AFS- ADVANCES IN FOOD SCIENCES

Contininuation of CMTL founded by F.Drawert

Production by PSP-Vimy Str. 1e,85354 Freising,Germany in

cooperation with PRT-Parlar Research & Technology

Vimy Str 1e,85354 Freising

Printed in Germany-ISSN 1431-7737

AFS

Advances in Food Sciences(AFS) is abstracted/indexed in:

Chemical Abstract Service, BIOSIS, CAB International, Cambridge Scientific Abstracts, Food Science and Technology Abstracts(FSTA) , Current Contents/Agriculture, CSA Civil Engineering Abstracts, CSA Mechanical & Transportation Engineering,ISI(Current Contents)

CONTENTS

ORIGINAL PAPERS

EVALUATION OF TOTAL PHENOLIC, FLAVONOIDS AND ANTIOXIDANT ACTIVITY OF *Calendula Officinalis* (L.) EXTRACTS COLLECTED IN KOSOVO

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ABSTRACT

The antioxidant capacities, total phenolic and flavonoid content of five different extracts (diethyl ether, chloroform, ethyl acetate, n-butanol and water) of *C. officinalis* (L.) growing wild in Kosovo were analyzed. Antioxidant activity was determined by DPPH assay. Total phenolics and total flavonoids content in the extracts was determined spectrophotometrically. Statistical analysis was performed using SPSS version 13.0 and Excel 2010. The amount of total phenolics in *C. officinalis* (L.) extracts ranged from 7.30 mg/g to 37.60 mg/g (expressed as gallic acid equivalent, mg GAE/g of dried extract). The amount of total flavonoids in *C. officinalis* (L.) extracts ranged from 10.15 mg/g to 27.52 mg/g (expressed in rutin equivalent, mg RE/g dried extract). Ethyl acetate (EtOAc) and water (H_2O) extracts of *C. officinalis* (L.) expressed very strong scavenger activity, 15.13 µg/mL and 15.20 µg/mL, respectively. The observed differences in antioxidant activity could be partially explained by the levels of phenolics and flavonoids in extracts of *C. officinalis* (L.). This plant can be used to discover bioactive natural products that may serve as leads in the development of new additives for application in food technology.

KEYWORDS:
Extracts, Phytochemical, Antioxidant, *Calendula officinalis* (L.), Kosovo

INTRODUCTION

Several plant extracts and plant products have been shown to possess significant antioxidant potential, [1, 2, 3] *Calendula officinalis* (L.), an herb used in traditional medicine, has been reported to have several pharmacological activities including anti genotoxic effect [4]. This reported pharmacological activity might be related to the antioxidant activity of *C. officinalis* (L.) extract, although this was not fully substantiated. Based on our knowledge, there is only one report on the antioxidant activity of *C. officinalis* (L.) extracts [5]. The aim of this research was to determine phenols, flavonoids and antioxidant activities of different extracts as diethyl ether (Et_2O), chloroform ($CHCl_3$), ethyl acetate (EtOAc), n-butanol (n-BuOH) and water (H_2O) of *C. officinalis* (L.) growing wild in North-Western part of Kosovo.

MATERIALS AND METHODS

The aerial parts of *C. officinalis* (L.) growing in North-Western part of Kosovo, was collected in May 2018 (Figure 1). Voucher specimens were deposited in the herbarium (Nr. 15/2018) of the Department of Plant Protection, University of Pristina. The plants were dried at room temperature.

FIGURE 1
C. officinalis (L.) growing wild in North-Western part of Kosovo (photo taken from Arben Haziri)

Preparation of plant organic extracts. A portion of the finely powdered material (200 g) was extracted three times with 70% methanol (methanol, 4 L) during a 24-h period. After removal of methanol under reduced pressure, the aqueous phase was successively extracted with four solvents of increasing polarity, namely diethyl ether, chloroform, ethyl acetate and n-butanol. The extraction was carried out until a colorless extract was obtained. The residue was the aqueous extract. All five extracts (ethyl acetate, diethyl ether, water, n- butanol and chloroform)

were evaporated to dryness and then dissolved in 50% ethanol to make 10% (w/v) solutions. These solutions, either as such or in diluted state, were used in subsequent experiments.

Determination of total phenolic and flavonoid content. The amount of total phenolic contents in the extracts was determined spectrophotometrically with the Folin-Ciocalteu (FC) reagent using the method of Fukumoto and Mazza [6] with small modifications [7]. Gallic acid was used as standard [8]. Total phenolic content is expressed in mg gallic acid equivalents (GAE) per g dried extract. Different concentrations of gallic acid solution (5 mg/100 mL) were used to plot the calibration curve. All measurements were replicated five times. Total flavonoid content in the extracts was determined spectrophotometrically, using a method based on the formation of a flavonoid-aluminum complex with an absorbance maximum at 430 nm [9]. Content of total flavonoids is expressed in mg rutin equivalent (RE) per g dried extract. All measurements were replicated five times.

Antioxidant activity- DPPH assay. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as described previously [10, 11], following the transformation of the DPPH radical to its reduced, neutral form (DPPH-H). The same procedure was repeated with *tert*-butylhydroxytoluene (BHT) and *tert*-butyl-4- hydroxyanisole (BHA) as a positive control. For each sample five replicates were recorded. The inhibition was calculated using the following formula:

Inhibition (%) = 100 x (A_0 – A_1) / A_0

Where A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard, respectively. The percent inhibition values were plotted versus the concentration curve and the concentration of the sample required to achieve 50% inhibition was determined and expressed as the half maximal inhibitory concentration (IC_{50}). A lower the IC_{50} value indicates high antioxidant capacity.

STATISTICAL ANALYSIS

Data are presented as means with standard deviation (±SD). Statistical analysis was performed using SPSS version 13.0 and Excel 2010. Each experiment was repeated five times.

RESULTS AND DISCUSSION

Total phenol and flavonoid contents. The amount of total phenolics in *C. officinalis* (L.) extracts ranged from 7.30 mg GAE/g dried extract (in Et_2O extracts) to 37.60 mg GAE/g dried extract (in EtOAc extracts). A significant amount of these compounds also has been observed in the H_2O extracts 28.70 mg GAE/g dried extract (Table 1).

Furthermore, considerable total flavonoids content was determined in the H_2O and EtOAc of *C. officinalis* (L.). The amount of total flavonoids in *C. officinalis* (L.) extracts ranged from 10.15 mg RE/g dried extract to 27.52 mg RE/g dried extract. The less total flavonoids were determined in n-BuOH and in Et_2O extracts (Table 1).

TABLE 1
The amount of total phenolic contents and content of total flavonoids in *C. officinalis* (L.) extracts

Extracts	Et_2O	$CHCl_3$	EtOAc	n-BuOH	H_2O
Total phenolic content[1]	7.30±0.25	8.15±0.20	37.60±0.66	15.80±1.30	28.70±0.10
Total flavonoids content[2]	10.30±0.86	15.30±0.85	27.52±0.34	10.15±0.42	26.90±0.56

[1]Total phenolic content is expressed in expressed in mg gallic acid equivalents (GAE) per g dried extract;
[2]Content of total flavonoids is expressed in mg rutin equivalent (RE) per g dried extract.

TABLE 2
DPPH scavenging activity of *C. officinalis* (L.) extracts presented as IC_{50} values (µg/ml)

Extract	IC_{50} (µg/mL)[1]
Et_2O	40.20 ± 0.35
$CHCl_3$	37.60 ± 0.60
EtOAc	15.13 ± 1.10
n-BuOH	17.16 ± 0.78
H_2O	15.20 ± 0.46
BHT	16.30 ± 0.87
BHA	15.60 ± 0.80

[1]Each value in the table vas obtained by calculating the average of five analyses ± standard deviation.

Free radical scavenging activities. The antioxidant activity of *C. officinalis* (L.) extracts has been evaluated in a series of *in vitro* tests. The DPPH radical is one of the most commonly used substrates for fast evaluation of antioxidant activity because of its stability (in radical form) and the simplicity of the assay. In the DPPH assay, the ability of the investigated extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H was investigated (Table 2).

EtOAc and H_2O extracts of *C. officinalis* (L.) expressed very strong scavenger activity 15.13 µg/mL and 15.20 µg/mL, respectively. On the other hand, Et_2O and $CHCl_3$ extracts showed much weaker effect in the neutralization of DPPH, 40.20 µg/mL and 37.60 µg/mL, respectively. The whole assessed extracts of *C. officinalis* (L.) were able to reduce the stable, purple-colored radical DPPH to the yellow-colored DPPHH form. Comparison of the DPPH scavenging activity of the investigated *C. officinalis* (L.) extracts with those expressed by BHT (16.30 µg/mL) and BHA (15.60 µg/mL) showed that only the H_2O and EtOAc extracts expressed stronger antioxidant effects. Comparing with the DPPH test results of total flavonoids content in the extracts (Table 2), it could be concluded that only in case of the H_2O and EtOAc extracts of *C. officinalis* (L.) there is some correlation between the DPPH scavenger activity and content of flavonoids.

CONCLUSIONS

The results from this study showed that *C. officinalis* (L.) extract has antioxidant potential. Also *C. officinalis* (L.) extracts have high concentration of phenols and flavonoids. Based on these results, this plant can be used to discover bioactive natural products that may serve as leads in the development of new additives for application in food technology. Next work will be focused on the phenolic and flavonoids profile of extracts from *C. officinalis* (L.).

ACKNOWLEDGEMENTS

This work was supported by the Ministry for Education, Science and Technology of Kosovo under Grant No. 4851-1.

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Received: **08.01.2022**
Accepted: **20.03.2022**

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© by PSP Volume 44 – No. 1/2022 – pages 5-14 Advances in Food Sciences

COMPARATIVE STUDY ON GROWTH PARAMETERS, PROXIMATE ANALYSIS AND MINERAL COMPOSITION OF *Ganoderma lucidum* CULTIVATED ON DIFFERENT SUBSTRATES

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ABSTRACT

Mushrooms are usually cultivated for their medicinal and other attributes. The cultivation of *Ganoderma lucidum* is limited in Nigeria. The present study aimed to cultivate *G. lucidum* on four substrates and comparatively determine its growth parameters, proximate analysis, mineral composition. Cultivation used method of Adenipekun and and Fasidi (2005). The mineral comtents were determined using Atomic Absorption Spectrophotometer. Proximate analysis (%) was done using AOAC (2010) procedures. Growth paramerters showed that 10% wheat bran level of *Mansonia altissima* sawdust gave the highest yield (effect of fructification, days of fuctification and biological efficiencies). *M. altissima* showed higher yield than *Cordia. Millenii*. The pH ranges from 6.08±0.14 to 6.55±1.00. There was significant difference ($P<0.05$) in the days of fructification due to the varied % WBL of the substrates. Biological efficiency was highest at 10% WBL on *M. altissima* sawdust. Proximate analysis revealed the presence of moisture content (7.27± 1.00), crude protein (28.40±0.50), crude fat (3.49±1.00), fibre (29.12± 0.00), ash (9.94±0.20) and carbohydrate, 74.69±1.00). The mineral composition (Mg/100g) revealed the presence of calcium (90.6±3.01), magnesium (167.05±4.00), manganese (11.65±1.01), iron (50.75±1.93), sodium (86.45±2.00) and phosphorus (506.80±5.30).

KEYWORDS:
Ganoderma lucidum, cultivation, proximate analysis, minerals

INTRODUCTION

Mushroom is a macrofungus with a distinctive fruiting body which can be either epigenous (growing on or close to ground) or hypogenous (growing under the ground) and large enough to be visible to the naked eye and to be picked up by hand. They are grown or cultivated on substrates such as log of wood, sawddust, wood chips, termite hills, etc from which it derives it nutrients [1]. Over 200 species of mushrooms have long been used as functional foods anf supplements around the world [2], but only about 35 species have been commercially cultivated [3,4]. They are a rich source of nutrients, particularly proteins, minerals as well as vitamins B, C and D [5]. They have received overwhelming attention from food and pharmaceutical researchers due their bioactive constituents and preparation to prepare medical concoctions and drugs [6,7,8, 9,10].

Ganoderma spp are referred to as higher fungi because the carpophores are visible enough to be seen with naked eyes, although the real organism comprises intercillary microscopic bodies which could not be seen with ordinary eyes [12,13]. In West Africa, especially Nigeria, many health claims have been made on the effect that *Ganoderma* spp have on the immune system but it is rarely cultivated in Nigeria. The local traditional doctors among the Yoruba people of Southern Nigeria have used *Ganoderma* spp in treatment of skin disorder, high blood pressure and intestinal disorder [13] *G. lucidum* contains more than 400 bio-active elements. It has been nominated as fungus of the year in 2013 by the Journal Mycology [14,15]. It has been used as drug for years in Asian countries [16]. *G. lucidum* is grown and used as medicine and feed supplement in the form of tablets, powder and capsules [17,18].

MATERIALS AND METHODS

Substrates and supplement. The four substrates used for this study were sawdust and woodchips of *Mansonia altissima* and *Cordia millenii* and the supplement used was wheatbran. The sawdust and woodchips obtained from sawmill, Bodija market in Ibadan, Nigeria. The supplement was collected from Bodija market in Ibadan. The spawn of the *Ganoderma lucidum* was collected from Zero Emission Research Initiative (ZERI) University of Namibia, Windhoek, Namibia

Substrate Preparation. The modified method of Lawal (2011) was employed . *M. altissima* sawdust and woodchips and *C. millenii* sawdust and chips were soaked in water and excess water squeezed out. 0g, 50g, 100g, 150 of wheat bran and 500g, 450g, 400g, 350g of sawdust and woodchips respectively were thoroughly mixed together. 1% of $CaCO_3$ (additive) at different percentages (0%, 10%, 20% and 30%). The mixture is then packed into well labelled polyethylene bags. The bags were autoclaved at 121 ^oC for 15 minutes, cooled and inoculatedwith 10g of vigorously growing spawn of *G. lucidum* in the dark for mycelia growth. Each substrate was prepared in three replicates.

Determination of Biological Efficiency (BE). Stipe length, pileus diameter and mushroon height were measured in centimeters with a meter rule. The mushrooms were counted and weighed. At the end of second and/or third flush, total yield and biological efficiency (BE) were determined.

$$\text{Biological Efficiency (BE)} = \frac{\text{Fresh weight of flush}}{\text{Dry weight of substrate}} \times 100$$

PROXIMATE ANALYSIS

Moisture content, ash content, crude protein content, crude fibre, sugar content, lipid content were done according to standard methods.

pH OF G. *lucidum* EXTRACT. The electrodes were rinsed by dipping them in distilled water, removed from the water and blotted dry. The electrodes were rinsed with a portion of the sample in a small beaker. Sufficient *G. lucidum* extract was poured into the small beaker to allow the tips of the electrodes to be immersed to a depth of about 2 cm. The electrodes was at least 1 cm away from the sides and the bottom of the beaker. Readings were taken after the pH meter was switched on. The pH meter electrodes were each time rinsed in distilled water before taking the next reading [20].

MINERAL COMPOSITION

1g of sample was taken into a digestion tube, 20ml of digestion acid mixture was added and heated in a digestion block till a clear solution was obtained. The solution was filtered through glass wool and the volume was made up to 500ml with distilled water. **Atomic Absorption Spectrophotometer (AAS)** was used to determine sodium, potassium, calcium, magnesium [21].

RESULTS

Table 1 shows that the PL, SL and SC recorded significant ($p \leq 0.05$) reduction across (i.e. from flush 1 to flush 2) the concentration levels with WBL at 10% concentration level showing higher significance more than both the control and other wheat bran levels (20% and 30%). Similar result was observed in the second flush. The highest PL values (8.27 and 7.67) were recorded in 10% *M. altissima* and *C. millenii* sawdusts. The best SL yield (1.97) was recorded in 10% *M. altissima* wood chips while the best SG yield (2.60) was recorded also in 10% WBL of *M. altissima* sawdust.

Table 2 shows fructification, number of fruit bodies per flush, pH, biological efficiencies and moisture content of *G. lucidum.* Incubation periods range from 47 to 63 days. The lowest (47) being 10% wheat bran on sawdust of *C. millenii* while the highest (63) being 30% wheat bran on sawdust of *C. millenii.* Number of fruit bodies range fro 1 to 7, the highest was found in 10% wheat bran on sawdust of *C. millenii*. There was no significant difference in pH which ranges from 6.08 to 6.55. The biological efficiency was highest in the *M. altissima* substrate containing 20% wheat bran. Moisture content was lowest (1.18) in 30% wheat bran on the wood chips of *C. millenii* and highest was in 10% wheat bran on sawdust of *M. altissima.*

Table 3 shows the result of proximate composition of the mushroom showed significant ($p \leq 0.05$) difference across the varying concentration levels. Similar result was obtained across the substrates and the *M. altissima* sawdust. 0% WBL of *M. altissima* sawdust gave the highest carbohydrate of 74.69%. Also, 20% WBL of *M. altissima* gave the highest crude protein of 28.40% and crude fat of 3.49%. whereas, 0% , 20% and 30% WBL of *C. millenii* sawdust gave the highest moisture content of 7.27%, ash of 9.94% and highest crude fibre of 29.12% respectively.

Table 4 shows the result obtained from the mineral components showed higher significance of Ca at 10% (88.50) and 30% (90.60) WBL of *C. millenii* sawdust and wood chips respectively, while there is no significant difference between 20% concentration and the control. Mg was relatively high in 20% WBL of *M. altissima* sawdust (155.70), wood chips (167.50) and *C. millenii* sawdust (115.20) and wood chips (138.85) respectively. Mn was highest (11.65) in 20% WBL of *C millenii* wood chips. Fe was highest (50.75) in 30% WBL of *C. millenii* wood chips. Na was higher in 10% (86.45) WBL of *M. altissima*; 0% (86.10) and 20% (84.90) of sawdust and wood chips of *C. millenii* respectively. Phosphorus was high in 10% (506.80), 20% (427.40) of *M. altissima* sawdust; 10% (456.10) WBL of *C. millenii* sawdust and 0% (440.50), 10% (418.10), 30% (489.25) of *C. millenii* wood chips. In all, phosphorus was the highest

mineral, followed by magnesium, by sodium, by calcium, by iron and lastly by manganese.

DISCUSSION

The growth of *G. lucidum* was supported best by 10% wheat bran level (WBL) of *Mansonia. altissima* sawdust. This is in comformity to the work of [22] who observed that maximum biological efficiency of 17.4% was obtained from mango sawdust supplemented with 10% wheat bran. However, wood chips of *M. altissima*, sawdust and wood chips of *Cordia millenii* also supported the growth of *G. lucidum* which is in accordance to the works of [9,11,23] who reported that agricultural and agro-industrial residues could supoort the cultivation of mushrooms. These finding is in conformity with some authors who reported hardwood sawdust have been preferred for the commercial production [24] although *Ganoderma* can be cultivated on the sawdust which may originate from different kinds of trees [25]. Most of researches have focused on submerged media in obtaining mycelial biomass.

TABLE 1
Growth Parameters of *G. lucidum* on different substrates and substrate forms at a glance

Substrate	Substrate form	WBL (%)	First flush			Second flush		
			PL	SL	SG	PL	SL	SG
M. altissima	Sawdust	0	5.67^{b}	1.57^{a}	2.53^{ab}	5.17^{b}	1.40^{a}	2.30^{a}
		10	8.27^{a}	1.87^{a}	2.60^{a}	8.00^{a}	1.53^{a}	2.23^{a}
		20	4.80^{b}	1.23^{a}	1.90^{ab}	5.07^{b}	1.23^{a}	1.73^{a}
		30	4.70^{b}	1.47^{a}	1.80^{b}	4.37^{b}	1.57^{a}	1.93^{a}
	Wood chips	0	5.70^{ab}	1.80^{ab}	2.30^{ab}	5.83^{b}	2.03^{a}	2.47^{b}
		10	6.27^{a}	1.97^{a}	2.43^{a}	7.90^{a}	2.20^{a}	3.23^{a}
		20	4.37^{bc}	1.27^{bc}	2.30^{ab}	4.67^{bc}	1.33^{b}	2.23^{b}
		30	3.40^{c}	0.90^{c}	1.77^{b}	3.17^{c}	1.23^{b}	1.80^{c}
C. millenii	Sawdust	0	4.50^{b}	1.37^{ab}	1.67^{a}	5.50^{a}	1.90^{ab}	1.53^{a}
		10	7.67^{a}	2.03^{a}	1.77^{a}	6.53^{a}	2.20^{a}	1.90^{a}
		20	3.63^{b}	1.03^{b}	0.93^{a}	2.80^{a}	0.70^{c}	0.50^{b}
		30	3.63^{b}	0.97^{b}	1.17^{a}	3.13^{a}	0.90^{bc}	0.73^{b}
	Wood chips	0	3.60^{b}	0.90^{b}	0.97^{ab}	2.70^{b}	0.67^{b}	0.90^{a}
		10	6.67^{a}	1.77^{a}	1.73^{a}	5.50^{a}	1.50^{a}	1.23^{a}
		20	3.33^{b}	0.70^{b}	0.70^{b}	2.80^{b}	0.80^{b}	0.80^{a}
		30	2.73^{b}	0.67^{b}	0.63^{b}	3.13^{b}	0.90^{b}	0.93^{a}

Each value is a mean± standard error of three replicates. Means in the same column with different letters are statiscally significant at $p \leq 0.5$ using Duncan Mutiple Range Test (DMRT) for homogenity of means.
Key: WBL=wheat bran level, PL=Pileus length, SL=stipe length, SG=stipe girth

TABLE 2
Effect of ve on cultivation of Ganoderma lucidum on sawdust and wood chips of *Mansonia altissima* and *Cordia millenii*

Substrate	Incubation period (days)	Number of FB per flush		pH	Biological efficiency	Moisture content	
	F1	F1	F2	F1	F1	F1	F2
Sawdust + 0% WB of MS	55.00±0.20^{b}	2.00±0.00^{c}	2.00±0.00^{d}	6.24±0.23^{a}	3.40^{d}	2.34±0.00^{c}	3.54±0.670^{a}
Sawdust + 10% WB of MS	50.00±0.80^{d}	2.00±0.00^{c}	2.00±0.00^{d}	6.55±1.00^{a}	6.60^{b}	5.46±0.59^{a}	1.96±0.24^{a}
Sawdust + 20% WB of MS	59.00±2.00^{ab}	4.00±0.00^{a}	2.00±0.00^{d}	6.43±0.00^{a}	7.60^{a}	4.53±0.40^{ab}	2.55±0.80^{a}
Sawdust + 30% WB of MS	51.00±1.01^{bc}	2.00±0.00^{c}	1.00±0.00^{e}	6.14±0.20^{a}	4.50^{c}	3.18±0.87^{bc}	2.41±0.50^{a}
Sawdust + 0% WB of CM	50.00±1.00^{bc}	2.00±0.00^{c}	3.00±0.00^{c}	6.41±0.20^{a}	4.10^{c}	2.22±0.40^{ab}	1.93±0.60^{b}
Sawdust + 10% WB of CM	47.00±0.60^{c}	2.00±0.00^{c}	2.00±0.00^{d}	6.46±2.00^{a}	7.30^{a}	5.61±0.10^{a}	4.98±0.54^{a}
Sawdust + 20% WB of CM	48.00±2.00^{c}	1.00±0.00^{d}	2.00±0.00^{d}	6.16±0.70^{a}	2.90	1.66±0.30^{b}	1.89±0.22^{b}
Sawdust + 30% WB of CM	51.00±0.20^{ba}	1.00±0.00^{d}	2.00±0.00^{d}	6.43±0.80^{a}	2.70^{e}	3.04±0.00^{ab}	1.02±0.50^{b}
Wood chips + 0% WB of MS	55.00±0.10^{b}	3.00±0.00^{a}	2.00±0.00^{d}	6.14±0.40^{a}	4.20^{c}	2.19±0.40^{b}	4.57±0.15^{a}
Wood chips + 10% WB of MS	47.00±2.00^{c}	4.00±0.00^{a}	7.00±0.00^{a}	6.48±0.10^{a}	6.20^{b}	4.63±0.20^{a}	4.68±0.44^{a}
Sawdust + 20% WB of MS	47.00±0.10^{c}	4.00±0.00^{a}	4.00±0.00^{bc}	6.34±1.03^{a}	4.60^{c}	2.80±0.15^{b}	3.51±0.82^{a}
Wood chips t + 30% WB of MS	60.00±1.00^{a}	2.00±0.00^{c}	2.00±0.00^{d}	6.18±2.02^{a}	3.80^{d}	2.01±0.25^{b}	2.35±0.14^{a}

Wood chips + 0% WB of CM	56.00±1.00^{b}	1.00±0.00^{d}	1.00±0.00^{e}	6.38±1.00^{a}	3.00^{b}	2.11±0.21^{ab}	1.76±0.10^{b}
Wood chips + 10% WB of CM	48.00±0.30^{c}	3.00±0.00^{b}	2.00±0.00^{d}	6.32±0.00^{a}	6.20^{b}	3.53±0.58^{a}	2.88±0.20^{a}
Wood chips + 20% WB of CM	61.00±1.00^{a}	1.00±0.00^{d}	2.00±0.00^{d}	6.08±0.14^{a}	3.00^{d}	1.84±0.55^{b}	1.38±0.00^{b}
Wood chips + 30% WB of CM	63.00±0.70^{a}	1.00±0.00^{d}	1.00±0.00^{e}	6.48±1.50^{a}	2.00^{e}	1.18±0.11^{b}	1.19±0.10^{b}

Each value is a mean± standard error of three replicates. Means in the same column with different letters are statiscally significant at p≤0.5 using Duncan Mutiple Range Test (DMRT) for homogenity of means. Key: WB = wheat bran, MS = *Mansonia altissima*, CM = *Cordia millenii*, FB = fruit body

TABLE 3
Proximate analysis of *G. lucidum* cultivated on different substrates and substrate forms at a glance

Substrate	Substrate form	WBL (%)	%moisture content	%crude protein	%crude fat	%crude fibre	%ash	%carbohydrate
M. altissima	Sawdust	0	4.30^{b}	16.71^{d}	1.50^{c}	20.13^{a}	2.79^{d}	74.69^{a}
		10	4.32^{b}	17.95^{c}	2.25^{b}	18.94^{b}	7.61^{a}	67.87^{c}
		20	4.00^{c}	28.40^{a}	3.49^{a}	16.53^{c}	6.19^{b}	57.92^{d}
		30	5.59^{a}	18.42^{b}	1.01^{d}	14.92^{d}	4.20^{c}	70.79^{b}
	Wood chips	0	5.69^{d}	16.91^{d}	1.27^{c}	17.58^{b}	3.00^{c}	73.15^{a}
		10	5.99^{c}	20.21^{b}	1.50^{b}	18.90^{a}	8.58^{a}	63.72^{c}
		20	6.21^{b}	24.55^{a}	2.72^{a}	16.12^{c}	6.39^{b}	60.13^{d}
		30	6.91^{a}	19.67^{c}	1.27^{c}	15.48^{d}	2.99^{c}	69.16^{b}
C. millenii	Sawdust	0	7.27^{a}	20.13^{a}	1.77^{b}	13.39^{c}	3.19^{d}	67.64^{c}
		10	6.43^{b}	19.32^{b}	2.00^{a}	12.88^{d}	3.97^{c}	68.30^{b}
		20	6.32^{b}	18.54^{c}	1.50^{c}	18.23^{b}	9.94^{a}	63.70^{d}
		30	2.78^{c}	17.04^{d}	1.76^{b}	29.12^{a}	6.39^{b}	71.66^{a}
	Wood chips	0	6.00^{b}	17.92^{c}	1.99^{b}	15.11^{a}	3.40^{c}	70.68^{b}
		10	3.63^{d}	21.42^{a}	1.51^{c}	14.44^{b}	1.41^{d}	72.03^{a}
		20	7.21^{a}	18.80^{b}	1.50^{c}	13.18^{c}	6.21^{b}	66.28^{d}
		30	3.91^{c}	18.78^{b}	2.51^{a}	10.91^{d}	7.43^{a}	67.41^{c}

Data analysed were represented as Means±SD. Means in the same column with different letters are statiscally significant at p≤0.5 using Duncan Mutiple Range Test (DMRT) for homogenity of means.

TABLE 4
Mineral composition of *G. lucidum* cultivated on different substrates

Substrate	Substrate form	WBL (%)	Ca	Mg	Mn	Fe	Na	P
M. altissima	Sawdust	0	13.65^{c}	67.95^{d}	3.30^{a}	14.25^{d}	58.30^{c}	34965^{c}
		10	51.75^{a}	129.05^{b}	2.95^{a}	40.75^{a}	63.75^{b}	506.80^{a}
		20	12.90^{c}	155.70^{a}	4.25^{a}	20.85^{c}	69.65^{a}	427.40^{b}
		30	23.10^{b}	79.45^{c}	4.90^{a}	29.45^{b}	62.70^{b}	280.70^{d}
	Wood chips	0	8.85^{d}	73.80^{d}	3.65^{b}	24.95^{c}	46.90^{c}	276.75^{c}
		10	38.75^{a}	112.90^{b}	2.80^{c}	45.80^{a}	86.45^{a}	260.10^{d}
		20	12.75^{b}	167.05^{a}	2.40^{d}	26.05^{b}	69.10^{b}	350.15^{a}
		30	9.60^{c}	83.50^{c}	5.00^{a}	17.65^{d}	44.75^{d}	322.55^{b}
C. millenii	Sawdust	0	12.85^{c}	76.90^{c}	7.40^{b}	26.10^{b}	86.10^{a}	297.10^{c}
		10	88.50^{a}	92.40^{b}	6.65^{b}	15.65^{c}	64.15^{c}	456.10^{a}
		20	12.70^{c}	115.20^{a}	9.60^{a}	27.15^{a}	79.20^{b}	264.15^{d}
		30	77.65^{b}	59.35^{d}	2.90^{c}	26.10^{b}	42.60^{d}	355.20^{b}
	Wood chips	0	15.10^{c}	74.85^{c}	5.25^{b}	22.25^{c}	58.25^{b}	440.50^{b}
		10	45.90^{b}	108.95^{b}	4.95^{c}	26.50^{b}	78.15^{a}	418.10^{c}
		20	14.85^{c}	138.85^{a}	11.65^{a}	21.90^{c}	84.90^{a}	378.10^{d}
		30	90.60^{a}	61.80^{d}	3.95^{d}	50.75^{a}	40.70^{c}	489.25^{a}

Each value is a mean± standard error of three replicates. Means in the same column with different letters are statiscally significant at $p \leq 0.5$ using Duncan Mutiple Range Test (DMRT) for homogenity of means.

30% WBL of *Cordia millenii* sawdust gave the lowest moisture content (3.91). This is similar to the work of [26] who reported moisture of 3.38±0.01 and [27] who reported 2.78±0.05 moisture content. This variability was dependent on the mushroom species and other parameters such as environment temperature, relative humidity during growth and relative of metabolic water that may be produced or utilized during storage [28] . The less moisture content suggested the high shelf life of the sample and accounts for the toughness and hardness of the mushroom than other edible mushrooms as also previously confirmed by [29].

The results from proximate analysis of *G. lucidum* showed that notable variation in the carbohydrate, crude protein, crude fat, moisture content, ash, and crude fibre of *G. lucidum* suggest high nutritional values of the mushroom. This is consistent with the work of [30]. All the proximate contents in this research were higher than those reported by [28] except ash which was slightly (less than 2) lower. In the work reported by [30], protein, fat, ash contents were less and crude fibre and carbohydrate contents were higher than the findings in this research. In the same vein, moisture, crude protein, crude fat and crude fibre contents were less than while ash and carbohydrate contents were more than those reported by [31]. This variability might be due to difference in parameters such as environment temperature, relative humidity during growth and relative of metabolic water that may be produced or utilized during storage [29].

The negligible quantity of fat content of *G. lucidum* consists mostly of unsaturated fatty acids, which are less harmful to the health than the saturated fatty acids of animal fats [32]. The low fats content as reported in other plants [33] shows the health benefits of this mushroom and stressing its nutritional value, it is reported that extract from

this mushroom has cholesterol lowering properties [34] hence , its antihypertensive potentials in humans [35]. High content of protein supports nutritional importance of the mushroom as a food source [36]. High fibre and fat content makes the mushroom have low calorie value [37]. Although fibre is indigestible, it plays significant nutritional role since, it helps clean and maintains the proper motility of the intestinal tract [38]. High fibre diet reduces insulin requirement and stabilizes blood glucose profile, possibly by decreasing the rate of glucose absorption and delaying gastric emptying. The major reason for high ash content of the present species may be due to higher mineral contents. A recent study also reported the ash contens ranges similar to our findings [39].

There was significant difference in mineral composition of *G. lucidum* which is in agreement with the work of [40]. Phosphorus was the most abundant mineral which agrees with the work of [41]. Mg, Na and Ca were moderately high which agrees with the findings of [42,43,44]. Fe and Mn were relatively low. This variation might be due to the reason that the amount of minerals in mushrooms of the same species is directly related to factors such as origin, species and/or strains and cultivation conditions, genetic factors, substrates and distance from pollution sources [45,46,47].

The low fresh and dry weight in the cultivated *G. lucidum* was in consonance with the work of [48] who reported fresh weight of 19.0g, 9.4g, 10.0g, 24.0g and 30.0g and dry weight of 5.0g, 3.0g, 4.0g, 9.0g and 10.0g respectively. However, the result disagreed (i.e. lower) with the work of [49] who reported a higher fresh weight in oyister mushroom. This might be due to little moisture content contained by *G. lucidum* compared with other edible mushrooms. [50] repoted *Pleurotus florida* fresh weight of 27.85g and 22.85g cultivated on paddy straw and sugarcane molasses respectively.

The entire cultivation process from the time of incubation to the time of first harvest took 68 to 89 days. This is similar to the work of [51] reported 70 to 80 days. The difference in days of fructification could be due to different wheat bran levels and different substrates used. In the study conducted by [52] it was noted to be up to 92 days. [53] found that the average first harvest days of fruit bodies of *G. lucidum* for *S. mahagoni* with both supplements (rice bran and wheat bran) were 71 days and 60 days respectively, while it was 90 days and 66 days in case of *D. tarbinatus*. [52] reported 92 days.

The pH of the cultivated *G lucidum* which ranges from 6.14 to 6.55 agrees with the work of [31] who reported pH of 6.9. [54] reported pH of *G. lucidum* of 5.6. [55] reported pH from 4.0 to 7.0. This pH is slightly acidic, almost neutral, which might account for its consumption without any harm.

The biological efficiency of each treatment represents the conversion of the substrate into biomass for the mushroom development. This index is the most used by researchers because it makes the comparison of the results with the literature easier [56,57]. The result of the present study is in line with the work of [58] who reported biological efficiency ranging from 6.9% to 8.2%. [59] reported biological efficiency of 22.9%, 25% and 4.8% of *G. lucidum* cultivated on *Aruan*a grass, *Cynodon* spp and eucalyptus sawdust respectively. [60] obtained higher results than the others (BE, 72%) by cultivating *G. lucidum* in substrate based on elephant grass plus mango tree sawdust, supplemented with 10% of wheat branand 10% of crushed sugar cane.

ACKNOWLEDGEMENTS

This work was self-sponsored. I would like to thankGod and my supervisior Prof. Adenipekun O.Clementina for her immense support in many ways.

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Received: **10.01.2022**
Accepted: **24.03.2022**

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MILK FROM MONTENEGRO FARMS: MONITORING AND QUALITY OF RAW MILK AND DAIRY PRODUCTS

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ABSTRACT

Tourists come to Montenegro, stay on private farms, enjoy local specialties, and among other things enjoy domestic milk and dairy products. Milk production and processing in Montenegro is still organized in traditional way and routine, mostly in small private farms. Only a small part of milk (15 %) is processed in milk dairies. The aim of this research is to determine the quality of the raw milk and dairy products from private farms. The samples of milk and homemade cheese were taken from several different local manufactures from five cities: Podgorica, Danilovgrad, Kolašin, Pljevlja, Rožaje. Chemical and microbiological analysis were performed. The content of milk fat, protein, fat solids, total number of microorganisms, Coagulase, Sulfidoreduced clostridium, Proteus and *Escherichia coli*, PAHs, PCBs, pesticides, Pb, Cd and As were determined. Also, the milk samples were tested for presence of veterinary drugs, antibiotics: β-lactam (Penicillin G, Amoxicillin and Oxacillin) and Chloramphenicol. The paper summarizes the results that include samples taken in 2015, 2016, 2019 and 2020. The experimental results showed the presence of Benzylpenicillin above permitted level, only in one sample from Kolašin, while in other samples Benzylpenicillin was within the allowable concentration. The results show a good quality of milk and the microbiological safety of all analyzed samples.

KEYWORDS:
Milk analysis, Montenegro, ecotoxicology, veterinary drugs, PCB

INTRODUCTION

Milk is considered to be the most balanced food ever found in nature, which contains most nutrients. It is eaten by people of all ages and nationalities, and the quantity varies according to the eating habits and to its availability. Milk and dairy products are an important part of the daily diet of babies, children, elderly people, but these products are also the only source of animal protein in the diet of many vegetarians, whose number is continuously increasing all over the world [1].

In Montenegro, the total number of cattle in 2017 was 86 649 [2]. The farm structure is dominated by small family farms, which produce mainly for their own consumption. Their market share is limited. According to the data from Livestock Selection Service, average milk yield per cow in a controlled population is 5,077 kg, while the estimate for the total population is around 2,500 kg per cow [3]. In 2019, production of milk in Montenegro was 174,329 thousand tones. Though Montenegro production of milk fluctuated substantially in recent years, it tended to increase through 2010 - 2019 period ending at 174,329 thousand tones in 2019 (available on the knoema site: https://bit.ly/3cDCAMD).

Only 15 % of total milk production is delivered to dairies and industrially processed [4]. The rest is used for: own household consumption, or for direct sales (often at local markets) and for processing to the simple products. As we can see the big share of milk processing is on-farm, about 85 %, where home-made dairy products have been produced, for local market. Having in mind size of the farms it is hard and expensive to organize systematic quality control of milk and dairy products, and that is the reason why this research is important. Hand-made cheese has a good reputation in region and becoming more and more popular among the tourists.

Milk and the dairy products implicitly can be contaminated with different chemicals in different stages of its production and storage. The following types of chemical contaminants can find in milk: pesticide residues, other persistent organic pollutants (POPs) such as PCBs, PCDDs, PCDFs etc., some of metals, radionuclides, veterinary drugs and antibiotics, aflatoxins and mycotoxins, nitrites and nitrates,

detergents and disinfectants. Milk and dairy products are important sources of persistent organic pollutants (POPs) to humans [5].

The aim of this research is to examine quality of the raw milk at the private farms and quality of dairy products who are distributed through all Montenegro for domestic consumption and rural tourist offer, regarding the health safety standards and requirements in Montenegro and EU. The paper presents the results obtained in 2015, 2016, 2019 and 2020. The Northern region of Montenegro has the main agricultural land resources. Also, the north dominates with about 70 % of the total cattle population □3□. For this reason, most of the samples were taken in cities (Pljevlja, Kolašin, Rožaje) in the northern region. Pljevlja is well known for cheese production, 'Pljevlja cheese' has been a protected designation of origin in the EU. Kolašin is a popular tourist place in winter and summer, well know with its milk products domestically and internationally. On the other hand, larger dairies are located in the central region. Danilovgrad and Zeta (locations of farms from which some samples were also taken) are the locations very close to the capital Podgorica, where is the biggest consumption and production of milk.

Milk quality and nutritive values are evaluated through the following important indices: acidity, protein and fat content.

The safety indices studies include the microbial aspects and healthy aspects: antibiotic presence, detection of pesticide residues. Antibiotics are widely used in veterinary practice. The excessive and inconsiderate use of antibiotics may lead to the occurrence of drug residues in milk, bearing a risk to human health. Beta-lactam antibiotics are among the most frequently used antimicrobial agents. Chloramphenicol is a synthetic antibiotic, its use in animals is illegal in most countries [6]. The milk samples were tested for presence of β-lactam (Penicillin G, Amoxicillin and Oxacillin) and Chloramphenicol. We analyzed 5 samples of cow's milk for the presence of 150 pesticides.

Pb, Cd, As, PAHs and PCBs were measured in the samples of cow's milk, goat's milk and cow's cheese in order to evaluate the potential human health risk associated with their consumption. Non-essential heavy metals are toxic even at very low concentration. PAHs have been classified as genotoxic and possibly carcinogenic to humans. Fatty foods of animal origin, as a milk and dairy products are the main sources of human exposure to PCBs, which are lipophilic and have the propensity to bioaccumulate in biota. PCBs processing and distribution have been banned in almost all countries since 1980s. Nevertheless, PCBs are still present in the senvironment, and few studies investigated the presence of PCBs residues in milk samples [7].

MATERIALS AND METHODS

All samples were taken from private farms. Raw milk is poured into sterile glass bottles. An appropriate record was made for each sample: location, date, time. During transport all samples are stored in refrigerators.

Chemical and microbiological analysis of raw milk from Pljevlja. Raw cow's milk samples for chemical and microbiological analysis collected from 5 selected points in Pljevlja, were collected in 2015. The fat contents of whole-fat were analyzed using a Gerber method [8]. Samples were prepared in a Butyrometer, centrifuged in a Gerber centrifuge, then heated in a water bath. At least two determinations were performed on the same test sample. The difference between the results of two determinations carried out by the same analyst, simultaneously or immediately in succession, on the same sample, by the same method, under the same conditions and in the same laboratory, shall not exceed 0,1 % of the reading. Binder dryer was used for the analysis of dry matter in milk. To determine the protein, we used: FOSS mineralization system, type Digestor Auto; Kjeltec FOSS analyzer unit, type Kjeltec TM8200 and FOSS scrubber, type SR210 Scrubber.

Preparation of the milk sample to determine the presence of β-lactam (Penicillin G, Amoxicillin and Oxacillin) and Chloramphenicol. Raw milk samples from Danilovgrad, Kolašin, Podgorica and Pljevlja were analyzed. Samples were collected in 2016 and 2019.

The detection of β-lactam was performed using Shimadzu-LCMS 2010EV mass spectrometer. Internal standard solution of penicillin V was added in 5 mL of milk, afterwards 0.4 mL of 10 % acetic acid was added, too. Prepared sample was centrifuged, then 0.5 mL the clear solution was mixed with 0.5 mL methanol and filtered through a micro filter.

The analysis of Chloramphenicol was performed using liquid chromatography with mass spectrometry, Waters-LC MS/MS Quattro Micro Api. Standard solution of acetonitrile (20 mL) was added in 5 mL of milk sample. A clear layer of acetonitrile was transferred from the cuvette, evaporated to dryness under a stream of nitrogen and dissolved with 6 mL of deionized water. After purification in extraction columns in solid phase, filling the column is C18 as a suitable adsorbent for this type of antibiotics. The sample from the column is eluted with pure methanol and evaporate in the nitrogen stream. Before analysis all samples were filtered through micro-filters.

Methods used for analysis PAHs, PCBs and metals (Pb, Cd and As). Three samples of cow's milk, one sample of goat's milk and two samples of caw cheese were used for analysis of PAHs, PCBs

and metals (Pb, Cd and As). Samples were taken at four different private farms in Rožaje, Montenegro, in 2019. Metal content analysis was performed by applying atomic absorption spectrophotometry to an AAS Shimadzu instrument, AA6800. Samples were prepared by microwave digestion in microwave oven model MSW-4 (Berghoff, Germany). Determination of PAHs and PCBs were at the Center for Ecotoxicological Research, Podgorica. Samples were prepared in the usual and standard way for this type of instrumental techniques. The sample was analyzed on a gas chromatogram with a mass spectrometer (GCMS) manufactured by Shimadzu, the type of instrument is GCMS-QP 2010Plus which has a car sampler AOC-20i manufactured by Shimadzu. The type of column is ZB-5MS plus, length 30 m, diameter 0.25 mm and film thickness 0.25 um.

Analysis of pesticides . Samples of milk were taken from different locations in Podgorica, Pljevlja and Kolašin. Determination of pesticides in these samples were done using gas chromatography in Center for Ecotoxicological Research, Podgorica. For the analysis we used: LCMS/MS, UltiMate 3000, Thermo Scientific and GCMS QP2010 plus, Shimadzu. Analysis of organochlorine pesticides was performed on the instrument Gas chromatograph with electron capture detector-ECD Shimadzu GC-2020 Plus. Samples were prepared in the usual and standard way for this type of instrumental techniques.

RESULTS AND DISCUSSION

Chemical and microbiological testing. The main results of chemical and microbiological testing in milk collected from 5 points from Pljevlja (A, B, C, D and E) are shown in **Table 1**. The results taken for the protein content are little lower than the standard value. The standard range is considered 3.5-4 %. In most cases the reason for this is the presence of added water in milk [9]. The examined samples did not contain: Salmonella, Coagulase, Escherichia coli, Sulfidoreduced clostridium and Proteus. The results show a good quality of milk and the microbiological safety of all samples analysed [10].

Presence of β-lactam and Chloramphenicol. Several samples of cow's milk were provided to the laboratory in order to be examined for the presence of β-lactam and chloramphenicol. The samples were collected at private farms in three cities during the 2016 (Kolašin, Podgorica, Danilovgrad). The same analysis was done in 2019 in samples collected in Pljevlja. The results of the analysis of the Penicillin G, Amoxicillin, Oxacillin and Chloramphenicol are presented in Table 2.

Regulatory authorities such as the EU and US Food and Drug Administration, in order to ensure food safety, have enacted strict Maximum Residue Limits (MRLs) [11]. In accordance with these rules, in Montenegro is valid: Rulebook about maximum permitted concentrations of residues of pharmacologically active substances of veterinary drugs in food of animal origin [12]. In all tested samples, the obtained concentrations of β-lactam do not exceed MRLs. In view of the JECFA conclusions on the available scientific information, there is no safe level of residues of chloramphenicol or its metabolites in food that represents an acceptable risk to consumers. Based on all the obtained values, the tested milk is safe for use.

PAHs, PCBs and metals (Pb, Cd and As) in milk and cheese samples. Animals are constantly exposed to contaminants present in the environment and are able to accumulate pollutants in elevated concentration in their tissues, in particular in the fat. Therefore, the EU Scientific Committee on Food has set maximum level for certain contaminants in foodstuffs, periodically monitoring by the European Food Safety Authority (EFSA). Examined metals, PAHs and PCBs were measured in samples of cow's milk (A, B and C), goat's milk (D) and cow's cheese (E and F). The obtained results or the tested milk samples on Pb, Cd and As, are much lower than the allowed values (Table 3).

Our results show that Pb, Cd and As concentrations were not relevant in milks and cheese. Concentrations of Pb and Cu were below the regulation limit respect EFSA value [13], [14]. Arsenic, particularly in the inorganic form, is classified by IARC as carcinogenic to humans. The analysis of total arsenic in food has up to date suffered from difficulties with respect to accuracy and precision. Furthermore, specified data for arsenic are strongly needed because of the large differences in toxicity to humans of the various forms of arsenic.

Maximum levels of inorganic As in food for infants and young children is 0.1 mg/kg [15]. Codex Alimentarius has adopted through the years different MLs for tAs (10 µg/L for natural mineral water; 100 µg/kg for edible fats, oils (including fish oils), fat spreads and blended spreads; 500 µg/kg for food grade salt), and, more recently, for iAs (200 µg/kg for polished rice, and 350 µg/kg for husked rice) (FAO/WHO, 2018) [16].

The results of the analysis of milk and cheese samples for the presence of PAH are shown in the Table 4. Benzo(a)pyrene belongs to the group of polycyclic aromatic hydrocarbons (PAH) and is used as a marker for the occurrence and effect of carcinogenic PAH in food. But, the former system of using benzo(a)pyrene as the only marker for the group of PAHs, can not be maintained, based on the conclusions of EFSA. Therefore we determined the sum of four substances (PAH4): benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene. Such system provides that PAH levels in

food are kept at levels that do not cause health concern and that the amount of PAH can also be controlled in those samples in which benzo(a)pyrene is not detectable, but where other PAH are present [17]. Although there aren't PAHs limits referred specifically to raw milk or to milk products, the results in all tested samples are significantly lower than the maximum level set for foodstuff.

Analysis of milk and cheese samples for the presence of PCBs obtained the results shown in the Table 5. Since 1 January 2012, a maximum level of 40 ng/g of fat for PCBs (Sum of PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180) in raw milk and dairy products has applied throughout Europe [18]. The data here showed that the concentrations of this group of contaminants in all samples tested were below the legal limits.

Detection of pesticides residues. LC-MS and LC-MS/MS is an ideal, extremely specific and highly sensitive technique used for identification and quantification of pesticide residues. It provides information about analyte without derivatizing. It can compensate sample purity and it enables simultaneous analysis of the compounds with varying polarity [19]. Using the mentioned technique and the QUECHERS modified method, we analysed 5 samples of cow's milk from different regions of Montenegro (Podgorica, Kolašin and Pljevlja) for the presence of 150 pesticides. Given the huge number of results, only the results for selected pesticides are shown in the Table 6.

All levels of pesticide residues in analyzed milk were well below the maximum permissible limits given by FAO/WHO. The MRLs for all pesticides can be found in the MRL database on the European Commission website (https://ec.europa.eu/food/plants/pesticides/eu-pesticides-database_en) .

TABLE 1
Chemical and microbiological analysis raw milk from Pljevlja

	A	B	C	D	E
Milk fat content	3.66 %	3.8 %	3.72 %	3.75 %	3.69 %
Protein content	3.15 %	3.25 %	3.22 %	3.20 %	3.19 %
Contents of dry matter without grease	8.5 %	8.5 %	8.5 %	8.5 %	8.5 %
Acidy	7.1 SH	7.4 SH	7.6 SH	7.5 SH	7.4 SH
Temperature	10 °C	10 °C	11 °C	11 °C	12 °C
The total number of microorganisms	690000/mL	850000/mL	710000/mL	810000/mL	690000/mL
Salmonella	Not isolated	Not isolated	Not isolated	Not isolated	Not isolated
Coagulase	Not isolated	Not isolated	Not isolated	Not isolated	Not isolated
Sulfidoreduced clostridium	Not isolated	Not isolated	Not isolated	Not isolated	Not isolated
Proteus	Not isolated	Not isolated	Not isolated	Not isolated	Not isolated
Escherichia coli	Not isolated	Not isolated	Not isolated	Not isolated	Not isolated

TABLE 2
Content of β-lactam and chloramphenicol

Sample origin	Penicillin G		Amoxicillin		Oxacilin		Chloramphenicol	
	Conc. (µg/kg)	MRL (µg/kg)	Conc. (µg/kg)	MRL (µg/kg)	Conc. (µg/kg)	MRL (µg/kg)	Conc. (µg/kg)	MRL (µg/kg)
Kolašin	< 2.5	4	< 2.5	4	< 2.5	4	□ 0.1	-
Podgorica	< 2.5	4	< 2.5	4	< 2.5	4	□ 0.1	-
Danilovgrad	< 2.5	4	< 2.5	4	< 2.5	4	□ 0.1	-
Pljevlja	< 2.5	4	< 2.5	4	< 2.5	4	□ 0.1	-

TABLE 3
Concentration (mg/kg) examined metals in milk and cheese samples

	Milk samples					Cheese samples		
Determined metal	A	B	C	D	method label	E	F	method label
Pb	<0,010	<0,010	<0,010	<0,010	MEST EN 14084:2009*	<0,020	<0,020	AOAC 999.11*
Cd	<0,010	<0,010	<0,010	<0,010	MEST EN 14084:2009*	<0,010	<0,010	AOAC 999.11*
As	<0,06	<0,06	<0,06	<0,06	AOAC 986.15*	<0,06	<0,06	AOAC 986.15*

TABLE 4
The content of PAHs (µg/kg) in milk and cheese samples

	Milk samples					Cheese samples		
determined PAHs	A	B	C	D	method label	E	F	method label
benzo (a) pyrene	<1	<1	<1	<1	PAH-1	<1	<1	PAH-1
The sum of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene	<5	<5	<5	<5	PAH-1	<5	<5	PAH-1

TABLE 5
The content of PCBs (ng/g) in milk and cheese samples

	Milk samples					Cheese samples		
determined PCBs	A	B	C	D	method label	E	F	method label
The sum of PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180 (ICES-6)	<10	<10	<10	<10	EPA 1613-mod.	<10	<10	EPA 1613-mod.

TABLE 6
The content of selected pesticides (mg/kg) in 5 milk samples

Selected pesticides	Podgorica I	Podgorica II	Pljevlja	Kolašin	Podgorica III
Aldrin	<0.005	<0.005	<0.005	<0.005	<0.005
Dieldrin	<0.005	<0.005	<0.005	<0.005	<0.005
Chlordane (sum of cis- and trans-chlordane)	<0.002	<0.002	<0.002	<0.002	<0.002
p,p′-DDT	<0.01	<0.01	<0.01	<0.01	<0.01
p-p′-DDE	<0.01	<0.01	<0.01	<0.01	<0.01
p,p′-DDD	<0.01	<0.01	<0.01	<0.01	<0.01
Fipronil (sum fipronil + sulfone metabolite)	<0.008	<0.008	<0.008	<0.008	<0.008
Hexachlorobenzene	<0.005	<0.005	<0.005	<0.005	<0.005
Hexachlorocyclohexane (HCH), alpha-isomer	<0.005	<0.005	<0.005	<0.005	<0.005
Hexachlorocyclohexane (HCH), beta-isomer	<0.005	<0.005	<0.005	<0.005	<0.005
Lindane	<0.005	<0.005	<0.005	<0.005	<0.005

CONCLUSIONS

Based on obtained results from the samples the presence of veterinary drugs was under the standards. Regarding the pesticides presence, it can be confirmed that there is no any problem in these areas. The measurement of PCBs, PAHs, Pb, Cd and As, indicating environmental contamination, in milk and cheese samples showed that these compounds presented values below the reference limits established by the European Community. This means that milk products are good quality and safe for consumption These results are important not only because of the local population but also because of the tourists. The tourists are mainly interested in our local food: meat and dairy products, especially yogurt, cheese and cream cheese. Tourism is growing sector in Montenegro, and each year we are recording more and more tourists from EU. As a small state we can offer combination of see and mountain tourism with combination with local food in packages of 5, 7 or 10 days. Based on interviews with the local tourist workers, there is great interest of foreign tourists for mountains for hiking and staying at traditional farms. Old traditional ways productions are not still compromised with market values. But with the more tourists and higher demand for milk products, the bigger and modern farms will be needed, and stricter quality control of products should accompany these trends

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Received: **25.01.2022**
Accepted: **21.03.2022**

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NUTRACEUTICAL: FLAVONOIDS AND CANCER PREVENTION

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ABSTRACT

Flavonoids are plant substances that have a range of health advantages. There are six primary types of flavonoids, each with health-promoting effects. The best way to obtain all six types of flavonoids is to consume a variety of fruits and vegetables. Many plant-based foods and beverages like tea and wine contain flavonoids. Numerous studies have shown the many benefits of these phytonutrients. Researchers discovered that consuming a flavonoid-rich diet lowers the risk of cardiovascular disease, diabetes, and certain tumors.

KEYWORDS:
Flavonoids, diet, cancer, prevention

INTRODUCTION

Nutraceuticals, health foods, and functional foods are applied to substances that may or may not be considered foods or components of foods, but are beneficial to health when consumed [1].

The term "Nutraceuticals" is now commonly applied to a very wide variety of preparations, considered to have medicinal but not necessarily dietary value, such as: amino acids, essential fats, dietary fibers and fiber-rich foods, pigments of plant or animal origin, antioxidants, vitamins, minerals, sugar and fat substitutes, lean meats, skim milk, genetically engineered synthetic foods, herbal products and processed foods such as cereals, soups and drinks [2].

Similarly, some practitioners consider that fruits and vegetables should be included in functional foods because they are so rich in nutrients, while others would prefer to reserve this term for foods fortified in some way, such as orange juice. Others would prefer to reserve the term for foods that are fortified in some way, such as orange juice fortified with calcium.

Phytonutrient for medicinal plants with no apparent nutritional value. Phytomedicines refers to therapeutic agents derived from plants or parts of plants or preparations made from them but not pure chemicals [3].

Contrary to pharmaceuticals products, which can only be prescribed by a physician, nutritional supplements are much less expensive, and have been touted as a new and cost-effective health system, so plant-based vitamins and a wide variety of chemical constituents of fruits and vegetables have many of the benefits of herbal medicines [4].

PHARMACEUTICAL PRODUCTS OF PLANT ORIGIN

Modern pharmacology relies on refined chemicals, both plant and synthetic. The first pure medicinal substance derived from plants was morphine, which was extracted from the opium poppy in the early 19th century. Often, chemicals extracted from plants are modified to produce medicines [5].

For example, diosgenin is extracted from various species of Igname (Dioscorea), from South America is converted into progesterone, the active ingredient in anovulants. In the past, aspirin-like chemicals were extracted from willow (*Salix* spp.) and meadowsweet (*Filipendula ulmaria*), but aspirin is synthesized in the laboratory. Approximately 50% to 60% of pharmaceuticals products are of natural origin or are synthesized from natural products [6,7,8]. The commercial value of bioactive compounds of plant origin is estimated to be around $30 billion per year worldwide [9]. Higher plants are the source of the origin of some 120 commercial drugs [10].

THERAPEUTIC IMPORTANCE OF NATURAL MOLECULES

Although only about 100,000 natural substances are currently available, about 40% of all medicines used are derived from nature and 80% of the world's population use medicinal plants to treat themselves [11].

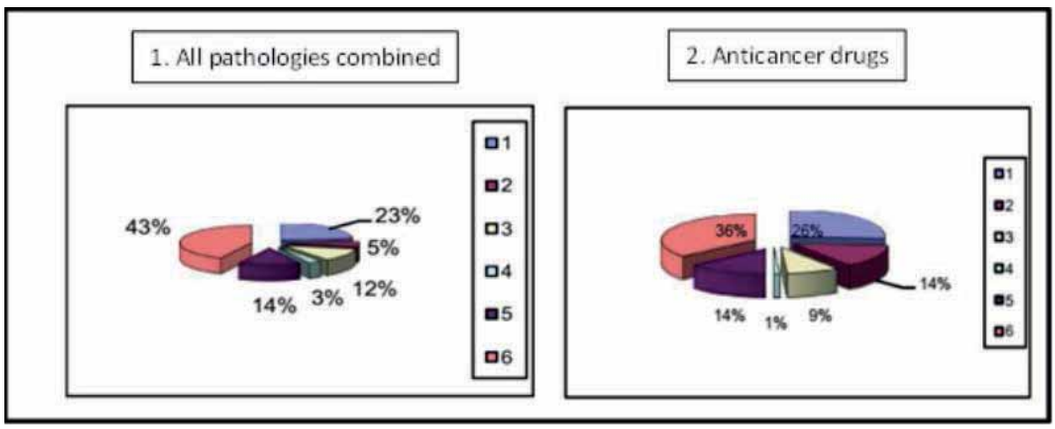


FIGURE 1
Importance of natural products in the pharmaceutical sector.
With 1: semisynthetic derivatives, 2: products of natural origin, 3: Drugs of biological origin (peptides, proteins), 4: Vaccines, 5: Synthetic drugs inspired by natural pharmacophore (example: β-agonists taking for model biogenic amines, 6: Totally synthetic drugs.

For clarification of the importance of the natural products in the pharmaceutical sector, I present in the following flowcharts a comparative statistical study of the different origins by the new medicines put on the market the last twenty years of the 20th century and beginning of the 21th century [from 1981-2003] [12].

Inspection of Figure 1 shows that, over the whole category of anticancer drugs, 14% products of natural origin, 24% between products of natural origin, drugs of biological origin (peptides, proteins) and vaccines, and 36% were totally synthetic drugs.

In category of all pathologies combined, just 43% were synthetic in origin, and 20% between products of natural origin, drugs of biological origin (peptides, proteins) and vaccines.

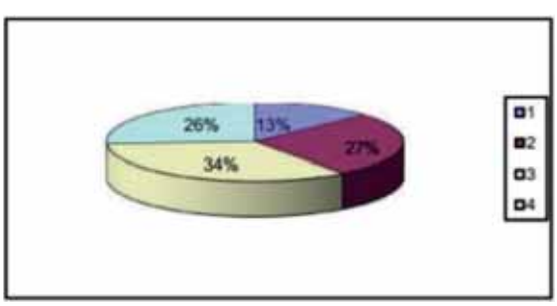


FIGURE 2
Ratio of the use of plants for the different pathologies.
With 1: semisynthetic derivatives, 2: products of natural origin, 3: Drugs of biological origin (peptides, proteins), 4: Vaccines.

According to many experts, the majority of the most important natural substances have already been found. Out of about 250,000 higher plants, maximum 10% have been examined phytochemically, out of about 1,000,000 insect species, very few (<0.05%) have been studied chemically. It is estimated that less than 10% of the existing bacteria and less than 5% of the existing fungi are only correctly identified. Marine organisms (algae, animals, microorganisms ...) have also been studied very little [12].

The comprehensive data sets in the Figures 1 and 2 displayed to above emphasize the ongoing significance that natural products and structures derived from/related to natural products from all sources have played and continue to play in the physician's contemporary therapeutic armamentarium.

An examination of the data reveals that natural products continue to play a significant role in drug development (the ratio of the use of products of natural origin is 27% and 87% between products of natural origin, drugs of biological origin and vaccines), despite the present decline in natural product-based drug discovery programs at pharmaceutical companies, with a few notable exceptions.

Studies (multivariate statistical research) comparing the structural properties of natural molecules, compounds from combinatorial chemistry, and medicines on the market have demonstrated the structural relevance of molecules of natural origin. The PCA graphs show that the compounds from combinatorial chemistry cover a smaller surface than natural products or drugs. There seems to be a much greater structural homology structural homology between natural products and drugs. Thus we notice that on average the natural products:

- Have higher molecular weights.
- Have fewer aromatic rings.
- Incorporate less nitrogen, halogen or sulfur but more oxygen than the products of combinatorial chemistry [13].
- Especially natural products are sterically more complex. Synthetic products are often limited to zero, one or two centers of asymmetry, while drugs from natural products can exceed nine centers of asymmetry.

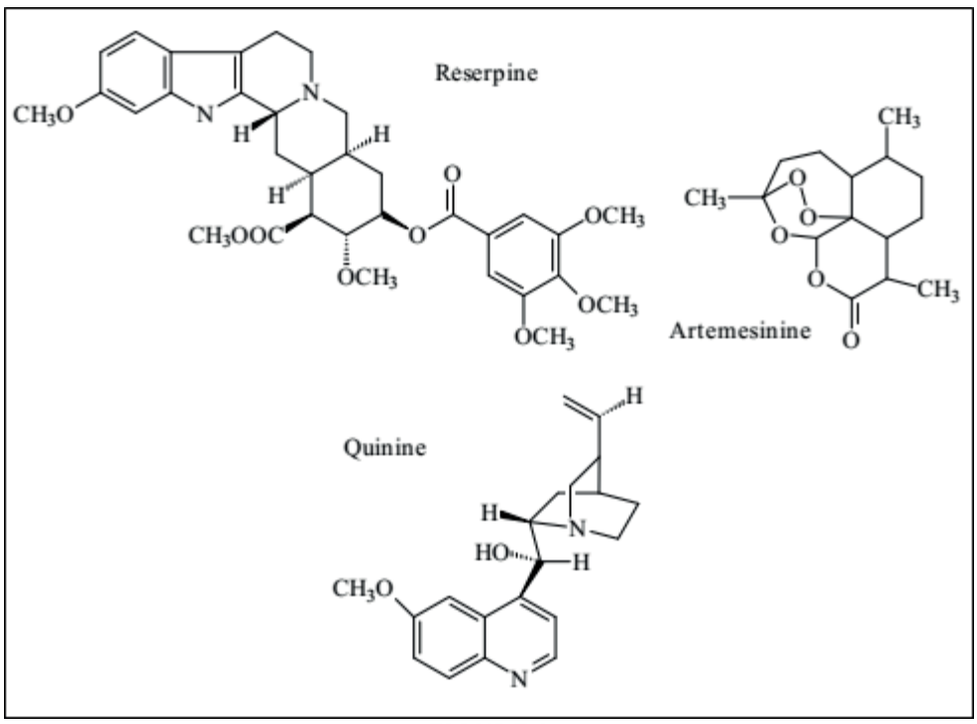


FIGURE 3
Some active pharmaceutical principles' structures.

TABLE 1
Examples for drugs of natural origin (or semi-synthetic)

Compound	Trade name	Year on the market	Therapeutical use
Venorelbine	Navelbine	1989	antitumor
Paclitaxel	Taxol	1993	antitumor
Docetaxel	Taxotere	1995	antitumor
Topotecan	Hycamptin	1996	antitumor
Irinotecan	Campto	1994	antitumor

Example structural diversity of active principles in Table 1 is recorded diversified examples for drugs of natural origin (or semi-synthetic).

Natural products have also served as a model for the development of many drugs; examples include:

- Local anesthetics, from cocaine.
- Cough suppressants, from codeine.
- Curariforms from tubocurarine and toxiferine.
- Chloroquine and quinoline antimalarials, from quinine.
- Etoposide, an antitumor from podophyllotoxin.

Okadeic acid has allowed the understanding of the importance of phosphorylation processes involved in eukaryotic cell metabolism and more particularly in the processes of tumor growth [14,15].

CHEMISTRY OF NATURAL SUBSTANCES OF VEGETABLE ORIGIN

Natural products are compounds formed by living systems. One of the most significant disciplines of organic chemistry is the comprehension of their structures, chemistry, synthesis, and biosynthesis. Natural products are divided into two main categories [16]:

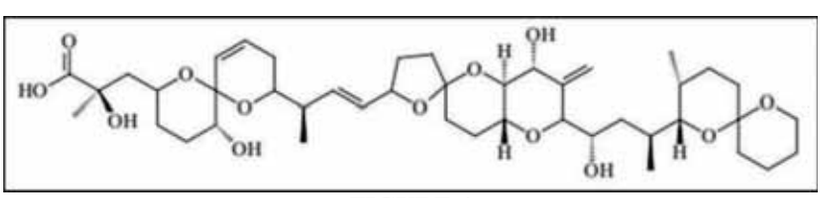


FIGURE 4
Okadeic acid.

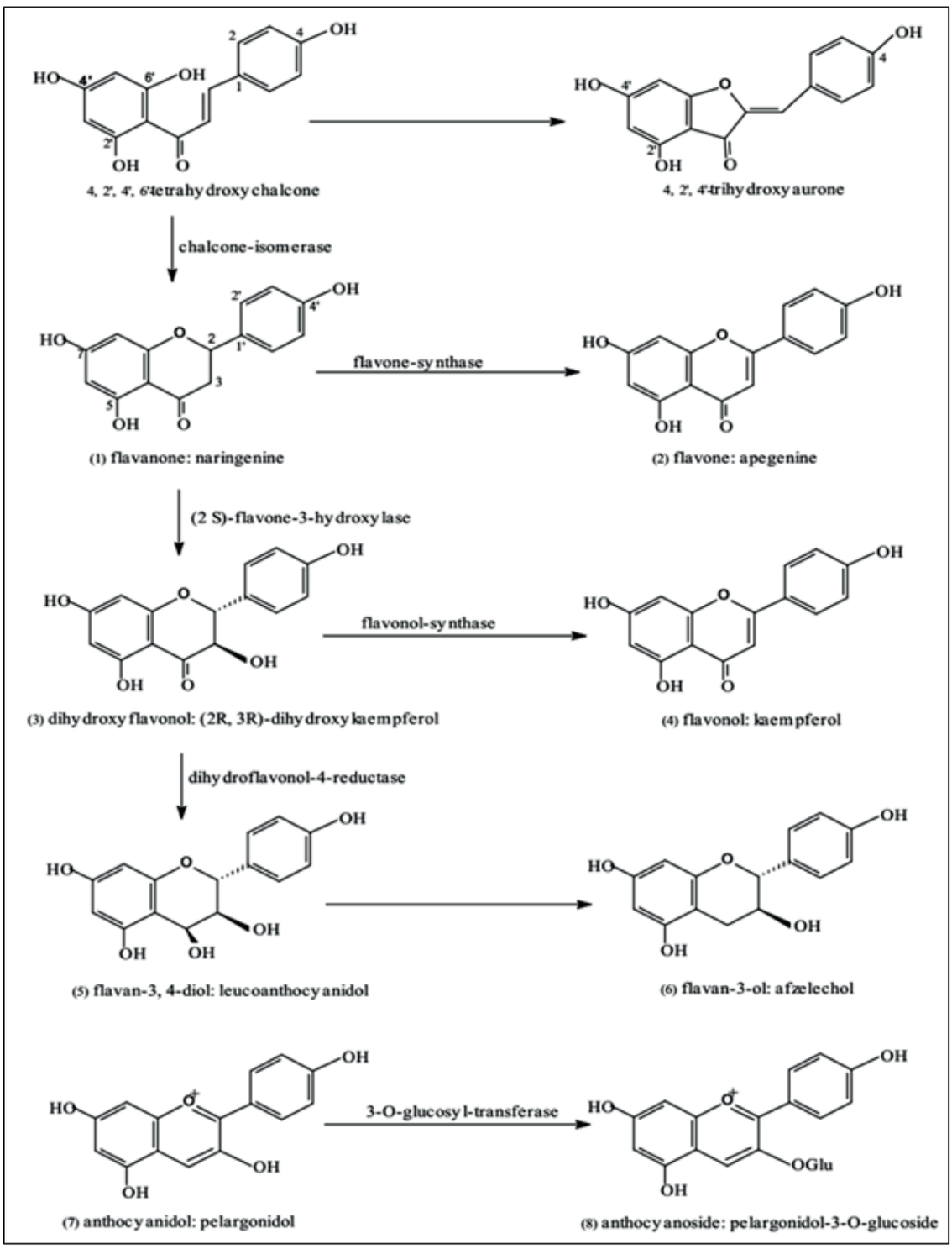


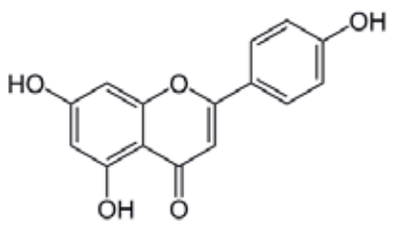
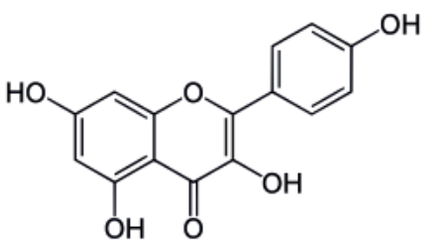
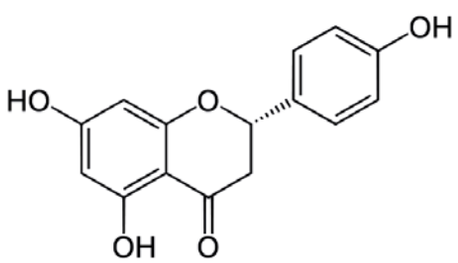
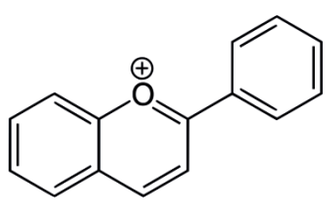
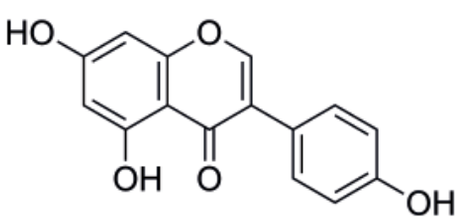
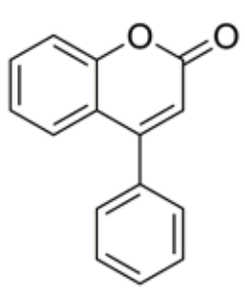
FIGURE 6
Biosynthesis of flavonoid derivatives [16].

- ***Primary metabolites:*** form the essential structure of the plant, and are involved in the translocation, storage, and respiration systems. Among these compounds, we distinguish proteins, carbohydrates and lipids [17,18].

- ***Secondary metabolites:*** these are compounds that protect the plant against herbivorous attacks and thanks to them, the plant can resist the different hydric and thermal stresses, these compounds exist in infinite quantities and their concentration varies from one plant to another, among these substances, we distinguish: flavonoids, alkaloids, tannins, saponins, etc. [19]

We describe the properties of flavonoids, whose properties and uses are very important.

TABLE 2
Classes of flavonoids [16].

Class	Example	Structure
Flavone	Apigenin : Trihydroxy-5,7,4' flavone	
Flavonol	Kaempferol : Trihydroxy-5,7,4' flavonol	
Flavanone	Naringenin: Trihydroxy-5,7,4' flavanone	
Anthocyanidins	Tetrahydroxy-3,5,7,4' flavane	
Isoflavonoids	Genistein: Tri-hydroxy-5,7,4' isoflavanoe	
Neoflavonoid	Chromane(4-phenylcoumarins)	

FLAVONOIDS

Flavonoids are phenolic compounds derived from phenyl-2-chromone, having a certain number of free or sugar-stabilized phenols. They are almost universal pigments in plants, responsible for the coloring of flowers, fruits and sometimes leave. Flavonoids are very common in the plant kingdom, generally in the heteroside state, where the osidic part can be mono-, di- or trisaccharide [20].

Chemical properties. All flavonoids, more than 4000 have a common biosynthetic origin (Figure 5), and therefore, have the same basic structural element, C6 - C 3 - C 6 comprising two aromatic rings A and B and a heterocycle.

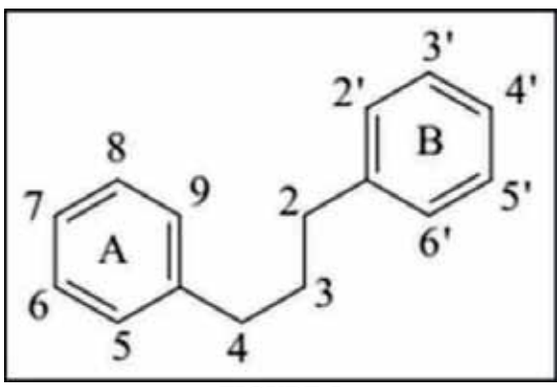


FIGURE 5
Basic skeleton of "genin" flavonoids.

Their biosynthesis (Figure 6) is based on a common precursor, 4,2',4',6'-tetrahydroxychalcone. [21,22] Through the action of enzymes, this chalcone is metabolized into different classes of flavonoids: flavanone (10), aurone (9), 2,3-dihydroflavonol or flavanonol (12), flavone (11), anthocyanidin (15), flavonol (13), catechin (14)... Subsequent steps, mainly glycosylation and acylation, bring flavonoids to the final form in which they are found in vivo. The compounds of each subclass are distinguished by the number, position and nature of substituents (hydroxyl, methoxyl and other groups) on the two aromatic rings A and B and the intermediate C3 chain [16].

According to the nature of the heterocycle “C” we distinguish different classes of flavonoids (Table 2).

Table 2 shows the six primary types of flavonoids and some examples with their structures, each with health-promoting effects. The best way to obtain all six types of flavonoids is to consume a variety of fruits and vegetables.

The important numbers of flavonoids extracted from plants are heterosides or the osidic part can be mono-, di- or trisacharisidic, glucose and rhamnose are the most frequently met sugars in this class of natural substances.

The bond between the genin and the ose is preferentially established:

- With the OH function in position 3 of the flavonol.
- With the OH function in position 7 of the flavone.

The detection of flavonoids in the vegetable drug is done primarily by reaction " of cyanidine ", (only chalcones and isoflavones do not react). The reaction consists in treating the extract of the plant by magnesium in chlorohydric acid medium; one obtains a red coloration with flavanols, cherry red with flavonols and purplish red with flavanones. If the initial coloration of the extract marks the developed coloration, the reaction should be done on the hydroalcoholic extract of the plant extract [16,23].

Biological properties. One of the major functions of flavonoids is to contribute to the color of the plants and in particular to the color of the flowers or it is by the color of its flowers that the plant exerts an attractive effect on the insects and the birds pollinator ensuring a fundamental stage of its reproduction. Flavonoids have fungicidal and insecticidal properties that protect the tree against predators. Also the UV absorption of flavonoids is important which protects the plant from harmful radiation [16,24].

Pharmacological properties. Flavonoids are essentially used in the capillary-venous field, alone or associated, by forming vasoconstrictors and venotonics. They act by decreasing the permeability and increasing the resistance of capillaries (vitamin P action). They are mainly indicated in the treatment of capillary fragility disorders in the skin (petechiae, ecchymosis) as well as in the mucous membranes (epistaxis, gingivorrhagia); in the treatment of functional signs of hemorrhoidal crisis; in that of metrorrhagia related to intrauterine devices, disorders related to the retinal and/or choroidal circulation in association with other drugs . Finally, they are used in the treatment of symptoms related to veno-lymphatic insufficiency: oedema, cramps, heavy legs [16,25,26].

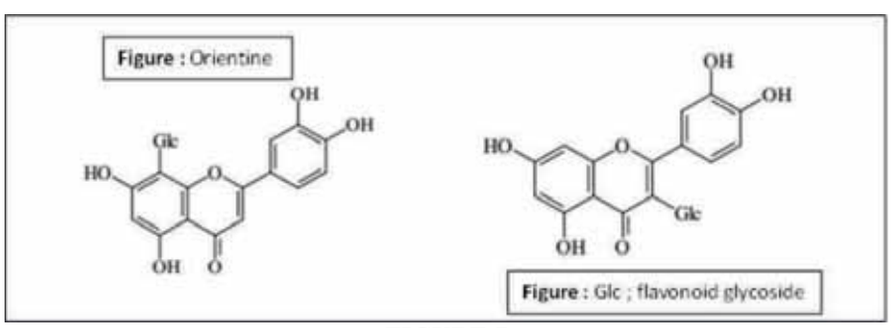


FIGURE 7
Orientine and flavonoid glycoside.

Why do you require flavonoids? A plant-based diet is beneficial to the body in a variety of ways. Flavonoids, for example, have anti-inflammatory properties and protect your cells from oxidative damage, which may lead to illness. These dietary antioxidants have been shown to reduce the risk of cardiovascular disease, diabetes, colon tumor [27], and cognitive disorders such as Alzheimer's and dementia.

Other health advantages include:

 Cancer Prevention: A published study of all flavonoid research conducted over an eleven-year period indicated that a flavonoid-rich diet reduces the incidence of various malignancies. According to these researches, flavonoids' antioxidant activity protects against breast, prostate, and colorectal cancers. It is crucial to note that these studies show that various flavonoids have a protective effect against particular cancer types. Anthocyanidins, for example, reduce the risk of lung cancer, but flavonols diminish the risk of prostate cancer. As a result, it's preferable to eat a variety of plant foods to get a variety of flavonoid subtypes. [28]

 Treatment for Viral Infections: Flavonoids have been shown to have antibacterial and antiviral properties. Numerous laboratory studies have shown that certain flavonoids prevent cell replication of H1N1 flu, HIV, SARS, and RSV viruses. More study is needed to discover how flavonoids fight viruses in the body and whether they might be used as a preventive strategy. [29]

HOW CAN CANCER BE AVOIDED OR DETECTED EARLY?

Cancer refers to illnesses in which aberrant cells develop uncontrollably and can infect other tissues. Cancer cells can travel to different regions of the body via the circulatory and lymphatic systems. Cancer is a group of illnesses, not just one. There are more than 100 kinds of cancer [30, 31].

- **Screening Tests:** Screening means checking your body for cancer before you have symptoms. Getting screening tests regularly may find breast, cervical, and colorectal (colon) cancers early, when treatment is likely to work best. Lung cancer screening is recommended for some people who are at high risk [32].

- **Vaccines:** Vaccines (shots) also help lower cancer risk. The human papillomavirus (HPV) vaccine helps prevent most cervical cancers and several other kinds of cancer. The hepatitis B vaccination may help reduce the chance of developing liver cancer [33].

- **Healthy Choices:** You may lower your risk of cancer by making healthy choices such as maintaining a healthy weight, avoiding cigarettes, limiting your alcohol use, and protecting your skin [34].

FOODS THAT COMBAT CANCER

As researchers continue to fight cancer, many have turned their attention to what may be the most promising weapon yet: diet. When it comes to a cancer-fighting diet, most experts agree that it should consist mostly of plant-based nutrients. That seemingly simple advice could mean a drastic change in diet for many people. You might aim to continue with some of the foods listed below, as they all show promise as cancer-fighting agents.

- **Tea:** If you love drinking tea, you'll be pleased to know that it looks to be effective against several types of cancer. Tea, like many plant-based meals, includes flavonoids, which are recognized for their antioxidant properties. One flavonoid in particular, kaempferol, has been proven to be anti-cancer. A large-scale research of over 66,000 women's kaempferol consumption found that those who took the most of it had the lowest chance of getting ovarian cancer. A research suggests that consuming between 10 milligrams and 12 milligrams daily of kaempferol -the amount found in four cups of tea- offers protection against ovarian cancer. A separate study found a connection between flavonoids and a lower risk of breast cancer. The research, which looked at almost 3,000 people's lifestyle patterns, discovered that postmenopausal women who consumed the most flavonoids were 46 percent less likely to get breast cancer than those who consumed the least. Flavonoid intake, on the other hand, had no influence on breast cancer risk in premenopausal women [33].

- **Wholegrains:** There is compelling evidence that consuming whole grains can help prevent against colon cancer. Wholegrains, which include brown rice, wholegrain bread, quinoa, spelt, rye, and oats, are high in nutritional fiber. They are known to aid digestion and lower cholesterol levels, as well as protect against several forms of cancer (https://www.wcrf.org/dietandcancer/wholegrains-vegetables-and-fruit/) [34].

- **Citrus fruits:** Research suggests that citrus intake may significantly reduce risk of esophageal cancer. A review looking at nine studies revealed that eating more citrus fruits was associated with a lower risk of pancreatic cancer. Another study found that eating at least three servings of citrus fruit each week lowered the incidence of stomach cancer by 28%. To make sure you're eating enough citrus

fruits, try putting lemon slices in your tea, dressing a salad with lime and eat grapefruits for breakfast [35, 36].

- **Garlic:** Not only does garlic add flavour to many meals, it is also anti-carcinogenic. According to research by Cancer Council Australia (https://wiki.cancer.org.au/policy/Obesity/Related_resources), high levels of allium vegetables (such as onions, garlic and shallots) reduce the risk of stomach cancer. Garlic is shown to "possibly" protect against colon cancer. Add more garlic into your diet, by making fresh, homemade dishes for lunches and dinners rather than shop-bought ready meals. Meals like stir-fries, chicken hot pots (a warm broth with chicken, vegetables and noodles or potatoes cooked in a single pot), and oven-baked fish dishes can all be made using plenty of garlic [37-40].

- **Chocolate:** Flavonoids are recognized to be present in fruits, vegetables, and some beverages such as tea; however, they are also present in chocolate. In reality, dark chocolate contains a high proportion of flavonoids. A study published in The Lancet showed that chocolate contained four times as much catechin, a type of flavonoid, as tea [41].

FLAVONOIDS MAY HAVE POTENTIAL FOR KILLING CANCER CELLS

A study from the University of Illinois showed that celery, artichokes, and herbs, especially Mexican oregano, contain flavonoids that killed human pancreatic cancer cells under lab conditions. The flavonoids act by inhibiting an important enzyme. However, the research team said that the trick seemed to be in using the flavonoids as a pre-treatment instead of using them and the chemotherapeutic drug simultaneously, since “flavonoids can act as antioxidants and taking antioxidants supplements on the same day as chemotherapeutic drugs may negate the effect of those drugs” [42].

CONCLUSIONS

In conclusion, the anti-inflammatory and antioxidant effects of flavonoids have also encouraged researches to study their potential as anticancer drugs. Certain flavonoids have been proven in studies to help prevent cancer cells from proliferating. Including flavonoid-rich foods in your diet and maintaining a healthy diet may reduce your chance of developing some malignancies. Still, more studies are needed to confirm whether flavonoids can be used as an effective cancer therapy.

ACKNOWLEDGEMENTS

Disclosure statement. The author declares no conflict of interest. The author alone is responsible for the content and writing of the article.

Funding. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Received: **21.10.2022**
Accepted: **30.03.2022**

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Title page: The first page of the manuscript should contain the following items in the sequence given:A concise title of the paper (no abbreviations). The names of all authors with at least one first namespelled out for every author.The names of Universities with Faculty, City andCountry of all authors.

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