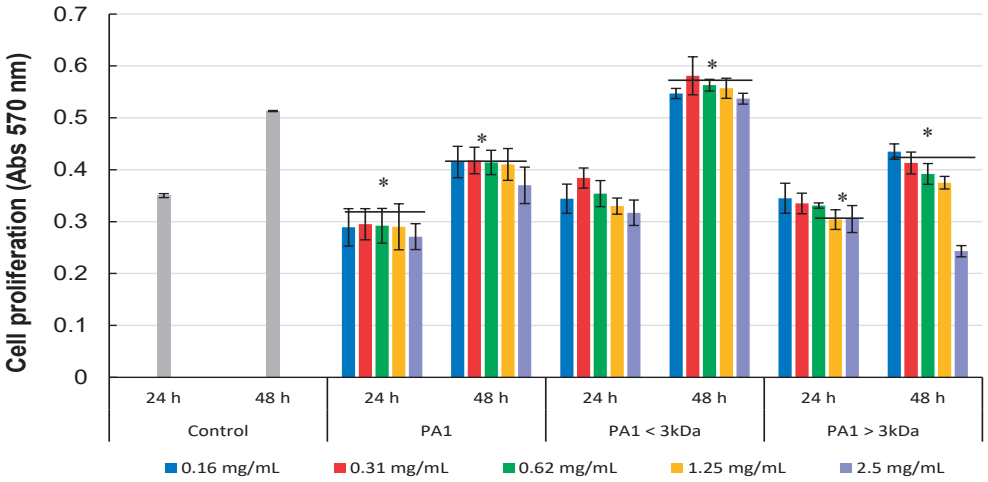

Figure 2. Antiproliferative activity of alcalase hydrolysate (PA1) and peptide fractions from *C. vagabunda* green alga. The results represent mean ± SD (n=3). *$p<0.05$, compared to control.

Previous studies have reported that two peptides VPGTPKNLDSPR and MPAPSCALPRSVVPPR identified in trypsin hydrolysate of *Porphyra haitanesis* protein extract revealed inhibition of human MCF-7 breast cancer cells and human HepG-2 liver cancer cells proliferation and cell cycle arrest inducing cell apoptosis (Fan et al., 2017). The study on papain hydrolysate of *Pyropia haitanensis* protein extract showed antiproliferation effect of QTDDNHSNVLWAGFSR peptide on human HepG-2 liver cancer cells at a concentration of 500 µg/mL that induced low inhibition of human normal liver cells (Mao et al., 2017).

CONCLUSIONS

Green biotechnologies based on combined UAE and EAE using specific neutral proteases (proteinase K, alcalase) were established to extract bioactive peptides from *C. vagabunda* green macroalga. Alcalase was selected as optimal enzyme for the isolation of peptide fractions from *C. vagabunda* green macroalga due to higher degree of hydrolysis, compared to proteinase K. The hydrolysate obtained by algal protein digestion using alcalase and peptide fractions isolated after centrifugal ultrafiltration showed significant antioxidant capacity as free ABTS radicals scavenging potential. The experimental model *in vitro* demonstrated that the isolated bioactive peptides could inhibit cell proliferation in human tumor HT-29 epithelial cells.

In conclusion, the algal peptides isolated from *C. vagabunda* had significant biological activity and are recommended for further testing as therapeutic agents.

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MICROSCOPIC CHARACTERISTICS OF RHIZOMES OF *Curcuma longa* AND *Zingiber officinale* (Zingiberaceae) - A SELECTION OF LIGHT MICROSCOPY IMAGES

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Abstract

The Zingiberaceae, the ginger family, is a family of monocotyledonous herbaceous plants with creeping horizontal or tuberous rhizomes. The plants are aromatic, characterized by the presence of volatile oils and oleoresins. The Zingiberaceae are especially abundant in Southeast Asia and they have been widely used as spices, ornamental, or medicinal plants. The most important commercial spices in the family are ginger (Zingiber officinale), turmeric (Curcuma longa), green cardamom (Elettaria cardamomum) and black cardamom (Amomum subulatum), that also have a great number of health properties. Due to their use in the food and pharmaceutical industries, the microscopic analysis and other parameters of the whole rhizomes/powder of turmeric and ginger provide valuable information in the identification of the plant material, followed by biochemical analysis. In the current study, a microscopic analysis of ginger and turmeric rhizome was carried out to record some of the parameters of taxonomic relevance using a simple method.

Key words*: ginger, light microscopy, microscopic images, rhizome, turmeric.*

INTRODUCTION

Zingiberaceae is a family of monocotyledonous plants in the Order Zingiberales that includes perennial aromatic plants with horizontal rhizomes, comprising of about 53 genera and more than 1200 species, with pantropical, but mainly Indomalayan distribution (Pandey et al., 2023). The type genus of the family is *Zingiber* (ginger), the source of the aromatic "ginger root", which is an important spice. The rhizome (underground stem) of the edible ginger (*Zingiber officinale*) is harvested every year and is used fresh or dried and ground into a powder. Similarly, aromatic rhizomes of *Curcuma longa* (turmeric) are used fresh or boiled in water and dried, then grounded into powder. The orange-yellow turmeric powder, rich in curcumin, has been used as a flavour and colouring agent in many Asian dishes since ancient times.

Some other Zingiberaceae species have seeds used as a spice, for example *Elettaria cardamomum*, commonly known as green cardamom or true cardamom and *Amomum subulatum*, known as black cardamom.

The rhizomes of *Zingiber officinale* and *Curcuma longa* contain volatile compounds (essential oil) and non-volatile compounds, including oleoresin and other biological active compounds, some compounds have application in the pharmaceutical and cosmetics industries (Gîrd et al., 2009).

Other plants of the family have also important economic uses as source of food, condiments and spices, medicinal plants used in traditional medicine or the pharmaceutical industry, or as horticultural ornamentals.

Various parameters, including plant morphology, microscopy and phytochemical profiles of plant material or their powder are used to characterise the true plant material and ensure its quality using standard techniques (several reference books are listed in Amel, 2015).

The current study presents a microscopic analysis of fresh rhizomes of *Zingiber officinale* and *Curcuma longa* to record some of the parameters of taxonomic relevance using a simple method.

MATERIALS AND METHODS

The biological material was obtained commercially in June 2021 and in May-June 2022 and was brought fresh to the Laboratory of

Biology of the Faculty of Biotechnology (UASVM Bucharest) where the practical activity was carried out. A Micros Austria optical microscope equipped with an S-Eye 2.0 digital camera was used for the microscopic analysis, but some of the pictures were taken with a Sony digital camera (Sony Cyber-shot®, Carl Zeiss Vario-Tessar 5× zoom lens).
Wet mount slides of hand section or surface preparations were used according to the techniques described by Andrei & Paraschivoiu (2003) with or without staining (toluidine blue and iodine tincture). Relevant microscopic structures were identified using the descriptions found in the literature for *Curcuma longa* and *Zingiber officinale*: Abraham et al. (2018) for *Curcuma longa* and Aye (2020) and Prakash et al. (2011) for *Zingiber officinale*.

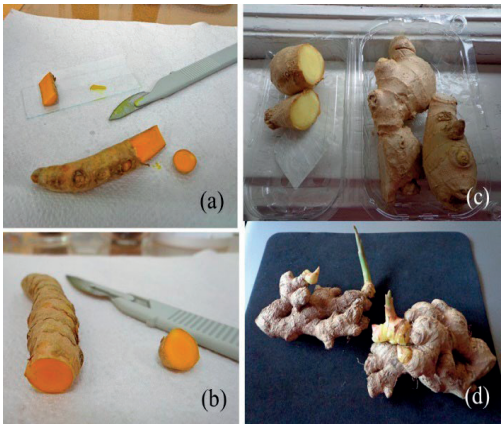

Figure 1. The biological material used in the present work: (a) rhizome of *Curcuma longa* (turmeric); (b)-(d) rhizome of *Zingiber officinale* (ginger)

RESULTS AND DISCUSSIONS

Microscopic analysis of turmeric rhizome

Images of the hand sections through the rhizome of *Curcuma longa* show several structures, as follows:
In transverse sections:

- epidermis formed by epidermal prosenchymatous cells can be seen;
- cork - made of 5-6 layers of cells (Fig. 2);
- cortical parenchyma - consisting of large, thin-walled parenchymal cells (Figs. 3-5);
- distinct endodermal cells (Fig. 6);
- the pith consists of parenchymal cells similar to those in the cortex.

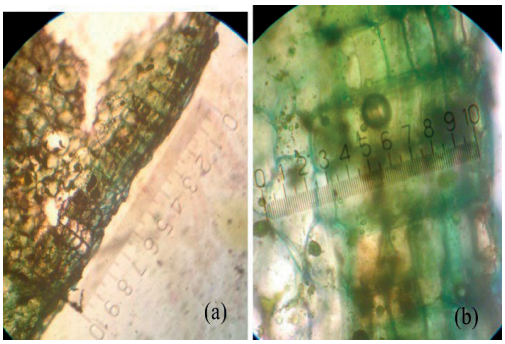

Figure 2. Microscopic images showing cork layers in a transverse section through the rhizome of *Curcuma longa* (toluidine blue stain), (a) ob. 10×, (b) ob. 40×

The microscopic images show parenchymal cells with bright orange oily content - oleoresin (curcumin and volatile oil) (Figs. 3, 4, 5, 7), as well as parenchymal cells containing yellow lipid droplets, which almost fill the cells (Figs. 3, 5, 8, 9).

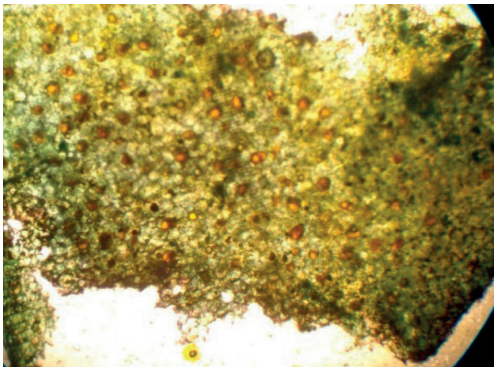

Figure 3. Transverse section through *Curcuma longa* rhizome - cork and cortical parenchyma

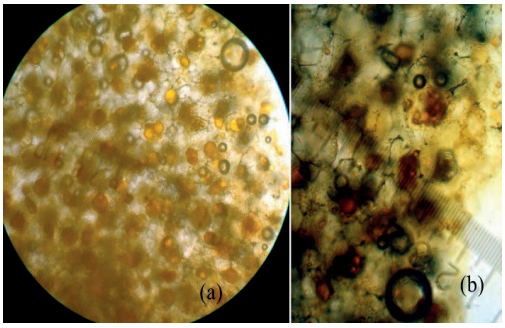

Figure 4. Transverse sections through *Curcuma longa* rhizome showing parenchymal cells with bright orange oily content due to curcumins, (a) ob. 10×, (b) ob. 40×

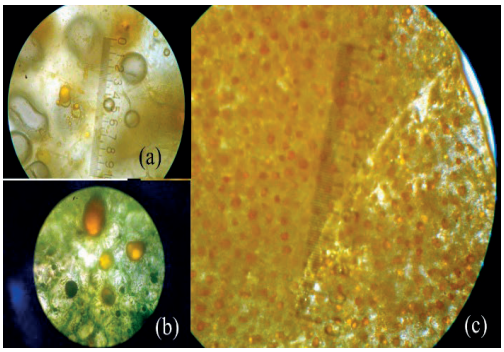

Figure 5. Transverse sections through *Curcuma longa* rhizome: microscopic images show the characteristic bright orange colour of curcumins (oleoresin) and the yellow colour of lipid droplets, (a)-(b) ob. 40×, (c) ob. 4×

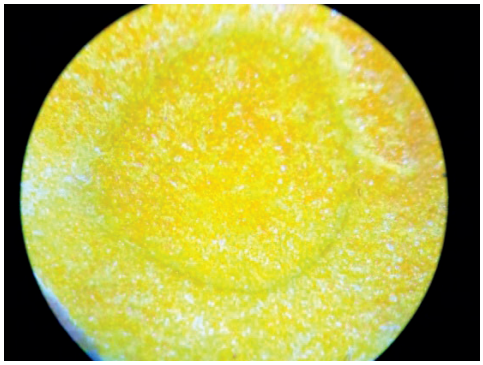

Figure 6. Transverse section through the rhizome of *Curcuma longa* - the endoderm is visible

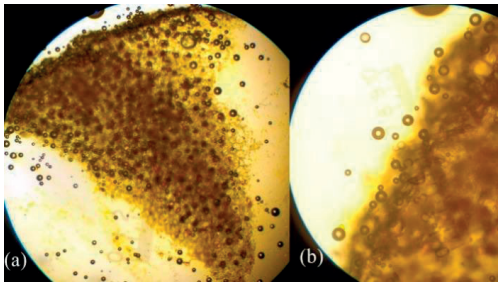

Figure 7. Light microscope images of transverse sections through *Curcuma longa* rhizome: (a)-(b) numerous dispersed oil droplets

Numerous starch granules are also present (Figs. 8, 9). Starch granules are simple, elongated, with the hilus towards the narrow end of the granule. The number and size of starch granules is an important character in the identification of *Curcuma* species (Abraham et al., 2018).

In the longitudinal sections, wide vessels with spiral thickening can be seen.

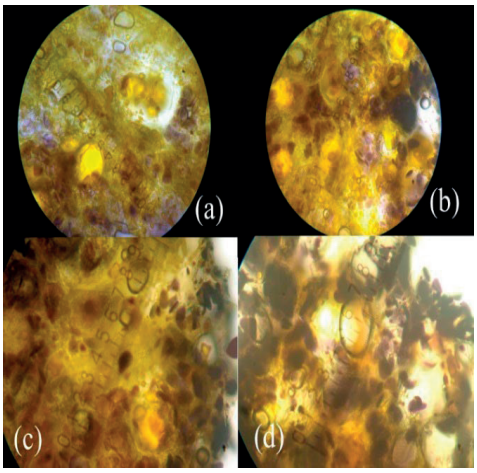

Figure 8. Light microscope images of transverse sections through *Curcuma longa* rhizome: (a)-(d) parenchymal cells with starch granules (stained violet with iodine tincture); (detail - ob. 40×)

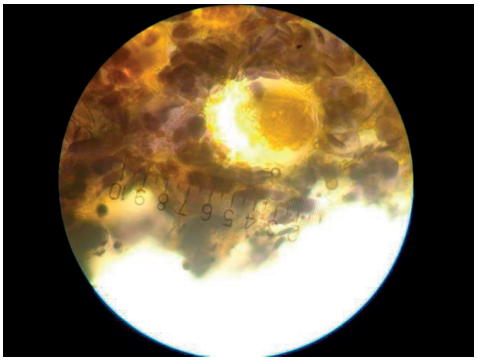

Figure 9. *Curcuma longa* rhizome – microscopic image of transverse section showing a parenchymal cell with yellow oily content and parenchymal cells filled with starch granules (iodine tincture, detail - ob. 40×)

Microscopic analysis of ginger rhizome

Surface preparations/hand sections of ginger rhizome (*Zingiber officinale*) showed several structures:

- cork on the the outside (Fig. 10);
- parenchyma that includes cells with yellow oil (oleoresins) and cells with starch granules (Figs. 11-14);
- abundant, simple, globoid starch granules, with a size of ~ 11 µm (Figs. 12, 14);

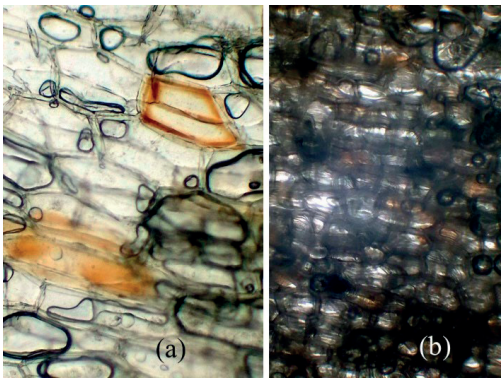

Figure 10. Light microscope images of ginger rhizome (*Zingiber officinale*): (a)-(b) cork cells in apical view

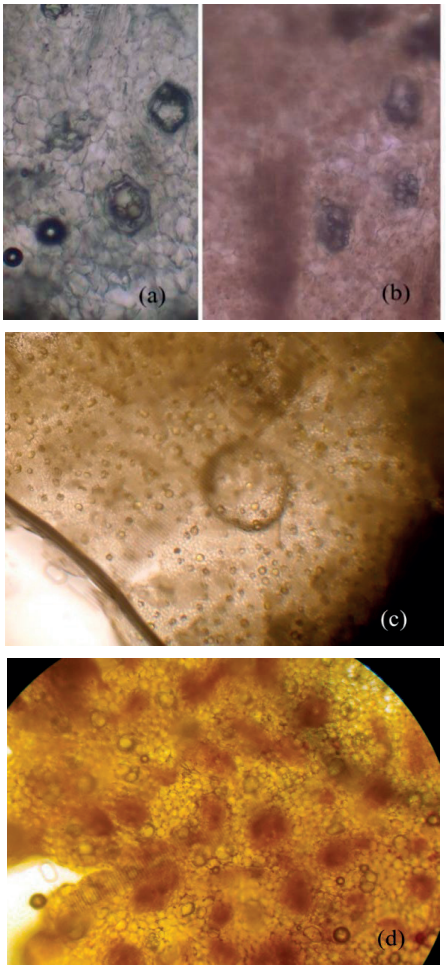

Figure 11. Different microscopic images in transverse sections through the rhizome of *Zingiber officinale:* (a)-(d) parenchyma cells with yellow oil and cells with starch granule

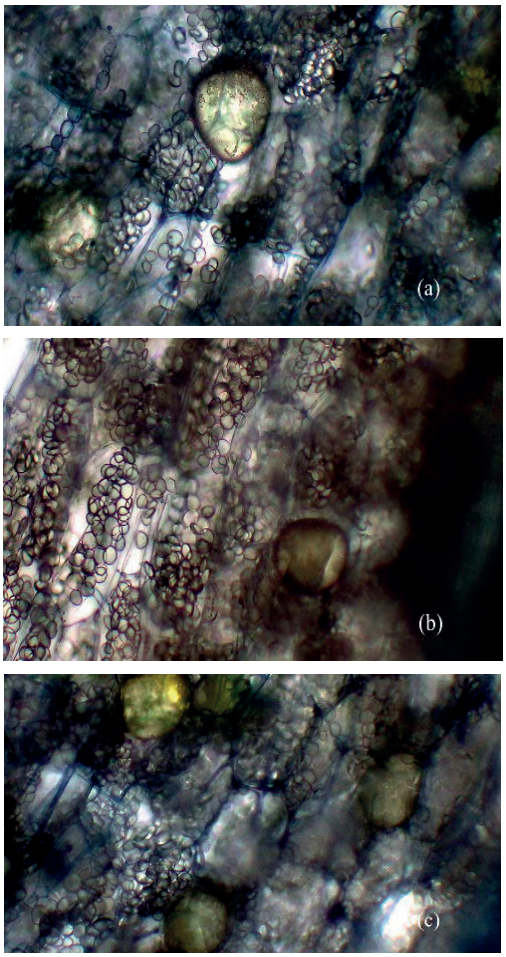

Figure 12. Light microscope images of longitudinal sections through the rhizome of *Zingiber officinale*:. (a)-(c) amyliferous parenchyma and yellow oleoresins

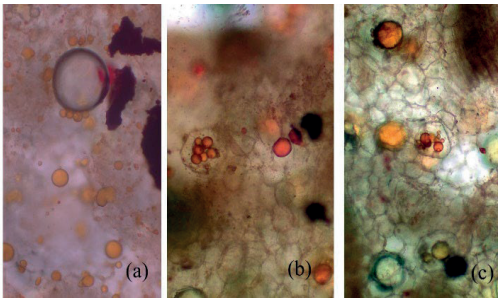

Figure 13. Sections through ginger rhizome: (a)-(c) microscopic images after histochemical staining for lipids (Sudan III)

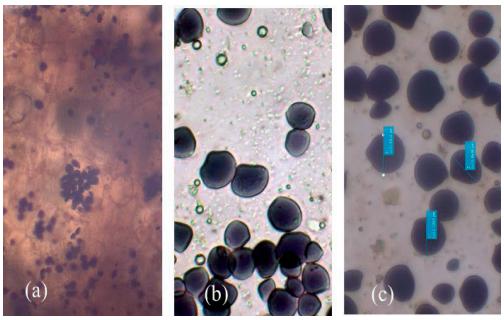

Figure 14. Section through the rhizome of *Zingiber officinale* (iodine stain): (a) amyliferous parenchyma; (b)-(c) simple, globoid starch granules with a size of ~ 11 µm

Figure 15 shows conducting vessels with spiral and annular thickening in longitudinal sections through the rhizome of *Zingiber officinale*.

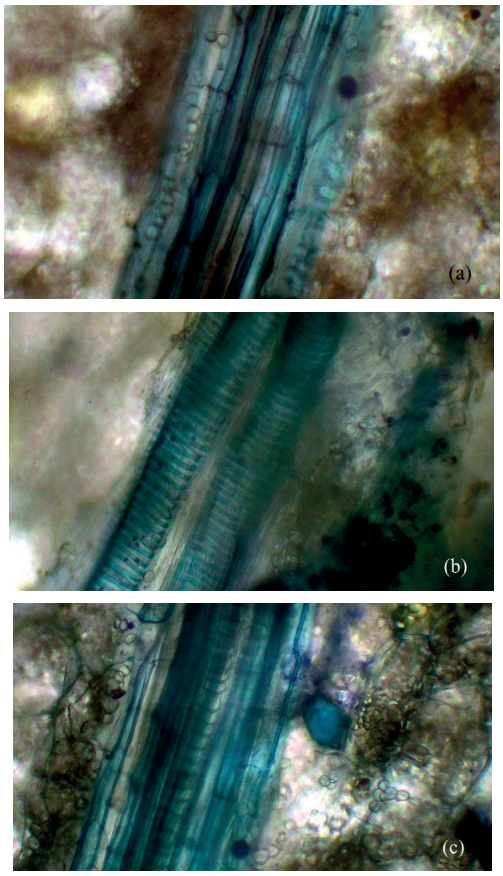

Figure 15. Microscopic images of longitudinal sections through the rhizome of *Zingiber officinale:* (a)-(c) conducting vessels (toluidine blue staining); (b) vessels with spiral thickening;(c) vessels with annular thickening

CONCLUSIONS

In this paper, several microscopic characteristics mentioned in the specialized literature for turmeric rhizome and ginger rhizome were shown, as follows:

In *Curucuma longa* (turmeric): the epidermis; cork; cortical parenchyma; parenchymal cells with bright orange oily content - oleoresin (curcumin and volatile oil); other parenchymal cells containing yellow lipid droplets, which almost fill the cells; amyliferous parenchyma, starch granules; endoderm; and conductive vessels.

In *Zingiber officinale* (ginger) section we detect: cork; cells with yellow oil (oleoresin); amyliferous parenchyma, starch granules; conductive vessels.

The substances in the composition of turmeric and ginger or the rhizomes as such are used in the food industry, but also in the pharmaceutical industry and the microscopic, macroscopic and organoleptic analysis of the rhizomes/powder of turmeric and ginger constitutes the first stage of the qualitative analysis aimed at the botanical identification of the plant material, followed by biochemical analysis, all of these analyses being carried out using standardised methods. Thus it is possible to detect the falsification of some products or the presence of some impurities in the plant material intended for food consumption or use in the pharmaceutical industry.

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BIOSURFACTANT PRODUCTION BY *Pseudomonas fluorescens* STRAIN

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Abstract

Biosurfactants are surface-active compounds synthesized by microorganisms as secondary metabolites with important applications in medicine, cosmetics, food, oil, agriculture, and the pharmaceutical industries. In the present study, the Pseudomonas fluorescens ICCF 392 strain was screened to determine its ability to produce extracellular biosurfactants. The strain was cultivated on M44 liquid medium (5% (v/v) glycerol as a carbon source) and also, on M44 modified medium (by replacing the glycerol as a carbon source with 5% (v/v) waste cooking oil). The supernatants obtained at the end of the bioprocesses were evaluated, to confirm the ability of the strain in biosurfactant production, using the drop collaps method, oil spreading technique, and emulsification activity determination (E_{24}). The best results were obtained in the case of the M44 liquid medium. The partially purified bioactive compounds were analyzed by the TLC method, which indicated the nature of the biosurfactants produced as rhamnolipids. Therefore, our results showed that the Pseudomonas fluorescens ICCF 392 strain was efficient in biosurfactant production, using glycerol or waste cooking oil as carbon sources in the biosynthesis process.

Key words*: Pseudomonas fluorescens,* rhamnolipids, glycerol, waste cooking oil, submerged fermentation.

INTRODUCTION

Biosurfactants are natural surface-active compounds produced by a variety of microorganisms, including bacteria, yeast, and fungi (Deshmukh et al. 2023; Roy, 2017).
Among them, species of the *Pseudomonas* genus are mostly utilized in the production of this significant class of bioactive compounds (Mohanty et al., 2021; Stancu, M.M., 2017).
Biosurfactants present several advantages over synthetic ones, such as lower toxicity, biodegradability, biocompatibility, high selectivity, stability over a broad pH and temperature range (Ciurko et al., 2023), and production from cheaper substrates (Balakrishnan et al., 2022).
Also, these bioactive molecules lower the interfacial and surface tensions of liquids, also are more stable and eco-friendly (Sharma et al., 2022).
Due to these physico-chemical characteristics, biosurfactants present potential applications in the biomedical field as antibacterial, antiviral, and antifungal compounds, in the cosmetic, food, detergents, textiles, petroleum recovery, bioremediation, and agriculture sectors (Anaukwu et al., 2020; Mandalenaki et al., 2021).
Usually, biosurfactants are typically amphipathic compounds that have both hydrophilic and hydrophobic elements. While hydrophobic compounds consist of a lengthy chain of fatty acids, hydrophilic compounds are typically composed of positive, negative, or amphoteric charged ions (Kumar et al., 2021).
Based on their molecular structure, biosurfactants can be glycolipids, lipopeptides, lipoproteins, phospholipids, or polymeric surfactants (Gurkok et al., 2021).
Of these, the most widely investigated class of biosurfactants are rhamnolipids, which belong

to the glycolipids group (Sarubbo et al., 2022). Parameters that play an important role in biosurfactant production are represented by the strain used, which must be non-pathogenic, the incubation conditions (temperature, aeration and agitation, time), and the carbon and nitrogen sources (Mandalenaki et al., 2021).

Presently, the high cost of the media components required in bioprocesses, such as glucose, limits the actual production of biosurfactants (Eras-Munoz et al., 2022). As an alternative, the use of glycerol or vegetable cooking oils as substrates in the fermentation processes becomes very important, taking into account that the amount of this waste increases year by year (Silva et al., 2010). Several studies from the literature have reported that *Pseudomonas* strains (especially *Pseudomonas aeruginosa)* are able to produce biosurfactants by using a variety of inexpensive substrates as carbon sources (Praveesh et al., 2011; Cássia et al., 2017).

Therefore, in this paper, potential substrates for the production of biosurfactants by the *Pseudomonas fluorescens* strain were assessed. These substrates, including glycerol and waste sunflower oil, were utilized as carbon sources in two different liquid media.

MATERIALS AND METHODS

Biologic material

The *Pseudomonas fluorescens* ICCF 392 strain used in this study, maintained on M44 agar medium, belongs to the Collection of Microorganisms of Industrial Importance-CMII–ICCF-WFCC 232.

Fermentation media and cultivation conditions

The bacterial strain used as the pre-inoculum was cultured on M44 agar medium with the following composition % (g/v): glycerol 5.0, yeast extract 1.0, bacto-peptone 1.0, and agar 2.0, and incubated for 48-72 hours at 30°C.

In order to prepare the inoculum, 2.0 mL of pre-inoculum containing $9x10^8$-CFU/mL (McFarland Standard No. 3) was seeded into 100 mL of liquid medium in a 500 mL Erlenmeyer flask. The inoculum medium was then incubated for 24 hours at 30°C and 220 rpm on a rotary shaker.

For biosurfactant production, the following liquid media were used:

- M44 medium (without agar) % (g/v): glycerol 5.0, as a carbon source; yeast extract 1.0, and bacto-peptone 1.0, as nitrogen sources.
- M44 modified medium % (g/v): waste sunflower oil 5.0, as a carbon source; yeast extract 1.0, and bacto-peptone 1.0, as nitrogen sources.

The chemicals were purchased from Difco (USA) and Sigma-Aldrich (Germany). The culture media were prepared with distilled water, pH-adjusted to 6.5-7.0, and sterilized for 30 minutes at 115°C.

The biosurfactant production was performed in 500 mL Erlenmeyer flasks containing 100 mL of the liquid medium inoculated with 10% (v/v) of inoculum. The flasks were incubated at 30°C, 220 rpm, for 72 hours. Bacterial cell growth was determined by measuring the pH and optical density of the culture medium at 550 nm.

Detection of biosurfactant production

Three methods were used for the detection of biosurfactant production, as follows:

Drop collapse method

The drop-collapse test was performed in accordance with Ghazal et al. (2017): a droplet containing 25 µl of extracted biosurfactant was pipetted onto parafilm and allowed to flatten and spread over the surface for a few seconds or minutes. The water-stain droplet was then mixed with methylene blue, which had no effect on the droplet shape. After a period of drying, the diameter of the droplet was measured. The presence of biosurfactant in solution gave flat drops, while the absence of biosurfactant gave rounded droplets.

Oil displacement assay

After adding a biosurfactant-containing solution to an oil-water interphase, the diameter of the clear zone can be measured using the oil displacement method (Balakrishnan et al., 2022). In a Petri dish, 30 mL of distilled water were added and 1 mL of sunflower oil was added to the center of the plates. Over the oil layer, 20 µl of the culture supernatant obtained from the bacterial broth was added.

A zone of displacement in the oil was carefully observed in the Petri dishes, and the diameter of

displacement was measured and compared with the control using the uninoculated medium, after 30 seconds (Arora et al., 2015).

Emulsification activity

The production of biosurfactants by the *Pseudomonas fluorescens* ICCF 392 strain was assessed through submerged fermentations using 500 mL flasks containing 100 mL medium on a rotary shaker at 220 rpm. The strain was grown in M44 and M44 modified liquid media for 72 hours at 30°C.

After the culture broths were centrifuged for 20 minutes at 4°C and 9000 rpm, the emulsification index (E24) was calculated. In different tubes, 4 mL of the supernatant was mixed with 6 mL of heptane, octane, and sunflower oil. The tubes were then vigorously mixed for five minutes and stored for twenty-four hours (Pathak et al., 2014).

Using the formula E_{24} % = (height of the emulsified layer/total height of the liquid column) × 100, the emulsification index was determined.

The experiments were performed in triplicate.

Biosurfactant isolation

Bacterial cells were removed by centrifugation at 9000 rpm, 4°C, for 20 min.

In order to precipitate the biosurfactant, the cell-free supernatant was acidified with HCl concentrated to pH 2.0 and left overnight at 4°C.

The precipitate thus obtained by centrifugation in similar conditions was extracted using a 4-fold volume of ethyl acetate.

The lower organic phase, containing biosurfactant, was collected by using a separating funnel.

By using rotary evaporation at 40°C and low pressure, the solvent was removed.

A partially purified viscous honey-colored rhamnolipid product was collected after solvent evaporation and used for characterization (Invally et al., 2019).

Biosurfactant characterization by thin-layer-chromatography

The preliminary characterization of biosurfactants was performed by TLC analysis. Silica gel 60 F_{254} TLC plates (10 x 20 cm; Merck Millipore) were used for further analysis. Rhamnose was separated on silica gel plates using water, methanol, glacial acetic acid, and 1.2-dichloroethane (10:15:25:50, v/v/v/v) as the mobile phase.

Rhamnose spots were visualized by spraying with a thymol-EtOH-H_2SO_4conc. (0.5g, 95mL, 5mL) reagent. Plates were heated at 130°C for 10 minutes after the application of the spraying agent (Schenk et al., 1995).

Also, lipids were separated on silica gel plates using n-heptane, ethyl ether, acetic acid (70:25:5, v/v/v) as the mobile phase. Lipid spots were visualized by spraying with a 0.5% solution of vanillin in a mixture of 5 mL of sulfuric acid, 5 mL of phosphoric acid, and 90 mL of ethyl alcohol. Plates were heated at 130°C for 10 minutes after the application of the spraying agent (Avinesh et al., 2016).

RESULTS AND DISCUSSIONS

Drop collapse method

According to the drop-collapse method, an oily surface will cause a drop of liquid containing a biosurfactant to collapse and spread. The results of the collapse test revealed that the *Pseudomonas fluorescens* ICCF 392 strain was capable of producing biosurfactant on both substrates evaluated. For distilled water (control), no activity was detected. The droplets remain round, with a diameter of 0.4 mm. The biosurfactant droplets do result in collapsed droplets with a diameter of 0.8 mm in the case of media obtained using glycerol as substrate and 0.7 mm for waste cooking oil substrate, respectively, showing their effects on the reduction of surface tension (Figure 1).

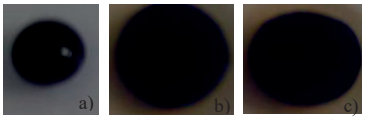

Figure 1. Detection of biosurfactant production by *Ps. fluorescens* ICCF 392 strain: drop collapse assay: a) control; b) M44 medium; c) M44 modified liquid medium

Oil displacement assay

The oil displacement method is used to measure the surface activity of a surfactant solution tested against oil; higher surfactant surface activity is indicated by a larger diameter of the displaced circle. The results of the oil displacement assay are shown in Figure 2. Both

samples produced by *Pseudomonas fluorescens* ICCF 392 strain had significantly displaced the oil layer and spread in the water; the largest diameter value of displaced circle of 7 cm was obtained in the case of media containing glycerol 5%, while the cultures obtained on media containing waste cooking oil 5% produced a displaced circle measuring 6.5 cm.

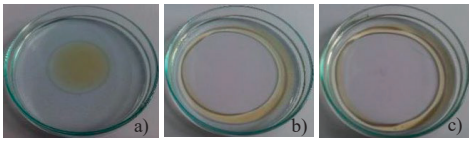

Figure 2. Detection of biosurfactant production by *Ps. fluorescens* ICCF 392 strain: oil displacement assay: a) control; b) M44 medium; c) M44 modified liquid medium

Emulsification Index

For 72 hours, bioprocesses were performed at 30°C. At the end of the process of biosynthesis, the media were centrifuged, and the ability of the supernatants to emulsify heptane, octane, and sunflower oil was assessed (Figure 3).

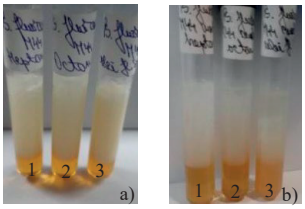

Figure 3. Emulsions obtained with 1-heptane, 2-octane, and 3-sunflower oil with the supernatants of *Pseudomonas fluorescens* ICCF 392 strain cultivated on: a) M44 liquid medium (b) M44 modified liquid medium

In Figure 3, the emulsions obtained with the supernatants of the *Pseudomonas fluorescens* ICCF 392 strain cultivated on M44 and M44 modified liquid media are presented.

Supernatants of *Pseudomonas fluorescens* ICCF 392 grown in M44 liquid medium produced stable emulsions with heptane, octane, and sunflower oil. After 24 hours, in the case of M44 liquid medium, the values of the emulsifying index were 72.46% for heptane, 75.71% for octane, and 72.58% for sunflower oil.

Regarding the M44 modified medium, the strain produced biosurfactant that emulsified heptane and octane, with an emulsifying index of 63.6% and 62.5%, respectively. Instead, regarding the emulsions obtained with sunflower oil, they registered slowly low values of the emulsifying index, at 61.3%. The emulsification index results (E_{24}%) obtained are shown in Figure 4.

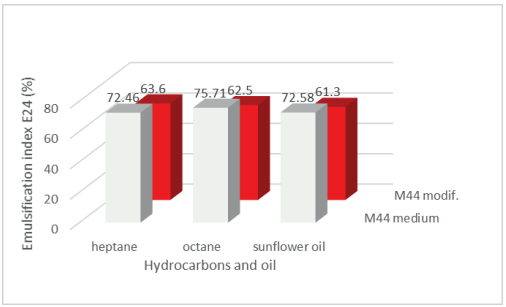

Figure 4. Emulsification index for heptane, octane, and sunflower oil obtained with supernatant of *Pseudomonas fluorescens* ICCF 392 cultivated on M44 and M44 modified liquid media

Therefore, the supernatants of the *Pseudomonas fluorescens* strain formed emulsions with heptane, octane, and sunflower oil, with the best values of the emulsification index obtained on M44 liquid medium containing glycerol at 5%, followed by those obtained using waste cooking oil at 5%.

The results show that the values of the emulsifying indices obtained are in accordance with the findings of Matátková et al. (2022), who reported an emulsification index of 70% for sunflower oil, and also with the results reported by Joice and Parthasarathi (2014), who obtained an emulsifying index of almost 70.0% for heptane. The emulsions obtained remained stable for over a month, and according to the literature, it is considered that emulsion stability is one of the most important characteristics of a biosurfactant (Anyanwu et al., 2011; Diab et al., 2016).

Biosurfactant characterization

The biosurfactants extracted were characterized by the TLC method. The analyzed samples in UV light show spots characteristic of the rhamnose compound, brown-violet-colored, identified at Rf~0.45 (Figure 5).

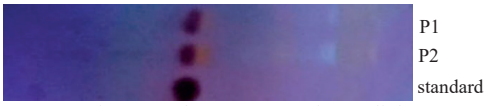

Figure 5. Rhamnose spots in UV light: P1- M44 medium; P2 –M44 modified medium; L(+)- Rhamnose - standard

Also, the biosurfactant fraction showed a positive reaction with the vanillin reagent, indicating the presence of lipid moieties. The chromatogram visualized after spraying with the identification reagent presented, in the two studied samples, multiple spots, pink-grey in color and of different intensities, at the following values: R_f~0.10; R_f~0.12; R_f~0.15; R_f~0.41; R_f~0.50; R_f~0.52; R_f~0.75; R_f~0.80; and R_f~0.92. Over time, the color of the spots changes to blue-grey and fades (Figure 6).

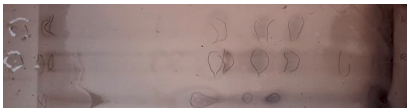

Figure 6. Spots of lipids after spraying with reagent: P1 - M44 medium; P2 - M44 modified medium; cholesterol - standard

The above results of TLC analysis demonstrated that the biosurfactant was a glycolipid composed of sugars and lipids. Similar reports of the production of rhamnolipids by *Pseudomonas* species (Rf values 0.4 and 0.37) are then in the literature (Moon et al., 2002; Rekadwad et al., 2019).

CONCLUSIONS

Our results demonstrate that the *Pseudomonas fluorescens* ICCF 392 strain is a good producer of biosurfactants on substrates containing glycerol and waste cooking oil as carbon sources at a concentration of 5%, suggesting the possibility of industrial production of biosurfactants using economically cheaper substrates. The biosurfactants isolated from the fermentation media were identified by TLC analysis as rhamnolipids and showed high emulsification activity, which makes them suitable for various industrial and environmental applications.
Thus, these preliminary results show that it is possible to reduce the costs associated with the raw materials used for biosynthesis, obtaining glycolipids with added industrial value. The biosurfactant-producing capacity of this strain will be further increased by optimization studies.

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EXPLORING CURRENT FRONTIERS OF ENVIRONMENTAL CHALLENGES BY BIOINDICATORS AND BIOMARKERS

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Abstract

Anthropic pressure on the environment threaten its biodiversity and subsequently, its sustainability. Nowadays, the research focusses on the development of a new efficient screenings regarding contaminants toxic impact on aquatic and terrestrial environments based on specific biological models, as bioindicators. Cellular and molecular biomarkers are considered to appear very fast under a pollution or climate change stress, before any significant changes at the organism level, and to be specific and sensitive indicators of environmental quality and adaptation mechanisms. Changes at the molecular level could provide rapid information and prediction patterns regarding the occurrence of lethal, sub-lethal, or adaptive effects on biotopes under the influence of anthropogenic stressors. The combined use of bioindicators and biomarkers could provide a comprehensive picture of aquatic and terrestrial ecosystems health status and they could facilitate to identify the environmental issues. In this review, we aimed to establish a relationship between pollution and specific adaptation mechanism responses at the molecular level such as biomarkers. Moreover, we analyzed how biomarkers respond to exposure to different toxic substances and exposure levels, determining dose-response relationships, and biomarker response time.

Key words*: bioindicators, biomarkers, ecotoxicity, biodiversity, anthropogenic stress.*

INTRODUCTION

The environment is constantly exposed to various anthropogenic pollutants generated by industrial, domestic, and agricultural activities. A wide range of pollutants are susceptible to interact with physiological processes such as growth and reproduction from aquatic and terrestrial organisms. Unfortunately, pollutants could massively alter their life, leading to serious disruptions of populations number and diversity due to impairing reproductive functions and adaptation mechanisms. Environmental resources, resilience and sustainability are major concerns to governments and researchers which try to identify and turn polluted ecosystems into clean environments by new environmental policies and ecofriendly methodologies. In this respect, monitoring programs are used to evaluate the pollution status followed by mitigation strategies. A fast and reliable monitoring system of pollutants triggers an early warning long before environmental damage extension. The ecotoxicological tests could predict the possible pollutant impact on the environment. The field of ecotoxicology is a complex scientific discipline based on principles of toxicology, biology, chemistry, and ecology, studying the effects of chemical compounds on living organisms other than humans. Ecotoxicology could evaluate pollutant dynamics based on mobility, migration, transformation, and degradation of different environmental areas as well as the effect of toxic compounds (acute, sub-acute or chronic effect) on sensitive biological models correlated with field data.

The studies in environmental field are innovative due to the diversity of test substances available on the national market (such as pesticides, surfactants, pharmaceutical compounds, biocides), complex laboratory experiments (acute, sub-acute or chronic exposure tests using different organisms and high-performance chemical detection methods), and data analyses, such as risk characterization, REACH (Registration, Evaluation, Authorization and Restriction of Chemicals)

classification, threshold approach system, or natural water classification according to Persoone et al. (2003). The ecotoxicological tests could use various biological models from bacteria to plants and vertebrates which could be used as bioindicators in analyzing the toxic effect of a wide range pollutants/chemical. Nowadays, monitoring and solving different environmental pollution issues relies on the use of bio-indicators / biomarkers as an ecotoxicological a top field for fundamental and applied research. The use of bioindicators could be further used to monitor the biodiversity and indirectly to give information about the environmental health and sustainability. Bioindicators include biological processes, species, or communities and are used to evaluate the environmental quality and how it changes over the time. Environmental changes are often linked to anthropogenic and natural stressors, although anthropogenic stressors are the main focus of bioindicator characterization. The development and widespread application of bioindicators have initially described in 1960s. Since then, introduction of biomarker tools continuously developed as a response of finding more sensitive indicators for sub-lethal effects. The scientific progress uncovered the biomarkers as tools for faster and reliable characterization of toxic effects. The 'biomarker' definition mentioned it as a 'biochemical, cellular, physiological, or behavioral variations that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants' (Depledge & Fossi, 1994). The combined use of bioindicators and biomarkers can provide a comprehensive picture of aquatic and terrestrial ecosystems health status and therefore they can identify environmental issues. Pollutants effect could be fast monitored at the molecular level by biomarkers long before perceiving the toxic effect at the organism level. Biomarkers at transcriptional level (DNA) or translational level (proteins) fasten the toxic effect detection and therefore increase the rapidity of ecological risk assessment (ERA) followed by mitigation measures to protect the environment. Recent studies indicated that biomarkers could contribute to ERA framework where gene expression (such as proteomics) showed a particular promise due to their cost-effectiveness and reliable results (Ali et al., 2017; Lill et al., 2021). Challenges in using biomarkers/ bioindicators for ERA were linked to difficulties in identifying a specific relationship stressor-biomarker/bioindicator, in quantifying a specific stressor dose-response functions, and in extrapolating from cellular or subcellular bioindicators to higher-order ecological effects (Bartell, 2006). The use of bioindicators and biomarkers in the context of environmental assessment should be included in a common European legislation regarding the environmental protection, public health, and biodiversity conservation. In the European Union, for example, the Water Framework Directive and the Habitat Directive, include provisions regarding the use of bioindicators and biomarkers in the assessment and monitoring of chemicals impact on the environment and human health. Additionally, international norms and standards, such as those established by the European Chemicals Agency (ECHA) and the World Health Organization (WHO), may provide further guidance on the use and interpretation of bioindicators and biomarkers. In the United States, agencies such as the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) may issue regulations and guidelines regarding the use of bioindicators and biomarkers in the context of assessing risks to the environment and human health. By analyzing and synthesizing previous research, this review study aims to identify current gaps and challenges in the field, as well as opportunities and future research directions. Through this study, the goal is to provide an overview of recent progress in identifying, validating, and using biomarkers and bioindicators in assessing and monitoring environmental health. Additionally, it aims to highlight new technologies and methodological approaches that could enhance our understanding of the anthropogenic impact on the environment and the sustainability of ecosystems.

MATERIALS AND METHODS

A literature search was conducted to access relevant material for this review. We comprehensively searched the Web of Science

and Google Scholar databases from 1990 to 2024 using the following terms and phrases: "*bioindicators*" or "*ecotoxicological tests*", "*biomarkers in ecotoxicology*" or "*Systems Biology in Ecotoxicology*" or "*Omics technologies in ecotoxicology*" or "*adaptive markers*" or "*DNA as a biomarker of pollution*". No restrictions were imposed regarding language, document type, or data category. Preliminary selection was based on information regarding ecotoxicological testing and the relevance of biomarkers. Secondary screening involved verifying details in the field of biomarkers at the cellular and molecular levels, with an emphasis on intensifying studies involving highly advanced technologies. A total of 78 publications were selected.

RESULTS AND DISCUSSIONS

Ecotoxicological tests, bioindicators and biomarkers develop an integrated and complementary framework for assessing the impact of anthropic activities on the environment. These tools provide detailed information about the environmental status and organisms' responses to various stressors, contributing to the development of more effective strategies for protecting and conserving ecosystems and human health. Stressors such as chemicals are a top public concern, especially regarding their bioaccumulation in organisms from the ambient environment and food.

Traditional ecotoxicological tests are standardized laboratory experiments used to assess the potential harmful effects of chemical substances on organisms and ecosystems by exposing, in controlled conditions, biological components (cells, individuals, populations, communities) to various concentrations of chemicals, mostly a single compound. A large number of bioassays have been developed to assess the toxicity of contaminants on plants, animals and microbes from terrestrial, marine and freshwater environments (Breitholtz et al., 2006). Biological effects are recording as survival, growth or reproduction endpoints (Connon et al., 2012; Schuijt et al., 2021). Overall, ecotoxicological tests cover acute and chronic toxicity tests, bioaccumulation studies, genotoxicity tests, reproductive toxicity tests, behavioral toxicity tests, sediment and soil toxicity tests and microbial toxicity tests (Vosylienė, 2007). Ecotoxicological dose-response bioassays are an important line of evidence for ERA, because they provide experimental evidence of cause and effect. Short-term tests quantified lethal concentrations (LC50 or LD50), while long-term tests assessed sublethal effects by providing values of EC50 or ED50 and determining NOEC and LOEC (Rand, 1991). These tests are regulated and standardized by organizations like the EPA and OECD.

An optimally designed ecotoxicological test should meet reliability, cost-effectiveness, sensitivity, and relevance criteria. Designing a test that meets all these criteria is challenging and often involves very high costs. However, tests at this level are often reproducible in terms of obtaining similar results upon test repetition, thereby making the results comparable between laboratories and easier to implement in regulatory hazard and risk assessments. Even though endpoints such as mortality and reproduction are ecologically relevant, they rarely provide information about effects at ecologically relevant concentrations because these endpoints are often assessed after acute or semi-acute exposures, using relatively high concentrations of xenobiotics (Furuhagen, 2015). Tests at the community and ecosystem levels were largely absent for organisms other than microorganisms and algae (Castaño-Sánchez et al., 2020). In addition to the shortage in biological models, the ecotoxicological tests cannot be accomplished within a reasonable timeframe, especially due to a large number of chemicals produced in Europe, more than 80,000 chemicals produced in quantities exceeding one ton, in spite of testing a "representative" class of chemicals on "standard" species. Defining "safe" concentrations of contaminants for ecosystems often relies on the use of an arbitrary "safety factor" (typically dividing a toxicity metric such as LCx, ECx, or NOEC by 100 or 1,000), based on data often available for only a small number of species (e.g., in aquatic risk assessment, often only Daphnia, fish, algae) or at most species sensitivity distributions (SSDs) constructed with only a few dozen species (Posthuma et al., 2019). The increasing number of chemicals to be

tested by standard ecotoxicological tests, relying on mortality or reproduction as endpoints, pushed the ecotoxicological field to extend the testing on biomarkers/bioindicators due to their superior efficiency, reduced costs and less workload of the testing procedure. Bioindicators and biomarkers are two interconnected concepts in ecotoxicology, providing information about the health of ecosystems and living organisms which are exposed to toxic chemicals. Moreover, both aquatic and terrestrial environments have particularities that must be considered for an accurate ecological assessment. For example, measuring pollutant concentrations in water has some disadvantages, such as low concentrations and random spatial and temporal variations. Sediment serves as a long-term integrator of pollution, where concentrations are higher than in seawater, but contaminants are not always available to organisms due to their physico-chemical forms. Furthermore, sediment heterogeneity (particle size and organic matter) could make comparisons between sites difficult. Therefore, the use of living organisms called bioindicators is preferable for quantifying pollutants. The health of an ecosystem or its surrounding environment is reflected on bioindicators as living organisms such as plants (lichens, algae, and vascular plants assessing air pollution or soil quality), invertebrates (earthworms, water scorpions or aquatic insects assessing water quality), fish and other aquatic organisms (assessing water quality and its contamination levels) or birds and mammals (assessing the health of terrestrial ecosystems and exposure to contaminants). Over time, bioindicators have been defined in various ways, one of them highlighted that ecotoxicology is ultimately analyzing the effects of pollutants on populations not individuals. In addition, it was mentioned that sub-lethal effects on the environment can have a greater impact on population size than acute toxicity (Moriarty, 1988). In 1993, Rainbow and Phillips defined bioindicators as animal(s) or plant(s) which accumulate contaminants in a direct ratio with the contaminant concentration from the surrounding environment. Another definition of bioindicators defined them as species or group of species that reflect the abiotic or biotic state of an impacted environment based on a subset of taxa diversity or the whole diversity within an area (Gerhardt, 1999). The author considered that bioindicators are useful in three situations: (i) where the indicated environmental factor cannot be measured, (ii) where the indicated factor is difficult to measure, (iii) where the environmental factor is easy to measure but difficult to interpret based on its ecological significance. On the other hand, biomarkers are measurable biological indicators at the molecular, cellular, or physiological level that can indicate an exposure to toxic chemicals.

The use of bioindicators for pollution impact assessment is called biomonitoring, based on quantifying contaminants in aquatic organisms which is important from a human health perspective. However, they cannot provide information on the toxicological significance of accumulated pollutants or nor indicate the health status of organisms. Therefore, recent biomonitoring programs involved biomarkers, which are measurable parameters at different levels of biological organization (molecular, cellular, or physiological). Biomarkers reflect changes in regulatory metabolic processes resulting from the effect of anthropogenic stressors. Assessing published studies, it was found that tests at the whole-organismal level and biomarkers were most common for invertebrates and fish, whereas in vitro bioassays primarily relied on mammalian cell lines. Transitioning from bioindicators to biomarkers in ecotoxicology signifies an advancement in assessing the impact of chemicals on the environment and living organisms. While bioindicators provide insights into the overall condition of ecosystems, biomarkers offer detailed information at the molecular, cellular, and physiological levels regarding exposure to toxic substances and their effects on individual organisms. This shift allows for the detection of subtle changes resulting from exposure to toxic chemicals, including sublethal effects and molecular or genetic alterations (Rand, 1991). Furthermore, biomarkers are more likely to detect effects at lower and more ecologically relevant concentrations. Several chemicals interfere with organismal oxidative processes, causing an imbalance in oxidative status that can result in oxidative stress, a potentially harmful condition to organism health. Oxidative stress biomarkers

are therefore common in ecotoxicological studies to demonstrate exposure. While in theory, biomarkers could facilitate ecological risk assessments (ERAs), there are still considerable knowledge gaps regarding confounding factors and connections between biomarker response and effects at higher biological levels. These gaps limit the full potential of biomarker utilization in ecotoxicological studies and ERA (Furuhagen et al., 2014a).

The advantage of introducing biomarkers in environmental research

Biomarkers, through their distinct molecular structures, are essential tools for assessing environmental quality and biotechnological processes. Their importance lies in their ability to be identified and measured through various biochemical and molecular approaches. Many ecotoxicological biomarkers originated from the field of biomedical sciences and they were initially developed and validated in humans and model mammalian species before being applied in ecotoxicology. Biomarkers include enzymes (such as hepatic enzymes to evaluate the toxicity of chemicals on the liver), specific proteins (which can be expressed in a specific manner due an exposure to a particular contaminant), nucleic acids (changes at the DNA or RNA level to assess genetic damage caused by exposure to chemicals) or specific metabolites (specific chemical substances that are produced or metabolized in a specific manner following exposure to a contaminant). Molecular, biochemical, and cellular systems are often the most sensitive and can react quickly to environmental changes. These cellular processes are responsible for detoxification, adaptation, repair, and cellular integrity protection, ultimately contributing to organismal health in response to stress. Biomarkers linked to these processes could monitor very early and at lower levels of harmful exposure the environmental stress (Binelli et al., 2006; Furuhagen, 2014b). These early warning biomarkers can be used predictively, allowing the implementation of strategies before irreversible ecological damage occurs, acting as short-term indicators for long-term biological effects. Biomarker application range is very wide, they could be used in human clinical and risk assessments studies to make predictions about an individual's future health and/or response to medical treatment. In addition, they could be used to reach ERA goals in predicting the integrity and functioning of ecosystems (population and community levels) under various stress factors (Hommen et al., 2010; Forbes et al., 2006). Some biomarkers are specific to a particular chemical or type of stressor, while others are nonspecific, responding to a wide range of stressors (Rossnerova et al., 2020). Susceptibility biomarkers indicate an organism's capacity to respond to a specific xenobiotic (Silvestre, 2020; Gonçalves et al., 2021). Exposure biomarkers are indicators of exposure to a particular stressor, while effect biomarkers are associated with the health and fitness effects on the organism (Depledge, 2020; Schuijt et al., 2021). Most biomarkers used in ecotoxicology would be classified as exposure biomarkers, as only a few of them have well-described connections to changes at higher biological levels (Furuhagen et al., 2014b).

Proteomics as translational-level biomarkers in the environment

Proteomics, as a branch of molecular biology, covers the entire set of proteins (proteome) expressed in a cell, tissue, or organism at a specific moment. In the context of the environment, proteomics becomes extremely useful in identifying and evaluating biomarkers, which are molecules or biological compounds that can indicate the presence or condition of a particular phenomenon, such as pollution. The use of protein biomarkers in environmental proteomics becomes a promising method for detecting and evaluating the impact of pollution on organisms and ecosystems. Organisms or cells exposed to new environmental conditions, such as pollution, modulates their protein expression pattern which could be correlated with specific protein biomarkers, indicating the level of stress or damage caused by pollutants. These proteins as biomarkers may be involved in various biological processes, such as detoxification, stress response, inflammation, or cellular damage, and can be detected and quantified using advanced techniques, such as mass spectrometry and two-dimensional gel electrophoresis. Furthermore, proteomics can

provide a deeper understanding of the molecular mechanisms involved in organisms' responses to pollution and can help identify biological pathways that are disrupted by environmental stressors. Proteins are more relevant than transcripts, because they are direct mediators of the resulting phenotype. Proteins direct all levels of the phenotype: structural proteins dictate physical form, enzymes catalyze biochemical reactions, and proteins act as signaling proteins, antibodies, transporters, ion pumps, and transcription factors to control gene expression. Molecular initiating events (MIEs) of adverse outcome pathways (AOPs) predominantly occur at the protein level (e.g., ligand-receptor binding), and proteomics can elucidate new MIEs and map key events in AOPs (Allen et al., 2014). Recent developments in environmental proteomics have shifted towards identifying and characterizing protein biomarkers in response to environmental stress. There is a growing interest in integrating proteomic methodologies with ecological applications, aiming to leverage their potential for translational-level biomarker monitoring in environmental management. Numerous studies have delved into employing proteomic analysis for monitoring environmental stress in aquatic organisms, examining its capability to identify protein biomarkers signaling exposure to pollutants and evaluating their suitability for environmental risk assessment and management (Brockmeier et al., 2017). Heat shock proteins (HSPs) are examples of general biomarkers. Their function is to prevent protein denaturation, a common effect of many environmental stressors; therefore, HSP induction is considered a general stress response. Other responses are induced only by a specific group of xenobiotics or stressors. Acetylcholinesterase (AChE) is considered a specific biomarker because it responds to organophosphate and carbamate pesticides and not to general environmental stressors or xenobiotics. General biomarkers often have multiple confounding factors that interfere with the toxic response (Aronson & Ferner, 2017). These biomarkers respond not only to the xenobiotics of interest but also to numerous other stressors and environmental factors, such as nutritional status, temperature, and UV irradiation (Moreira-de-Sousa et al., 2018; Demirci-Cekic et al., 2022). Commonly used biomarkers, such as induction of cytochrome P450 1A enzyme, acetylcholinesterase activity, metallothioneins, and pigments, have been treated with great interest. Environmental markers for pollutants such as dioxins, furans, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons are necessary for pollution monitoring. Cytochrome P4501A (CYP1A) and ethoxyresorufin-O-dealkylase (EROD) are biomarkers used to detect the biotransformation of these pollutants in fish and marine bivalves (Kim et al., 2013, Cortés-Miranda et al., 2024). Metallothioneins (MTs) are biomarkers for oxidative stress, with the ability to chelate toxic metals from cells (Hemmadi, 2016). Pigments, such as chlorophylls and carotenoids, are biomarkers for the distribution and abundance of phytoplankton, also being used in cancer research (Mouzaki-Paxinou et al., 2016; Atta et al., 2018; Husayn & Guda, 2023). Other studies summarize recent advancements in the application of proteomics for environmental monitoring and assessment. Proteomic techniques can identify and characterize biomarkers in response to environmental stressors and pollutants, highlighting their potential as translational-level indicators of environmental health. This can facilitate the development of more efficient strategies for managing and reducing the impact of pollution on the environment and human health. Thus, proteomics as translational-level biomarkers in the environment represents an innovative and powerful approach for monitoring and evaluating pollution, offering significant opportunities for improving environmental management and protecting public health (Armengaud, 2016; Kumari & Kumar, 2021).

DNA Integrity /expression/mutation as a Pollution Biomarker (DNA biomarkers as transcriptional level)

Pollution and environmental changes are a stress factor for living organisms triggering changes at molecular level as a part of adaptation mechanisms. The adaptation mechanisms relied on genetic characteristics and physiological resilience which later modulates the phenotypic adaptive changes. Under the stress factors, the genetic markers are first to react, giving information about DNA changes and the

adaptive potential of certain populations to the environmental changes.

Genotoxic agents (as exogenous stress factors) can severely affected DNA integrity inducing DNA strand breaks, loss of methylation and DNA mutations. Exogenous agents can induce DNA strand breaks by a direct damage of nucleotide DNA sequence, impairing the DNA repair processes or by other physiological responses. Members of polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene (BaP) can form a complex with DNA and subsequently induced a direct chemical single-strand breaks due to ionizing radiation or oxidation-reduction chemical reactions (Paniagua-Michel & Subramanian, 2016). Studies on DNA integrity from marine snail *Planaxis sulcatus* have highlighted the impact of pollution in various harvesting locations contaminated with petroleum hydrocarbons from coastal waste (Sarker et al., 2018, D'Costa et al., 2017).

Environmental changes could also affect allele frequencies in populations with short reproductive cycles or they could be correlated to neutral and adaptive genetic markers. The neutral genetic markers give information about population decline under new environmental conditions (pollution or climate change). Neutral markers currently predominate in population genetics applications in the field of conservation and management, providing information about population demographic processes (Hohenlohe et al., 2021). However, neutral markers have limitations for monitoring the effects of environmental changes, but evaluating the adaptive genes directly involved in the response to environmental changes could provide more information about the nature of selection imposed by environmental changes and the potential of populations to respond through evolutionary adaptation (Chown et al., 2016). A useful set of adaptive markers should include loci that contribute significantly to the genetic variation of a trait within and between populations and undergo significant allele frequency changes with environmental changes (Stephan, 2016). Each locus must contribute significantly to genetic variation to be useful in monitoring adaptive changes; otherwise, the large number of required loci could be impractical, and the power to link genetic changes to environmental effects would be reduced. Pleiotropic effects can influence adaptive evolution by hindering the increase in frequency of favored alleles under selection, such as in the evolution of pesticide resistance (Baucom, 2019). Studies showed that polymorphisms in unique markers genes can influence population growth rate, and adaptive changes can be observed in an increasing number of populations (Meyer et al., 2018). To identify sets of candidate genes, it is important to focus not only on plastic responses to stress but also on comparisons between populations adapted to that stress (Agrawal, 2020; Noble et al., 2019). Quantitative trait loci (QTL) mapping can identify genomic regions involved in adaptive changes, including modifications to protein structures and gene expression (Abraham & Croll, 2023). These studies can highlight genomic regions that control resistance to different stresses and provide information about genes involved in environmental adaptation, such as flowering time in *Arabidopsis thaliana*.

Various genetic tools are used to identify candidate genes involved in adaptive changes in response to environmental changes, including microarrays for measuring expression changes, selection experiments, QTL mapping, and strain comparisons (Franks et al., 2007; Alvarez et al., 2015, Bourdon-Lacombe et al., 2015). Most methods lead to the identification of sets of candidate loci rather than specific alleles, although the involved alleles can eventually be identified, including in non-coding and transcribed gene regions (Jeremias et al., 2020). Genes such as *Adh* in *Drosophila melanogaster* and *Gly* in *Pinus edulis* are considered candidates for monitoring adaptation to thermal and moisture changes, respectively. Knowledge of physiological pathways, such as abscisic acid synthesis in plants, may suggest genes involved in adapting to environmental changes (Hoffman & Willi, 2008). As knowledge of relevant pathways and gene functions in model species increases, the functional roles of candidate genes in relation to specific traits can be tested (Gómez et al., 2015). Recently invaded and established latitudinal climates, as well as newly introduced plants, are ideal experimental systems for identifying candidate genes (Chown et al., 2016), while spatial models of long-term

differential adaptation can provide less information, as evolution has occurred over long periods of time, with multiple genetic drift events (Hoban et al., 2016; Bock et al., 2016). Microarrays and other techniques are used to compare genetic expression patterns between environments and between strains of the same species (Alvarez et al., 2015). These techniques can be applied to non-model organisms, both from field-collected material and laboratory conditions. Genetic expression studies can help identify stress response genes by comparing the plastic responses of populations exposed to different conditions (DeBiasse & Kelly, 2016; Rivera et al., 2021; Kelly, 2019). Examples include identifying genes involved in water stress response in *A. thaliana* and salinity variation response in flounders (*Platichthys flesus*) (Knight et al., 2006). New techniques allow for the generation of dense genetic marker maps, which can be used in association studies to link markers to quantitative traits. The use of dense genetic maps can help identify causal genes in natural hybrid populations, but it requires the analysis of a large number of individuals and can be costly. Methods for identifying genes under selection fall into two categories: those that test divergence between populations at specific loci and those that test genetic variation within populations (Bernatchez, 2016). Tests for selective sweeps do not require crosses and can be performed on field-collected material but require a high-density map (Hoffmann & Willi, 2008). The approach relies on examining allele divergence and variation in allele size at markers located near expressed genes. This approach can identify loci with reduced diversity and/or variation, potentially linked to candidate genes under selection. Most genes are conserved in related organisms, suggesting that sets of candidate genes have the potential to be applied in related species (Wright et al., 2020). The predictability of adaptation can be improved by considering ecological context and the type of environmental stresses (Kristensen et al., 2020). There are universal mechanisms present at the organism level to cope with environmental stresses, and genetic changes in microorganisms seem to have some degree of predictability (Deans, 2021). Changes in allele frequencies can indicate the presence and impact of stress factors on populations (Ament-Velásquez et al., 2022; Brennan et al., 2022). Genetic variation influences traits under selection in natural populations, and changes at candidate markers are associated with adaptive changes. Alleles in structural or regulatory gene sequences are favored in new environments, accelerating adaptation (Lasky et al., 2023). Technological advances allow for rapid identification of genetic changes and candidate genes (Singh et al., 2016). Developing a high density of polymorphic markers allows for the isolation of candidate genes through association studies and marker scans (Hoffman & Willi, 2008; Pang et al, 2020; Tibbs Cortes et al., 2021).

Transition to Omics Technologies

The transition to omics technologies in ecotoxicology represents a significant shift towards a more comprehensive and high-throughput approaches for assessing the effects of contaminants on the environment and organisms. Omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, enabled a simultaneous analysis of thousands of genes, transcripts, proteins, or metabolites within biological systems. This represents a holistic understanding of how organisms respond at molecular level to chemical exposures, providing insights into complex biological pathways and mechanisms of adapting to toxicity. Studies which integrated omics technologies into ecotoxicological studies could identify with greater accuracy and sensitivity biomarkers linked to stressors exposure and effects. These biomarkers can serve as early warning indicators of environmental stressors and predict ecological risks associated with chemical contaminants. Overall, the adoption of omics technologies in ecotoxicology enhances environmental health assessment, prediction of the potential ecological impacts, and development of more effective strategies for environmental management and conservation. The toxic effect of stressors could be assessed by Omics technologies combining toxicogenomics (Ankley et al., 2006) with transcriptomics and proteomics (De Wit et al., 2010). Biomonitoring of aquatic and terrestrial environments focuses on measurable biomarkers at different biological levels. Biomarkers can measure the effects of

pollutants or the host's response at biochemical, cellular, and molecular levels, and sometimes can also indicate effects at the whole organism level, such as behaviour, energy or metabolic processes. For example, marine mollusks are efficient indicators of xenobiotic impacts in the marine ecosystem. The term "exposure biomarkers" indicates that the organism has been exposed to pollutants, while "effect biomarkers" or "stress biomarkers" measure the organism's response to these pollutants (Paniagua-Michel et al., 2016). Technological advancements have led to the discovery and validation of new environmental biomarkers under the omics era (Garcia-Reyero & Perkins, 2010). Recent applications of omics technologies have redefined the roles of biomarkers in environmental biotechnology, with concurrent analyses of common biomarkers and new technologies for optimizing metabolic networks. Biomarkers, mainly molecular markers such as genes, proteins, and metabolites, are used for disease diagnosis and prognosis, as well as predicting response processes in living cells. Biomarkers include a variety of measures of the molecular, biochemical, cellular, and physiological responses of specimens of key species to exposure to contaminants or physical stressors (Kadim et al., 2022). Omics technologies, including genomics, transcriptomics, proteomics, and metabolomics, are used to identify and quantify network components and interactions (Ebner, 2021). Recent developments in the analysis of mixed microbial communities, using culture-independent molecular tools, have brought new perspectives on catabolism in extreme and fragile environments. These approaches pave the way for identifying new biomarkers, increasing biodiversity expectations by about 99% compared to conventional classification. The use of functional genes through metagenomic matrices will enhance understanding of microbial interactions and metabolism, facilitating the development of appropriate strategies for environmental bioremediation. Genomic studies and metagenomic sequencing provide holistic approaches to microbial communities, enabling biotechnological exploitation. The use of 16S ribosomal RNA often precedes metagenomic analysis and can guide technological choices. Culture-independent technologies and next-generation sequencing have revealed a high microbial diversity, amplifying the knowledge about these communities (Sunagawa, 2015; Charles et al., 2017).

An ecotoxicological system is vast complex, consisting of numerous components and interactions, which could not be analyzed and understood separately. Omics technologies allow a deeper understanding of system biology and the complex interactions among different molecular components in living organisms. The fundamental principle of systems biology is that the emergence of disease or exposure to chemicals disrupts a network of biological pathways in the organism (Tantardini et al., 2019). They are used in a wide range of fields, including biology, medicine, ecology, and biotechnology, and are of great importance in studying ecological phenomena such as ecotoxicology, by providing detailed and comprehensive data on organism responses to stress factors and the environment (Sharma et al., 2022; Ebner, 2021). Once network components and interactions are clarified, quantitative predictions could be made about organism adaptive response and recovery from chemical stressors.

Future trends

There is a need in ecology, and not only there, to have a comprehensive view of how chemicals and other stressors impact organisms and ecosystems. Systems biology in ecotoxicology employ systemic approaches by integrating information about interactions between biological molecules, metabolic pathways, genes, and the surrounding environment to better understand organisms' responses to toxic substances. This combined approach provides valuable information for systems biology to identify effects undetectable by isolated technologies (Ankley et al., 2006; Garcia-Reyero & Perkins, 2010). Systems biology, already in work for human health, is finding a growing place in ecotoxicology by tackling future directions such as i) development and application of mathematical and computational models to simulate and predict the effects of chemical on organisms and ecosystems. These models can be used to assess ecotoxicological

risk and guide environmental management; ii) utilization of omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, to identify biomarkers and molecular pathways involved in the response to ecotoxicological stress. These information can be used to develop diagnostic tests and pollution monitoring; iii) integration of data from different levels of biological organization, from the molecular level to the ecosystem level, to obtain a deeper understanding of the impact of chemicals on biodiversity and ecosystem functioning; iv) exploration of interactions between chemical stressors and environmental stressors, such as climate change and terrestrial pollution, to evaluate complex risks to the environment and human health. Ongoing research efforts aim to advance the development and application of bioindicators and biomarkers to enhance environmental monitoring, risk assessment, and biotechnological applications. Research directions in the development of bioindicators and biomarkers in ecotoxicology and biotechnology it should focus on identification of novel biomarkers and integration of omics technologies. Standardized protocols and assays for measuring biomarkers are also essential for ensuring reliability and comparability of results across different studies and laboratories. Research focuses on developing and validating robust methods for biomarker analysis. Future researches should aim to develop application of high-throughput screening and development of non-invasive biomonitoring techniques. Non-invasive biomonitoring techniques, such as remote sensing, bioimaging, and biosensors, are gaining attention for their ability to monitor environmental quality without harming organisms. The future research should to improve the sensitivity and specificity of these techniques for detecting biomarkers in situ. Having in view that bioinformatics and data analytics play a crucial role in processing and analyzing large omics datasets generated from biomarker studies the future research should focuses on developing computational tools and algorithms for data integration, interpretation, and visualization. Understanding the ecological relevance of biomarkers is essential for their application in ecotoxicology. Research explores the relationship between biomarker responses and ecological endpoints to assess the overall health and resilience of ecosystems. Last but not least field-based validation studies are necessary to assess the performance and applicability of biomarkers under real-world environmental conditions. Research involves conducting longitudinal studies in natural environments to evaluate the efficacy of biomarkers in predicting environmental health.

CONCLUSIONS

The levels of contaminants have increased in terrestrial and aquatic environments due to the raise of anthropogenic activities. Their complex harmful impact on the environment should be analyzed by a rapid assessment of the impact followed by an implementation of appropriate corrective measures. These continuous exposures to anthropogenic pollutants can also affect physiological processes which make more imperative to have a reliable bioscreening programs. The development and application of testing procedures at the experimental model level to highlight the impact of contaminants on both aquatic and terrestrial environments are essential for efficient screening of environmental matrix toxicity. Research and application strategies for biomarkers development in ecotoxicology are linked to specific environmental matrices, organisms and pollution. Thus, the development of non-invasive and non-destructive biomarkers will allow long-term monitoring without affecting the health or integrity of organisms. A series of steps must be taken, starting from the identification and validation of biomarkers, followed by the characterization of their responses for an efficient assessment of the anthropogenic stressors impact. Overall, long-term monitoring can be carried out to identify trends over time, and by combining biomarkers with other methods for assessing ecological risks, such as standardized toxicity tests and ecological modelling, a more comprehensive understanding of the impact of anthropogenic factors on the environment can be achieved. These studies contributed to the understanding of environmental adaptation and evolution to new anthropogenic challenges followed by new environmental policies.

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CLASSIFICATION AND INDUSTRIAL APPLICATIONS OF BIOSURFACTANTS – MINIREVIEW

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Abstract

A well-developed industrial sector based on biotechnology will significantly reduce dependence on chemical resources, contributing to climate change objectives and leading to a greener and more environmentally friendly growth. The key lies in developing new technologies for the sustainable transformation of renewable natural resources into bio-based products and biofuels. Bioeconomy involves the production of renewable biological resources and their conversion into food, feed, and bio-based products through innovative and efficient technologies provided by industrial biotechnology. This paper presents a general classification of biosurfactants used in industrial applications. The term "surfactant" is derived from "surface-active agent." Biosurfactants have become increasingly significant across various fields owing to their diverse properties, including enhanced biodegradability and reduced toxicity. They are categorized into high and low molecular weight molecules. Biosurfactants find applications in industries such as cosmetics, food processing, pharmaceuticals, and environmental bioremediation. While numerous surfactants are already in use in various industries, it's essential to develop indigenous technologies for the production of biosurfactants from local micro-organism. This would ensure their suitability for application in specific environments.

Key words: *bioremediation, biosurfactants, emulsifiers, industry, microorganisms.*

INTRODUCTION

Biosurfactants enhance the surface area of hydrophobic substrates that are insoluble in water, which organisms utilize for their growth and function effectively in processes such as biodegradation, bioremediation, and biocontrol processes.

Microorganisms that utilize hydrocarbons produce a surface-active agent known as biosurfactant, which accelerates these processes. Various methods exist to test the biosurfactant potential of bacterial isolates, including hemolytic activity, drop collapse test, oil spreading test, emulsification index test, blue agar plate test, CTAB agar plate method, and hydrocarbon overlay agar method. This article discusses the classification, properties, methods, and significance of biosurfactants (Amaral et al., 2009).

Shete et al. (2006) charted the patents related to biosurfactants and bioemulsifiers, revealing a significant number of patents in industries such as petroleum (33%), cosmetics (15%), antimicrobial agents and medicine (12%), and bioremediation (11%). Notably, sophorolipids (24%), surfactin (13%), and rhamnolipids (12%) represent a substantial portion of these patents.

The physiological role of biosurfactant production in microorganisms includes antimicrobial activity and facilitating the availability of substrates for cell absorption under adverse environmental conditions. Biosurfactants are classified based on molecular weight and chemical composition (Maneerat, 2005). They offer advantages such as biodegradability, reduced toxicity and enhanced surface and interface activity, yet challenges include limitations in production scalability and patent rights. Factors influencing biosurfactant production encompass the carbon source, nitrogen source, C:N ratio, temperature, aeration and pH. Biosurfactants find applications in agriculture, industry, medicine, and the petroleum sector.

Surfactants, the active ingredients in soaps and detergents, concentrate at air-water interfaces and are commonly used to separate oily matters from specific environments by increasing the water solubility of non-aqueous liquids and reducing surface/interfacial tension at air-water and oil-water interfaces.

The main classes of biosurfactants include glycolipids, phospholipids, polymeric biosurfactants, and lipopeptides (surfactin). Glycolipids, such as rhamnolipids, sophorolipids, and trehalolipids, are well-known, while surfactants,

widely used in various applications, are often chemically synthesized, posing potential environmental and toxicological risks. With advancements in biotechnology, there is increasing interest in environmentally friendly processes for producing biosurfactants from microorganisms (Cohen & Exerowa, 2007).

Classification of Biosurfactants

Biosurfactants are classified based on their molecular weight and chemical composition.

Classification based on Molecular Weight

Biosurfactants with low molecular weight, typically glycolipids or lipopeptides, reduce surface and interfacial tension at air/water interfaces. Examples include rhamnolipids, trehalolipids, and sophorolipids, which consist of acylated disaccharides with long-chain fatty acids or hydroxylated fatty acids. Often referred to as bioemulsifiers, biosurfactants with high molecular weight are more efficient in stabilizing oil-in-water emulsions and exhibit extended substrate specificity. They contain long-chain fatty acids or fatty acid derivatives in their hydrophobic moiety and can include carbohydrates, amino acids, phosphate, or cyclic peptides in their hydrophilic portion (Tahzibi et al., 2004).

Classification based on Chemical Structure

Glycolipids are carbohydrates, such as glucose, mannose, galactose, and rhamnose, combined with long-chain aliphatic acids or hydroxy aliphatic acids through ether or ester groups. Rhamnolipids, trehalolipids, and sophorolipids are prominent examples, with other glycolipids including cellobiolipids (Monteiro et al., 2007).

Rhamnolipids are composed of rhamnose molecules linked to one or two molecules of β-hydroxydecanoic acid. The glycosidic linkage involves the OH group of one of the hydroxydecanoic acids with the reducing end of the rhamnose disaccharide; the OH group of the other hydroxydecanoic is involved in ester formation (Monteiro et al., 2007).

Trehalolipids. Trehalose is a non-reducing disaccharide in which the two glucose units are linked by an α,α-1,1-glycosidic bond. It is the basic component of cell wall glycolipids in mycobacteria and corynebacteria. Several trehalose lipid structures are found.

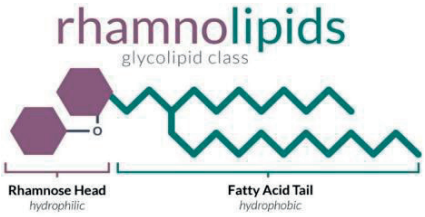

Figure 1. Chemical formula for Rhamnolipids

Sophorolipids. Many yeast species, such as *Torulopsis bombicola*, *T. petrophilum* (Cooper & Paddock, 1983), and *T. apicola*, mainly produce sophorolipids. Sophorolipids are dimeric sophorose carbohydrates linked to long-chain hydroxylated fatty acids, with a mixture containing at least six to nine different hydrophobic sophorosides. While sophorolipids are capable of reducing surface and interfacial tension, they are not highly effective as emulsifying agents (Cooper & Paddock, 1984).

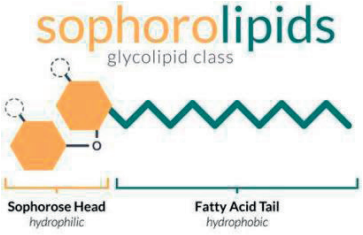

Figure 2. Chemical formula for Sophorolipids

Lipopeptides and Lipoproteins. Bacteria like *Bacillus brevis* and *Bacillus polymyxa* produce numerous cyclic lipopeptides, including decapeptide antibiotics (e.g., gramicidin) and lipopeptide antibiotics (e.g., polymyxin), which exhibit remarkable surface-active properties. These compounds consist of a lipid attached to a polypeptide chain (Desai & Banat, 1997; Muthusamy et al., 2008).

Fatty Acids, Phospholipids, and Neutral Lipids. Certain bacteria and yeasts produce significant amounts of fatty acids and phospholipid-like surfactants when grown on n-alkane substrates. These surfactants, resulting from microbial oxidations, include straight-chain acids and complex fatty acids containing hydroxyl and alkyl branch groups, such as corynomuolic acids (Rahman & Gakpe, 2008).

Phospholipids are major constituents of microbial membranes. When hydrocarbon-degrading bacteria or yeasts grow on alkane

substrates, the levels of phospholipids in their membranes increase significantly. For instance, when the bacteria *Acinetobacter sp*. HO1-N grows on hexadecane substrate, it primarily produces phosphatidylethanolamine (Muthusamy et al., 2008).

Polymeric Microbial Surfactants. Many biosurfactants are polymeric heteropolysaccharides containing proteins. Prominent examples include emulsan, liposan, manoprotein, and protein-polysaccharide complexes (Desai & Banat, 1997).

Particular biosurfactants. Certain bacteria produce extracellular membrane vesicles that facilitate the partitioning of hydrocarbons and the formation of microemulsions. These microemulsions play a crucial role in the absorption of alkanes by microbial cells, such as those produced by *Acinetobacter* sp. (Desai & Banat, 1997).

Properties of biosurfactants:

- Reduction of water surface tension;
- Excellent capacity for micelle critical concentration (CMC) formation;
- Low lethality;
- Good compatibility and digestibility.

Methods for Evaluating Biosurfactant Efficiency

Various methods are employed to assess the efficiency of biosurfactants, including the hemolysis test, oil spreading test, drop collapse method, emulsification index, hydrocarbon overlay agar method, and blue agar method (Amalesh et al., 2012).

Hemolytic activity. In this test, the isolate is streaked onto a blood agar plate and then incubated at 37°C for 48-72 hours. After the incubation period, the presence of a halo zone around the indicated colonies indicates a positive result. This halo zone is categorized as alpha, beta, or gamma hemolysis. Alpha-hemolysis manifests as a greenish zone around the inoculum, beta-hemolysis presents as a clear white zone around the inoculated colony, and gamma-hemolysis occurs when there is no change around the streaked colony.

Oil spreading test. Two layers are formed on an empty Petri dish: water as the first layer and hydrocarbons as the second layer. The cell-free extract of the 24-hour isolate is added to the surface of the Petri dish. A clear zone around the culture indicates a positive result, and the diameter of this clear zone is measured. A water drop serves as a control (Langer et al., 2006).

Drop collapse test. This qualitative test assesses biosurfactant activity. The isolated strains are placed on a hydrocarbon surface, and the collapse of suspension drops without cells indicates a positive result. A water drop serves as a control (Langer et al., 2006).

Emulsification index test. This is a quantitative process where 2 ml of hydrocarbon is mixed with 2 ml of a 48-hour grown culture suspension in test tubes. The mixture is then vortexed for 2 minutes and allowed to stand for 24 hours. After incubation, the emulsification index is calculated according to standard methodologies (Langer et al., 2006).

Blue agar plate method or CTAB agar plate method detects extracellular glycolipid production. A CTAB agar plate containing methylene blue (5 mg/mL) and cetyltrimethylammonium bromide (CTAB) (0.2 mg/mL) is prepared. The 24-hour bacterial isolate is inoculated in spots on the plate and then incubated for 24 to 48 hours. The formation of a dark blue color indicates extracellular glycolipid production (Langer et al., 2006).

Hydrocarbon overlay agar method. In this method, an LB agar plate covered with hydrocarbons is inoculated with a 24-hour culture. The plate is then incubated at 37°C for 48 to 72 hours. After inoculation, growth is observed on the LB plate, and a colony surrounded by emulsified halos is considered positive for biosurfactant production (Kitamato et al., 2002).

Importance of biosurfactants for microbial cells. Biosurfactant production provides a selective advantage to slow-growing microorganisms over fast-growing ones. These compounds are secreted extracellularly or attached to cell surfaces, primarily on water-immiscible substrates, and play a crucial role in increasing the availability of nutrient substrates. Additionally, biosurfactants exhibit antimicrobial activity, enabling microorganisms to increase their cellular biomass (Kitamato et al., 2002).

An organism produces different types of biosurfactants with extensive applications in the petroleum field, as described in Table 1.

Table 1. Microorganisms involved in bioremediation (Silva et al., 2014)

Type of biosurfactant*	Application	Microorganism
Glucolipids and trehalose lipids	Operations for cleaning oil stains	*Rhodococcus erythropolis* 3C-9
Trehalose tetra ester	Bioremediation in petroleum-contaminated environments	*Micrococcus luteus* BN56
Lipopeptide	Bioremediation of marine oil pollution Environmental applications	*Rhodococcus* sp. TW53 *Bacillus subtilis* BS5 *Nocardiopsis alba* *zotobacter chroococcum*
Rhamnolipid	Bioremediation in petroleum-contaminated environments Bioremediation of marine oil pollution Environmental applications Bioremediation of marine and soil environments	*Pseudomonas aeruginosa* S2 *Pseudomonas aeruginosa* BS20 *Pseudoxanthomonas* sp. PNK-04 *Pseudomonas alcaligenes* *Pseudomonas cepacia* CCT6659 *Pseudomonas cepacia* CCT665
Glycolipids	Bioremediation applications Bioremediation of marine and soil environments	*R. wratislaviensis* BN38 *Pseudozyma hubeiensis* *Nocardiopsis lucentensis* MSA04
Sophorolipid	Environmental applications Bioremediation of marine environments	*Candida bombicola* *Candida lipolytica* UCP0988

**The table presented outlines different types of biosurfactants, their applications, and the microorganisms involved in bioremediation according to a study by Silva et al. from 2014.*

Bioremediation of soil environments

Biodegradation by soil microorganisms. Soil contains various microbial communities that play key roles in breaking down organic pollutants. Bacteria, fungi and other microorganisms produce enzymes that degrade complex chemicals into simpler, less harmful compounds (Kitamato et al., 2002).

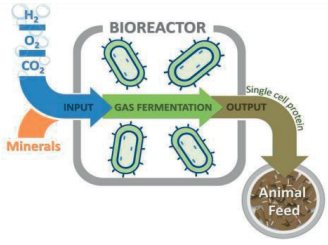

Figure 3. The stages of gas fermentation

Phytoremediation in soil: Similar to marine phytoremediation, plants can be used to extract pollutants from soil or water. Plant roots absorb contaminants, which are then either stored in the plant tissues or broken down into less toxic forms (Konishi et al., 2007; Csutak et al., 2017). *Bioaugmentation.* This involves introducing specific microorganisms into contaminated soil to enhance biodegradation processes. Selected strains of bacteria or fungi may be added to the soil to accelerate the breakdown of pollutants (Konishi et al., 2007).

These methods represent different approaches to bioremediation, each with its advantages and limitations. By harnessing the natural abilities of microorganisms and plants, bioremediation offers a sustainable and environmentally friendly solution to pollution problems in marine and soil environments

Bioremediation, in general, represents a sustainable and efficient approach in the fight against pollution, contributing to the regeneration and conservation of marine and terrestrial ecosystems. It is important to consider the local characteristics of the environment and the species involved before implementing specific bioremediation techniques. Microbial biosurfactants are compounds produced by microorganisms such as bacteria, fungi, or algae, and they possess surfactant properties. Surfactants are substances that enhance the water's ability to disperse and interact with other substances, such as oils and fats (Konishi et al., 2007).

Microbial biosurfactants have several key characteristics (Mukherjee et al., 2006):

- **Microbial origin**: they are produced by microorganisms such as bacteria (e.g., *Pseudomonas, Bacillus),* fungi (such as *Candida, Aspergillus*), or algae.
- **Chemical diversity**: there is a variety of chemical structures found in microbial biosurfactants, including glycolipids, lipoproteins, polysaccharides, peptides and phospholipids.
- **Surfactant properties**: these compounds possess the capability to decrease the surface tension of water and promote the dispersion of fatty substances in water or vice versa.
- **Role in bioremediation**: microbial biosurfactants are applied in various fields, especially in bioremediation, due to their ability to facilitate the breakdown and

removal of hydrophobic compounds, such as hydrocarbons, from soil or water.

- **Synthesis in diverse conditions**: a great advantage of microbial biosurfactants is their ability to be synthesised in various environments, including extreme conditions such as high or low temperatures and in the presence of high salt concentrations.
- **Biotechnological potential**: due to their distinctive characteristics, microbial biosurfactants are valuable compounds for biotechnology, but can also be used in other industries such as oil and gas, agriculture, food processing and bioremediation.
- **Favorable ecological effects**: the use of microbial biosurfactants in bioremediation processes offers numerous environmental benefits, because they can increase the bioavailability of contaminants and facilitate the activity of microorganisms implicated in their degradation. The use of microbial biosurfactants in bioremediation technologies is an environmentally friendly and sustainable approach to treating and removing pollutants from various environments.

The Relevance of Biosurfactants in Other Fields

Global production of biosurfactants has grown significantly, with an estimated annual growth rate of around 3 to 4%. The specific applications of surfactants are categorized based on their importance within their respective fields.

In **agriculture**, compounds with surface activity are used to hydrophilize heavy soils to maintain their nutrient retention capacity.

Agricultural productivity is a serious concern for all developing countries in meeting the ever-increasing needs of the human population. There is a need for the use of ecological compounds to achieve productive agriculture, and globally, on average, 35% of agricultural yield is lost due to pre-harvest pests. Today, conventional chemical pesticides used in agriculture are still under pressure to be phased out due to their harmful effects on the ecosystem, and resistance of bacteria and fungi also poses a significant barrier to their use (Mukherjee et al., 2006).

Recently, biosurfactants have become one of the promising biopesticides. Biopesticides have gained attention in managing fungi, pests and insects, and have been presented as potential alternatives to chemical pesticides. Scientific reports have mentioned the use of microbial biosurfactants in controlling diseases caused by fungi, in combating harmful insects. Lipopeptide and rhamnolipid biosurfactants have low toxicity to the ecosystem and are highly biodegradable, representing promising surface-active compounds that can be used as biopesticides. So far, rhamnolipid biosurfactants have been successfully studied and commercialised in the field of biopesticides. Growing environmental concerns about pesticides and agrochemicals are a real boost for the use of environmentally friendly chemicals in the agrochemical industry, where biosurfactants may be the best alternative. This section examines lipopeptide and rhamnolipid biosurfactants as biopesticides and promising alternatives to chemical pesticides (Mukherjee et al., 2006).

Biosurfactants hold considerable potential in **medicine** due to their unique properties and characteristics. Here are some potential applications of biosurfactants in the medical field (Sullivan, 1998):

- Biosurfactants can enhance wound healing by promoting cell migration, proliferation and angiogenesis. They can also aid in the removal of debris and promote tissue regeneration.
- Biosurfactants can be utilized to formulate drug delivery systems such as liposomes, micelles and nanoparticles. These systems can improve the solubility, stability and bioavailability of drugs, leading to enhanced therapeutic efficacy and reduced side effects.
- Some biosurfactants exhibit antimicrobial properties and can be used to develop antimicrobial formulations for treating infections caused by bacteria, fungi and viruses. They can disrupt microbial cell membranes, inhibit biofilm formation and enhance the activity of conventional antimicrobial agents.
- Biosurfactants have been shown to possess anti-inflammatory properties and can modulate inflammatory responses. They can be used to develop new therapeutics to treat inflammatory conditions such as arthritis, dermatitis, and inflammatory bowel disease.
- Biosurfactants can be incorporated into scaffolds and matrices used in tissue

engineering applications. They can improve the mechanical properties, biocompatibility and cell adhesion of tissue-engineered constructs, facilitating tissue regeneration and repair.

Biosurfactants have diverse applications in medicine and hold great promise for the development of new therapeutic strategies and medical devices. Continued research and innovation in this field are essential to fully appreciate the potential of biosurfactants in improving human health and well-being.

Biosurfactants have a wide range of applications in various industries due to their unique properties and environmental benefits. There are several significant **industrial applications** where biosurfactants play an important role, like (Tabatabaee et al., 2005):

- *Oil and gas.* Biosurfactants are used in enhanced oil recovery (EOR) processes to improve the efficiency of oil extraction from reservoirs. They can reduce interfacial tension between oil and water, alter the wettability of reservoir rocks and mobilize trapped oil, leading to increased oil production.
- *Agriculture.* Biosurfactants can be used as adjuvants in agricultural formulations to enhance the efficacy of pesticides, herbicides and fertilizers. They can improve the spreading, wetting and penetration of agrochemicals, resulting in better crop protection and increased yields.
- *Food and beverage.* Biosurfactants are used in food processing and manufacturing to emulsify, stabilize foam food products. They can improve the texture, appearance and shelf-life of food products, as well as reduce the use of synthetic additives and preservatives.
- *Cosmetics and personal care.* Biosurfactants are used in cosmetics and personal care products such as shampoos, soaps, and skin creams. They can act as gentle cleansers, moisturizers and foaming agents, providing mild and sustainable alternatives to synthetic surfactants.
- *Pharmaceuticals.* Biosurfactants have potential applications in pharmaceutical formulations as drug delivery systems, emulsifiers and stabilizers. They can improve the solubility, stability and bioavailability of drugs, leading to enhanced therapeutic efficacy and reduced side effects.
- *Environmental remediation.* Biosurfactants are used in bioremediation processes to degrade and remove pollutants from soil, water, and air. They can enhance the bioavailability of contaminants, stimulate microbial activity and promote the degradation of hydrocarbons, heavy metals and other pollutants.

Biosurfactants offer sustainable and eco-friendly solutions to various industrial challenges and have the potential to replace conventional surfactants derived from petrochemicals. Continued research and development in this field are essential to reveal the full potential of biosurfactants in industry and to promote sustainable development.

Biosurfactants are gaining more and more attention in the *food industry* due to their potential applications and benefits. The following describes the ways in which biosurfactants are explored in the food industry.

- *Emulsification and stabilization*: they can act as emulsifiers, helping to stabilize oil and water emulsions in food products such as salad dressings, sauces, and mayonnaise. They improve the texture, appearance and shelf-life of these products by preventing phase separation and maintaining a homogeneous mixture (Tabatabaee et al., 2005).
- *Foaming and whipping:* they can enhance the foaming and whipping properties of food products such as whipped cream, meringues and mousses. Biosurfactants increase the volume and stability of foam structures, resulting in lighter textures and improved mouthfeel (Tabatabaee et al., 2005).
- *Fat reduction*: they can be used to reduce the amount of fat or oil in food formulations without compromising sensory attributes such as taste and texture. By forming stable emulsions and reducing the surface tension of water, biosurfactants enable the dispersion of fat droplets throughout the food matrix, resulting in lower fat content and healthier food options.
- *Antimicrobial properties*: some biosurfactants exhibit antimicrobial properties and can be used to inhibit the growth of pathogenic bacteria and fungi in food products. They can

be incorporated into food packaging materials or applied directly to food surfaces to extend shelf-life and improve food safety.

- *Clean label ingredients*: they derived from natural sources offer clean label alternatives to synthetic emulsifiers and stabilizers commonly used in processed foods. Consumers are increasingly seeking clean label products with minimal additives and biosurfactants provide a sustainable and eco-friendly option for food manufacturers (Rodrigues et al., 2006).
- *Encapsulation and delivery of bioactive compounds*: they can be used to encapsulate and deliver bioactive compounds such as vitamins, antioxidants and flavors in food products. They improve the solubility, stability and bioavailability of these compounds, enhancing their functionality and nutritional value (Rodrigues et al., 2006).

Overall, biosurfactants offer versatile and sustainable solutions for improving the quality, safety and nutritional value of food products. Continued research and innovation in this field are essential to unlock the full potential of biosurfactants in the food industry and meet the evolving needs and preferences of consumers.

Biosurfactants play a significant role in the food industry due to their biocompatible, biodegradable and non-toxic nature, offering a range of beneficial properties. They function as emulsifiers, foaming agents, wetting agents, solubilizers, adhesive agents and antimicrobial agents, making them versatile additives (Cameotra & Makkar, 2004).

In food applications, emulsions play a crucial role, where biosurfactants contribute to their stability and texture. Emulsifiers are particularly valuable in low-fat products, enhancing their creaminess and texture (Ron & Rosenberg, 2002). Polymeric surfactants form stable emulsions, preventing coalescence, which is advantageous for cosmetics and food formulations.

Biosurfactants also serve as food stabilizers, aiding in consistency control in bakery and ice cream products. They act as fat stabilizers and anti-spattering agents during cooking with oils and fats (Kosaric, 1992). In food processing, rhamnolipid surfactants enhance the texture and shelf life of starch-containing products, influencing the rheological properties and stability of dough. Additionally, surfactants help control fat globule agglomeration, stabilize aerated systems and improve the texture of fat-based products.

Moreover, L-Rhamnose, derived from hydrolyzing rhamnolipid surfactants produced by *P. aeruginosa*, has industrial applications as a precursor to high-quality flavor components like Furaneol .

Biosurfactants are increasingly recognized as valuable food additives due to their multifunctional properties, including emulsifying, anti-adherent and antimicrobial activities. In food processing, where the aim is not only to ensure safety but also to maintain taste, appearance and aroma, additives play a crucial role in enhancing the final product's quality and appeal.

Emulsifiers are vital components in food manufacturing, facilitating the mixing of immiscible phases by reducing surface tension at their interface. While traditional emulsifiers like lecithin derived from soy and egg, and synthetic emulsifiers, have long been used, the growing demand for natural or organic ingredients in functional foods presents an opportunity for new alternatives. Biosurfactants, due to their natural origin, environmentally friendly nature and unique properties, hold promise as efficient emulsifiers in the food industry. Their ability to reduce toxicity and meet consumer preferences for natural ingredients further strengthens their potential market.

Moreover, biosurfactants exhibit antimicrobial properties against various microorganisms, including bacteria, yeasts, fungi, algae and viruses (Nitschke & Costa, 2007). Lipopeptides, a well-known class of biosurfactants, demonstrate notable antimicrobial activity, with surfactin from *Bacillus subtilis* being a prime example (Das et al., 2009; Fernandes et al., 2007). This antimicrobial action extends to other lipopeptides produced by *Bacillus* species, such as fengycin, iturin, bacillomycin and myco-subtilin (Das et al., 2007).

Aside from their applications in the food industry, biosurfactants find utility in other sectors such as petroleum and pharmaceutical industries. In petroleum recovery processes, biosurfactants aid in the degradation of hydro-

carbons, enhancing oil recovery by microorganisms and facilitating the cleaning of oil reservoirs and storage tanks (Perfumo et al., 2010).

Furthermore, biosurfactants demonstrate potential in pharmaceutical applications, including genetic manipulation techniques. Studies have shown that biosurfactant-based liposomes exhibit higher efficiency in genetic transfection compared to commercially available cationic liposomes, making them a promising tool in gene therapy and other biomedical applications (Gharaei-Fathabad, 2011; Kitamato et al., 2002). In the last decade, some techniques and methodologies have been developed for liposome-based gene transfection. Ueno et al. in 2007 examined liposome containing MEL-A for genetic transfection by introducing biosurfactants into this field.

Biosurfactants in Pollution Control

According to reported literature, biosurfactants have the ability to emulsify hydrocarbon-water mixtures (Zhang and Miller, 1992). In the current era, petroleum pollution accidents have become numerous and have caused social and ecological catastrophes (Burger, 1993; Burns et al., 1993), and in these cases, the emulsifying properties of biosurfactants make them potentially useful tools for controlling petroleum pollution, by enhancing hydrocarbon degradation in the environment (Atlas, 1993; Bertrand et al., 1994).

The Role of Biosurfactants in Bioremediation Process

Accelerating the natural process of biodegradation in contaminated environments by using microbial metabolism is known as bioremediation. Biosurfactants are involved in bioremediation by increasing the surface area of insoluble hydrophobic substrates in water.

Biosurfactants have a number of advantages, including: biodegradability, low toxicity, good digestibility and compatibility, good emulsifying properties and availability. Biosurfactants undergo easy degradation by microorganisms, promoting environmental sustainability. In contrast to certain chemically synthesized counterparts, they can be naturally decomposed.

Biosurfactants generally demonstrate minimal toxicity, rendering them safer for diverse applications compared to specific chemical surfactants that may pose environmental and health hazards.

Biosurfactants exhibit favorable compatibility and digestibility with other organisms, enhancing their suitability for application across various biological and ecological systems without causing harm.

They can be derived from inexpensive and readily accessible raw materials in substantial quantities, rendering biosurfactant production cost-effective and scalable.

Biosurfactants are good emulsifiers, facilitating more efficient dispersion and stabilization of immiscible substances (e.g., oil and water). This capability proves advantageous in numerous industrial processes.

Biosurfactants are environmentally acceptable because of their long-lasting properties. Their natural origin and eco-friendly attributes contribute to their embrace in green and sustainable practices.

These advantages emphasize the potential of biosurfactants as a more sustainable and environmentally friendly substitute for certain synthetic surfactants in a range of industrial, agricultural, and medical applications.

CONCLUSIONS

Creating new strategies and technologies is necessary to minimize the production cost of biosurfactants on a commercial scale and to make the production process economically competitive.

Leading scientists use approaches such as green chemistry and genetic engineering of microorganisms to improve the yield and quality of biosurfactant production. Pretreating renewable substrates makes it easy to grow organisms.

However, care must be taken not to lose the nutritional values of these substrates. Because both the quality and quantity of the product are mandatory to open wide industrial perspectives for microbial surfactants. There is huge potential for waste from food processing, animal fats, and dairy industry sectors that are still waiting to be explored.

In recent years, numerous biochemically derived compounds and their production methods have been patented, although only a handful have made it to commercialization.

The profitability of biosurfactant production hinges on various factors, including raw material expenses, the feasibility of an economical production process and the yield of the microorganism product. Consequently, cheaper substrates, including waste materials, can be utilized, alongside genetically modified strains and efficient bioprocessing techniques, to achieve cost-effective biosurfactant production.

In conclusion, the biosurfactant market is poised for growth as screening initiatives uncover new microbial strains harboring novel molecules with exceptional properties. Additionally, advancements in sourcing low-cost raw materials and refining strategies for scaling up processes promise to yield successful outcomes in the biosurfactant industry.

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ISOLATION OF MICROORGANISMS TO ENHANCE THE DIGESTIBILITY OF ORGANIC SUBSTRATE FOR BIOGAS PRODUCTION

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Abstract

Agro-industrial waste is an abundant and renewable resource being an alternative for fossil fuels by production of biogas. This substrate has a high content of organic polymers and other high-mass substances, such as: starch, lignocellulose, proteins, lipids, and other compounds. For the degradation of these compounds, several microorganisms (bacteria and fungi) were isolated from the soil and characterized in terms of enzyme production: amylases, cellulases, laccases, proteases, and lipases. The microorganisms that had the highest enzyme indices were multiplied by cultivation in liquid media in order to highlight the degree of decomposition of the organic matter in the substrate. The aim of the article is to obtain at least 5 microbial strains with high degradative potential that can increase the degree of the substrate degradation through specific hydrolysis reactions of organic compounds.

Key words*: microorganisms, organic substrate, enzyme production.*

INTRODUCTION

Climate change is among the main global concerns, which are directly related to the burning of fossil fuels that generate greenhouse gas emissions. In this context, biomass plays an important role in mitigating the problems associated with the increased burning of fossil fuels (Yu et al., 2021). Thus, in recent decades there has been a growing interest in energy recovery from organic sources, such as animal waste, food waste, lignocellulosic biomass, and sewage sludge (Karrabi et al., 2023).

Biomass includes all organic matter that derives from different categories of feedstock, namely biomass from agro-zootechnical sector, forestry, food industry waste, algae and organic fraction of municipal solid waste (Janiszewska & Ossowska, 2022; Perea-Moreno et al., 2019). The biomasses generated by various agro-industries sectors have great potential as renewable energy sources since they can be used to produce biofuels that can be used for transportation, heating, and power generation (Nazar et al., 2022). In addition, biomass is one of the renewable resources with almost zero carbon dioxide (CO_2) emissions, because when it is formed it absorbs CO_2 from the atmosphere, so when it is burned, it does not contribute to global CO_2 emissions (Tursi 2019).

Anaerobic digestion process presents a promising solution to generate clean and sustainable energy through biogas production. This source of renewable energy offers multiple environmental benefits that are reflected in the reduction of greenhouse gas emissions, in the agronomic quality of the fertilizer obtained, and in the recovery of organic waste from landfills (Chaib et al., 2024; Scano et al., 2014).

Lignocellulosic biomass is an abundant raw material for conversion into biogas (Chaib et al., 2024). Agricultural by-products, mainly com-posed of straw, are common sources of lignocellulosic biomass. Such by-products are potential feedstocks for biofuel production due to their high availability and low cost (Nazar et al., 2022). Biogas production from lignocellulosic biomass has significant benefits, not only from the point of view of producing renewable energy, but also from the perspective of waste management (Hosseini Koupaie et al., 2019). The main problem of using lignocellulosic biomass for biogas production is its complex nature, which represents the resistance of the biomass to chemical and biological degradation (Ferdes et al., 2020). The main components of lignocellulosic

biomass are cellulose (crystalline and linear polymer with a rigid structure, difficult to decompose), hemicellulose (an amorphous heteropolysaccharide) and lignin (a heterogeneous phenylpropanoid macromolecule which is bound to both hemicellulose and cellulose, forming a tight physical structure that acts as an impenetrable barrier in the plant cell wall, giving it resistance against microbial attack), their proportions depending on the substrate used in the fermentation process (Amin et al., 2017; Chaib et al., 2024; Andlar et al., 2018).

Thus, the pretreatment of lignocellulosic biomass before the anaerobic digestion process is considered an important step for improving its biodegradability as well as biogas production.

When properly chosen, pretreatment methods can improve methane concentration and/or anaerobic digestion rate, thereby improving digester performance (Carrere et al., 2016). Various pretreatment techniques have been recommended to improve the digestibility of lignocellulosic biomass in order to enhance anaerobic digestion efficiency. The pretreatment methods are classified into physical, mechanical, chemical, and biological methods, these can be applied individually or in combination (Banu et al., 2021; Ferdes et al., 2020).

It is well known that microorganisms could produce complex enzymes that are crucial to the conversion of lignocellulose. Thus, biological pretreatment represents an attractive pretreatment method, with significant advantages, such as: it is conducted in mild environmental conditions, with low energy consumption, minimal or no inhibitor formation and no requirement to remove solvents after pretreatment (Gao et al., 2022). Fungal and bacterial pretreatment techniques are widely utilized for lignocellulosic biomass pretreatment. Cellulase or laccase enzymes are applied as biological pretreatment in order to improve the biogas yield (Rahmani et al., 2022).

Therefore, the main aim of this work is to obtain at least 5 microbial strains with high degradative potential that can increase the degree of substrate degradation through specific hydrolysis reactions of organic compounds. The efficiency of these strains was determined through enzyme activities: amylolytic, cellulolytic, proteolytic, lipolytic and laccases enzymes.

MATERIALS AND METHODS

Culture media. For isolation and storage of microbial culture nutritive agar medium (NA) was used.

To select the most productive microbial strains from the total of 16 isolated from the soil, the following culture media were used:

(1) for highlighting the amylolytic enzymes produced by the microbial colonies a solidified culture medium containing starch was used: yeast extract 4 g/L; soluble starch 10 g/L; K_2HPO_4 1 g/L; Mg $SO_4.7H_2O$ 0.5 g/L; agar – agar 20 g/L. Adding Lugol solution, the zone of hydrolysis remained uncoloured, whereas the non-hydrolysed starch was coloured dark blue.

(2) for cellulolytic enzymes the culture medium was prepared from: ammonium sulfate 1.4 g/L; peptone 0.5 g/L; K_2HPO_4 2 g/L; $CaCl_2$ 0.03 g/L; $MgSO_4$ 0.03 g/L; yeast-extract 0.75 g/L; CMC 10 g/L; agar 17.5 g/L. The medium is then colored with a Congo red solution, and the producing colonies become surrounded by a colorless area. If the tested microorganism produces extracellular cellulolytic enzymes, they diffuse into the culture medium and produce a clarification of the agar around the active colonies.

(3) for highlighting the proteolytic enzymes, a nutrient medium with casein was used: casein 2.5 g/L; Ca $(OH)_2$ 0.15 g/L; $CaCL_2$ 0.05 g/L; agar-agar 15 g/L. The colonies producing proteolytic enzymes determine around them a clear area of casein lysis in the culture medium.

(4) nutritive agar medium (NA) was used for highlighting the lipolytic enzymes. The lipolytic enzymes produced an opaque zone due to the formation of calcium salts in culture medium (NA) supplemented with Tween 80 and $CaCl_2$.

(5) the biosynthesis of laccases was assessed on Potato Dextrose Agar (PDA) supplemented with 0.04% guaiacol. The reddish-brown zones around fungal colonies are due to the oxidation of guaiacol to a coloured product (Ferdes et al. 2018; Ferdes and Ungureanu, 2009).

All the culture media were sterilized by autoclaving 15 minutes at 121°C then 15-20 mL are distributed in Petri dishes and allowed to solidify.

Methods

Isolation procedure. Soil collected from orchard, solar garden and fertilizer sample were used for the isolation of the potential enzyme producing microorganisms. All samples were collected from the Faculty of Biotechnical Systems Engineering, National University of Science and Technology Politehnica Bucharest. The isolations were carried out in Petri dishes, using the streak technique.

In this study, were initially isolated 16 strains from the three types of soil previously presented. After assessing the extracellular enzyme activity, were selected in the end five the most productive microbial strains.

Enzyme activity testing by the screening method

The culture media specific to the method were poured into Petri dishes and allowed to solidify. The inoculation of the media was done with the hook by central inoculation with a fragment of the inoculum from the stock cultures in test tubes. The Petri dishes were thermostated for 3-4 days until the complete development of the colonies.

Then, the diameters of the degradation zones of the substrate and the diameters of the colonies were measured, and their ratio was made (enzyme indices).

RESULTS AND DISCUSSIONS

After the first stage, that of isolating the microorganisms from the soil, several isolated microbial colonies were obtained along the striations from which a fragment of biomass was collected.

This was used for inoculating the culture medium in test tubes (Figure 2).

In Figure 1 it can be seen the isolation of microbial colonies from the solar garden, orchard and fertilizer that developed on the nutrient agar.

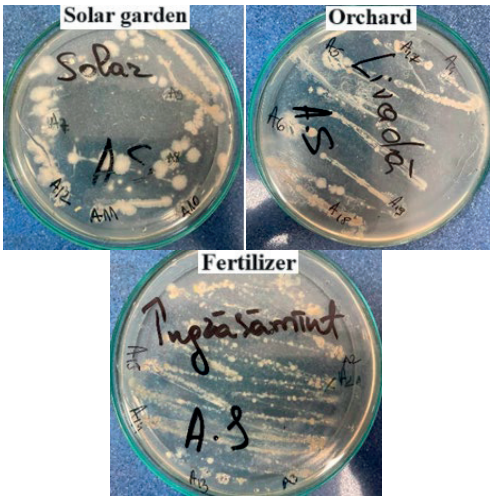

Figure 1. Isolation of the potential enzyme producing microorganisms

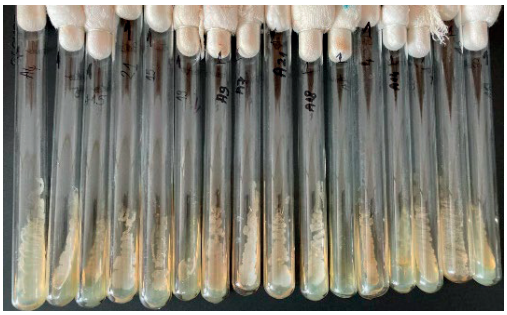

Figure 2. The isolated 16 strains from the three types of soil (in tubes)

Selection and identification of enzyme producing microorganisms.

In the preliminary stage, all 16 microbial strains were tested from the point of view of enzyme production, namely: amylolytic, cellulolytic, proteolytic, lipolytic and laccases. From these, depending on the enzyme indices, only 6 were selected, the best producers. Next, the 6 isolated strains were retested with the results shown in Table 1.

According to the result of extracellular enzyme activity assays, the most productive strains were selected (Figure 3).

Figure 3. The most productive microbial strains selected

The formation of a clear visible zone around the colony on the solid media supplemented with the suitable specific indicators demonstrated that isolated strains have enzymatic activity.

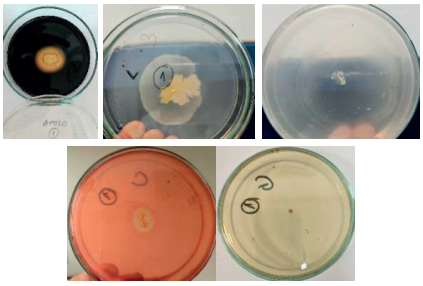

Figure 4. Appearance of colonies isolated from microbial strain no. 1 producing enzymes: amylase, lipase, protease, cellulase and laccase

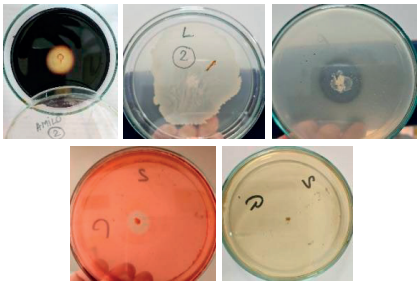

Figure 5. Appearance of colonies isolated from microbial strain no. 2 producing enzymes: amylase, lipase, protease, cellulase and laccase

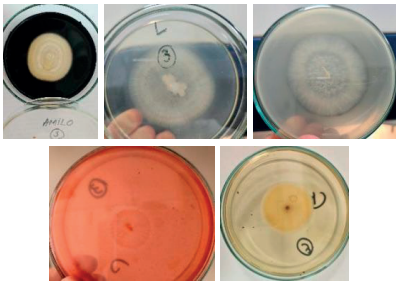

Figure 6. Appearance of colonies isolated from microbial strain no. 3 producing enzymes: amylase, lipase, protease, cellulase and laccase

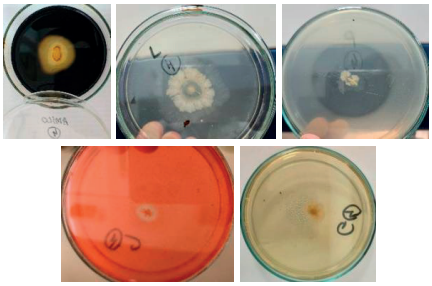

Figure 7. Appearance of colonies isolated from microbial strain no. 4 producing enzymes: amylase, lipase, protease, cellulase and laccase

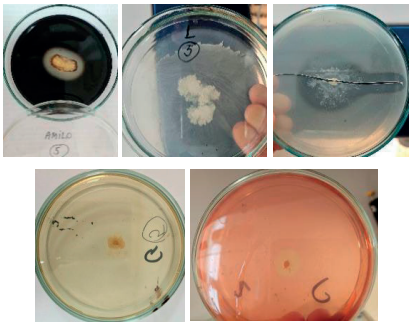

Figure 8. Appearance of colonies isolated from microbial strain no. 5 producing enzymes: amylase, lipase, protease, cellulase and laccase

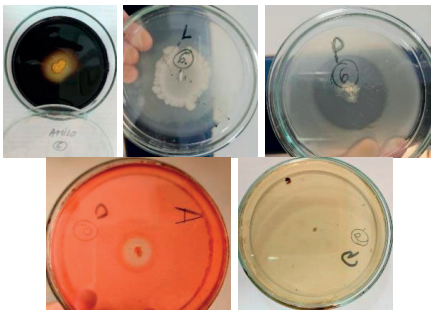

Figure 9. Appearance of colonies isolated from microbial strain no. 6 producing enzymes: amylase, lipase, protease, cellulase and laccase

For producing – laccase colonies, a slightly reddish colour is observed on the reverse side of the colony.

The selected bacterial strains exhibited different levels of enzyme activity. The enzymatic indices defined as the ratio of the diameter of hydrolysis zone and the diameter of colony has been calculated (Table 1).

Table 1. Enzymatic indices of selected strains (diameter of highlighted zone/ diameter of colony), mm mm^{-1}

Microbial strain no	Amylase index	Protease index	Lipase index	Cellulase index	Laccase production
1	2.5/1.2	0	0	1.2/0.8	-
2	2.7/1.6	2.4/1.3	0	1.3/0.4	+
3	3.3/2	3.6/3.5	6.8/5.6	2.1/2.1	+
4	4.5/1.5	4.5/0.9	0	1.1/0.5	+
5	3/1.2	3.2/2.4	0	1.2/0.6	+
6	3.5/1.2	4.4/1.2	8.2/5.2	1.4/0.5	+

After determining the enzyme indices, it was found that all the tested microbial strains showed amylase and cellulase enzyme activity. Proteolytic enzymes were absent in microbial strain no. 1. Only strains 3 and 6 synthesized lipolytic enzymes. Laccase biosynthesis was relatively low, but almost all microorganisms (except for microbial strain no. 1) had laccase activity (reverse colored reddish-brown). As shown in Table 1, bacterial strain no 6 showed the best enzymatic activity. Similar studies were carried out by Zhang et al. 2021, Wang et al., 2020 and Hossain et al. 2021.

Life cycle assessment of products and services is an environmental management technique to assess potential environmental impact associated with all life stages of a product, from the extraction of raw materials through material processing, production, distribution and use to waste management (Finnveden & Potting, 2014).

A lot of studies regarding the energy used and the environmental impact of bioenergy production were carried out in the literature (Osman et al. 2023; Yang et al., 2023; Ugwu et al. 2022; Ramirez-Arpide et al. 2018; Hijazi et al. 2016; Pacetti et al., 2015).

In the case of our study, the use of microorganisms increases the yield of the process because it hydrolyzes the substrate to a higher amount of nutrients which, probably, favors better growth of the microorganisms in the following stages.

Therefore, the final yield should be higher in the presence of hydrolytic and oxidation-reduction enzymes. We also appreciate that the CO_2 footprint should decrease when using these enzymes, thereby reducing the impact on the environment.

In addition, from an economic point of view, the isolation of these microorganisms from natural soils is reflected in the reduction of costs related to the pretreatment of the substrates and implicitly in the shortening the required time for the first stage of anaerobic digestion, that of hydrolysis.

CONCLUSIONS

In the present study, 16 bacterial strains were isolated from natural soils (orchard, solar garden and soil fertilizer) and the production level of amylolytic, cellulolytic, proteolytic, lipolytic and laccases enzymes was compared.

The selection of enzyme-producing microorganisms was carried out in several stages, namely: isolation from the soil, preliminary testing and then the selection of the best 6 strains that will be used in the following studies.

The best producer was microbial strain no. 6, which had an enzymatic index of 2.9 for amylolytic enzymes, 3.6 for proteolytic enzymes and 2.8 for cellulolytic enzymes.

The isolated enzymes producing microorganisms may be used for the effective pretreatment of lignocellulosic agricultural wastes for the production of biogas by anaerobic fermentation process.

The identification of such bacterial strains in natural soils is a process of actuality and is

necessary for the development of a sustainable and economically technology.

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HORSERADISH, A RESERVOIR OF USEFUL BIOACTIVE COMPOUNDS

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Abstract

Horseradish (Armoracia rusticana) is a plant of the Brassicaceae family, known for its strongly aromatic and spicy root. Horseradish, like many other root vegetables, is rich in biologically active compounds with antibacterial, anti-inflammatory, antioxidant and anticarcinogenic properties. The composition of the biologically active compounds of horseradish varies depending on the species of horseradish and the environmental conditions in which it is grown. The aim of this work was to determine the composition of some bioactive compounds from horseradish roots and leaves from the Romania and central part of Serbia. The elemental composition was determined by the XRF method, it being known that many mineral elements and trace elements are essential for the normal functioning of human bodies, reducing the risk of chronic diseases. The compounds with antioxidant activity from plant matrices are known as protectors of cells against oxidative stress, with a role in supporting the immune system. Consequently, the total content of polyphenols and antioxidant activity were determined from horseradish extracts by spectrophotometric methods. The results obtained recommend the tested horseradish as a beneficial food for health.

Key words*: horseradish, bioactive compounds, elemental composition, antioxidant capacity.*

INTRODUCTION

Horseradish (*Armoracia rusticana*) is a plant of the *Brassicaceae* family, rich in biologically active compounds with antioxidant properties. The composition of the biologically active compounds of horseradish varies depending on the species of horseradish and the environmental conditions in which it is grown.

Polyphenols are chemical compounds with antioxidant properties that are found in large quantities in vegetables. The content of polyphenols and compounds with antioxidant properties differs from one plant to another, but it is also influenced by a number of factors related to the cultivation conditions (soil type), the growth and development stage of the plant, or the processing of it. Among the polyphenols identified in horseradish, the most important are glucosinolates and flavonoids. The roots and leaves of horseradish also contain numerous organic acids, vanillin, amino alcohols, tannins, amino acids, vitamins, minerals, protein and enzymes, carbohydrates [Negro et al., 2022, Knez et al., 2022].

Glucosinolates are sulfur compounds, the most widespread in horseradish being gluconasturtin, sinigrin and glucobrassicin [Prieto et al., 2019]. Through the enzymatic hydrolysis of glucosinolates, mainly isothiocyanates and thiocyanates, nitriles are formed. [Popović et al., 2020].

Mineral elements such as potassium, calcium, magnesium and phosphorus contained by horseradish are essential for the general health and functioning of the plant but also of the human body, following its consumption. But horseradish can also contain transition metals (zinc, copper, iron, manganese etc.), and their excessive content can create toxicity and have adverse effects on human health.

All the biologically active compounds contained in horseradish made it to be used since ancient times for pharmaceutical purposes and for food improvement and preservation [Agneta et al., 2013, Knez et al., 2022, Petrović et al., 2017].

Determining the content of minerals, polyphenols and antioxidant capacity of horseradish is important for evaluating its quality as food, understanding its potential for health and protection against chronic diseases

and the relationship between nutrition and health.
The aim of this work was to evaluate the elemental composition, the polyphenols content and antioxidant capacity of horseradish roots and leaves from western Romania and central part of Serbia.

MATERIALS AND METHODS

Folin-Ciocalteu's phenol reagent, gallic acid (GAE), Trolox, neocupreine, bovine serum albumin (BSA), copper chloride, sodium carbonate and ethanol were purchased from Merck. All the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification.
Mature horseradish collected from 2 locations, one from Romania and the other one from Serbia, were harvested in October 2023. The vegetables (roots and leaves) were cleaned and washed with distilled water. The leaves were removed from the roots. The roots were ground, the bark being collected separately from the inner part. The vegetable parts (leaves, root's inner part and root's bark) were lyophilised and stored until use at 4^0C, in closed vials. The freeze drying was carried out until a constant mass of vegetable samples.
The horseradish extracts for the polyphenols, antioxidant capacity and protein analyses were obtained in an alcoholic medium (ethanol 50%) by mixing lyophilised vegetable parts with the extraction medium in a ratio of 1:10. The extraction time was 30 minutes, under magnetic stirring, 22^0C.
The total phenolic content was assayed by Folin-Ciocalteu method by using galic acid as standard [Folin et al., 1927, Singleton et al., 1999]. The Folin–Ciocalteu reagent was reduced by phenolic compounds contained by horseradish alcoholic extract in an alkaline environment. The absorbance of the reduced product containing a blue chromophore was measured at 750 nm. The total phenolic content was expressed as mg GAE/g dry weight of horseradish.
The total antioxidant capacity of the lyophilised horseradish extract was measured by CUPRAC method by using Trolox as standard [Apak et al., 2004]. In CUPRAC reaction the ligand copper (II)-neocuproine is reduced to a complex containing Cu^+. While the redox reaction takes place the colour changed from light blue to yellow-orange. The absorbance of the reduced CUPRAC product was measured at 450 nm. The total antioxidant capacity was expressed as μmol Tolox/g dry weight of horseradish.
The protein content was assayed according to the Lowry method, using the Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) as standard [Lowry et al., 1951]. The protein content was expressed as mg_{BSA}/g dry weight of horseradish.
The elemental analysis of the horseradish was assayed by X-ray fluorescence (XRF). The vegetable parts (leaves, root's inner part and roots bark) used for XRF analysis was dried by lyophilization and milled in a mortar to a powder in order to minimize the particle size effects.
Statistical analysis was completed using PAST Version 2.17c software (Hammer et al, 2001).

RESULTS AND DISCUSSIONS

The lyophilised horseradish from Romania and Serbia was analysed to determine the total antioxidant activity, polyphenol and protein content, minerals and heavy metals. The analyses were carried out both for the internal part of the horseradish roots, obtained after removing the bark, as well as for the bark and leaves.
The horseradish samples had different content of water, the dry substance ranging from 19 to 46%. Our results are consistent with those obtained by Biller at al., 2018 and Agneta et al., 2014, they measured values for dry substances in the range 13-30% and 25-36%, respectively.
In plants there is a great diversity of compounds with antioxidant activity which, together with enzymes, have the role of neutralizing free radicals in cells. The individual quantitative analysis of antioxidants is very difficult because it involves their separation and then their qualitative analysis. Considering that the molecules with antioxidant activity act synergistically, the quantification of each one separately would not lead to significant results. So that the total antioxidant capacity is more proper in evaluating the positive effects on the human health.

In this work the antioxidant activity was assayed by using cupric reducing anti - oxidant capacity method (CUPRAC). The CUPRAC assay is based on the oxidoreduction reaction between the CUPRAC reagent ($Cu(NeoCu)_2^{2+}$) and the antioxidants (AOX_S)from the horseradish extract, forming the reduced form blue $Cu(NeCup)_2^{1+}$ and the oxidized form of antioxidants $oxAOX_s$

$$Cu(NeCup)_2^{2+} + AOX_S \leftrightarrow Cu(NeCup)_2^{1+} + H^+ + oxAOX_s$$

The results can be found in the Figure 1.

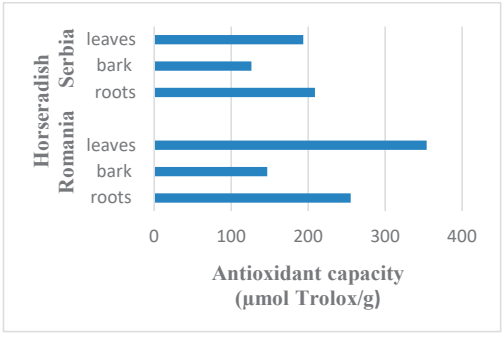

Figure 1. The antioxidant capacity of tested horseradish parts

It can be seen that the antioxidant capacity measured in the horseradish harvested in Romania is higher than that from Serbia, for all three parts of horseradish tested. The highest oxidizing activity was in the leaves of horseradish harvested in Romania and is 1.4 times higher than that in the internal part of roots.

The results obtained by us agree with those obtained in other studies and which show that the antioxidant properties can be influenced by the area where the horseradish is grown and the genotype of plants [Biller et al., 2018].

The total phenolic content was measured in the parts of horseradish plants by using the Folin-Ciocalteu method (Figure 2), the most used method for the quantitative analysis of plant extracts. The phosphomolybdic /phosphotungstic acid complex from the Folin-Ciocalteu reagent was reduced by the polyphenols from horseradish and a blue chromophore was formed.

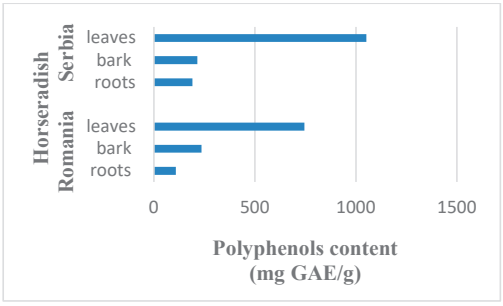

Figure 2. The polyphenol content of tested horseradish parts

The highest content of polyphenols was assayed in horseradish harvested in Serbia. Internal part of the horseradish roots from Serbia contains polyphenols in an amount 1.8 times higher than the same part of horseradish collected in Romania.

The polyphenol content of leaves is 5-7 times higher than that in internal part of roots of the same plant. Our results are in accordance with the results of Tomsone et al., 2020 and prove that the polyphenols are in different amounts in different parts of the same plant.

The differences measured for the biochemical parameters analysed in the tested horseradish could be due to the differences between the culture environments where the plants were grown and their potentially different varieties. According to studies of Biller et al., in many countries, the horseradish plants grew semi-wild and their varieties are not known [Biller et al., 2018].

The protein content was measured in leaves, root's internal part and roots bark extract. The horseradish collected from Serbia had a higher protein content measured in leaves and roots, but the values are quite closed.

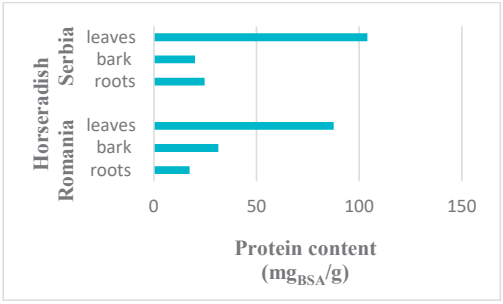

Figure 3. The protein content of tested horseradish parts

The elemental analysis of three different parts of horseradish was investigated by XRF method (Figure 4). The X-ray fluorescence method is a very used technique for evaluation of elements in plants materials, being an economical and less consuming time procedure.

This study followed two main directions: (1) determining the content of essential minerals for the growth and health of plants, minerals that can be beneficial to the human body after consuming horseradish; (2) establishing the potential toxicity due to the presence of heavy metals.

Cluster Analysis was performed (Figure 4) based on Paired Group Algorithm using Euclidean distance as Similarity measure shows a high correlation regarding mineral content of investigated samples (Cophenetic correlation coefficient= 0.991).

Principal Component Analysis (PCA) was performed using Variation-Covariation Matrix with the role to reduce the number of data while preserving the most important trends or patterns (Figure 5).

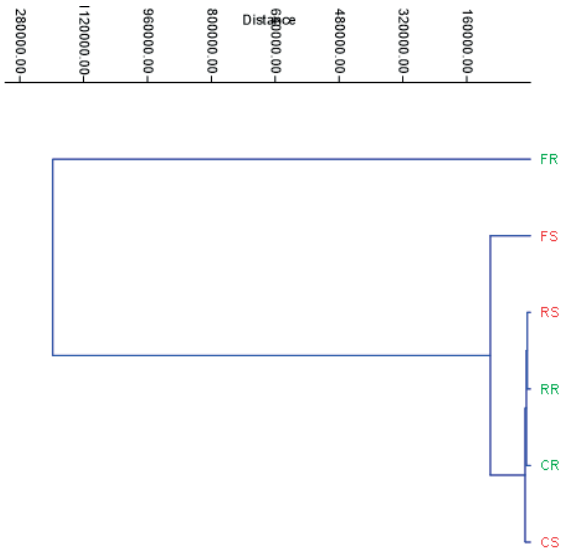

Figure 4. Cluster Analysis representation based on Euclidian Distances (RR-horseradish roots harvested in Romania, CR- horseradish bark harvested in Romania, FR- horseradish leaves harvested in Romania, RS- horseradish roots harvested in Serbia, CS- horseradish bark harvested in Serbia, FS- horseradish leaves harvested in Serbia)

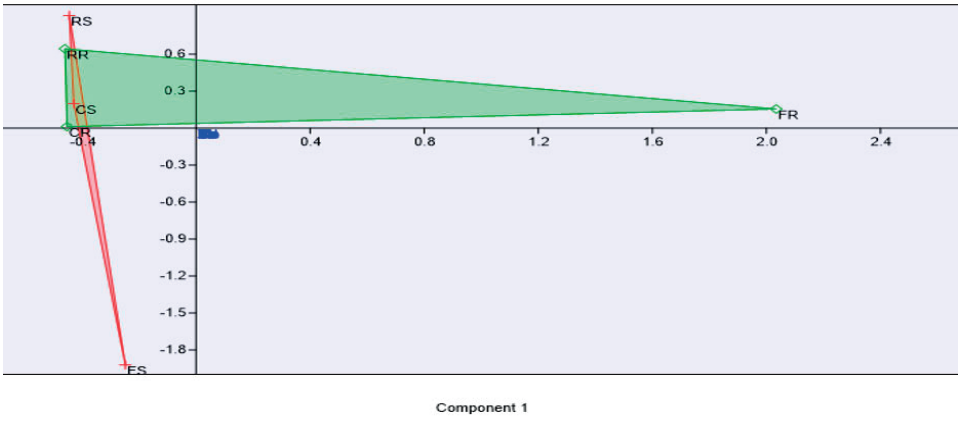

Figure 5. PCA Scatter Diagram of mineral composition data

Same like Cluster Analysis PCA shows also that the elemental data corresponding to the horseradish samples of Serbian origin are similar to the horseradish samples of Romanian origin, but the variation of data is much higher in the samples originated from Romania.
The trace elements with essential roles in physiological processes have been measured in all horseradish samples, in all tested parts of the plants. The content of K, Ca, Fe, Zn, Mn, Mg, Cu was found in concentrations similar to those from the scientific literature data, for all tested samples [Jabeen et al., 2010].
Roots have the ability to absorb heavy metals from the soil, such as Cd, Pb, Hg, As, etc. These metals, depending on their concentration, may have toxic effects as a result of consuming of the plants. None of these metals were in detectable doses in the horseradish tested samples.

CONCLUSIONS

The results of our research showed that the total antioxidant capacity and the content of polyphenols were affected by the environment where the plants grown and the variety of plants. All parts of horseradish had an important content of antioxidants, especially the leaves.
The horseradish grown in Romania has a higher antioxidant activity than the one in Serbia.
No toxic doses of heavy metals were detected in any of the tested samples.
The results obtained recommend the tested horseradish as a beneficial food for health.

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