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ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *GENTIANA LUTEA* L. ROOTS

SUMMARY

In this study, the antimicrobial and antioxidant properties of yellow gentian roots (*Gentiana lutea* L.) were investigated. Root samples were collected at the Vranica Mountain, situated in the central part of Bosnia and Herzegovina. The yellow gentian roots were evaluated for antimicrobial activities against four bacteria, namely, Gram-positive bacteria: *Staphylococcus epidermidis*, *Enterococcus faecalis* and Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. Total antioxidant capacity of root extracts was evaluated using ferric reducing antioxidant power (FRAP) assay. Total phenolic and total flavonoid contents were also evaluated. The antimicrobial assays indicated that the yellow gentian roots were more effective against *Staphylococcus epidermidis* and *Escherichia coli* than against *Enterococcus faecalis* and *Klebsiella pneumoniae*. The results also showed that the extracts of yellow gentian roots contained relatively high amounts of phenolic compounds and high antioxidant activity rates. The antioxidant activity of the root extracts was found to be positively associated with the total phenolic and flavonoid contents.

Keywords: bacteria, flavonoids, health, mountain habitats, phenolic compounds

INTRODUCTION

Gentiana lutea L., commonly known as yellow gentian, is distributed in the mountain areas of central and Southern Europe, including the Balkan Peninsula. It mostly occurs on grassy mountain meadows and grows at an elevation of 600-1700 m, and even more. The plant blooms from June until August.

Yellow gentian is a perennial herbaceous plant, rising to 1-2 m tall, with round, strong and vertical flower stem. Leaves are opposite, lanceolate to elliptic,

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10-30 cm long and 4-12 cm wide. Most of them belong to a basal rosette until flowering; however, basal leaves are slightly bigger than others, 30 cm long and 15 cm wide. The flowers, placed on the upper half of the stem are yellow, with the corolla distributed nearly to the base into 5-7 narrow petals. Fruit is a capsule, approximately 6 cm long, with plenty of seeds (Prakash *et al.*, 2017).

Yellow gentian root is well developed and branchy. It occurs as single or branched sub-cylindrical parts of various lengths and usually 10 - 40 mm in thickness. Dried fragments of gentian roots are identified by disagreeable smell and bitter taste. They contain some important bitter glycosides (gentiopicrin, gentianin) and alkaloids (gentiomarin) that have a wide range of pharmaceutical and medicinal utilities, primarily in treatments of various human digestive disorders (Olennikov *et al.*, 2015). Yellow gentian roots are also used as a bitter flavoring for alcoholic drinks, and in traditional medicine to stimulate the appetite and improve digestion (Catorci *et al*., 2014; Karalija *et al*., 2021).

Generally, the pharmacological activities of yellow gentian roots have been scientifically validated; however, the knowledge about their antioxidant and antimicrobial properties is still not well explored, particularly in the contexts of yellow gentian from natural habitats in Bosnia and Herzegovina. Therefore, the main aim of the present study was to evaluate the antioxidant and antibacterial properties of yellow gentian roots. This study focuses on the wild populations of yellow gentian at Mountain Vranica (Bosnia and Herzegovina).

MATERIAL AND METHODS

Study area

Mountain Vranica is part of the Dinaric mountain range, situated in central part of Bosnia and Herzegovina between the town Gornji Vakuf in the west and the town Fojnica in the east. The highest peak is Nadkrstac at 2112 m altitude, followed by Krstac (2069 m) and Rosinj (2059 m). Approximately 20 km east of the Nadkrstac, at 1660 m altitude, glacial lake ‘Prokoško Jezero’ is located (Figure 1).

Figure 1. Study area location

Lake Prokoško jezero and the surrounding area received a protected status due to the exceptional richness of flora and fauna, including some endemic plants such as *Edraianthus niveus* G. Beck, *Euphorbia gregersenii* K. Maly and *Alchemilla vranicensis* Pawlowsky.

Climatic patterns in this area are primarily mountainous with cold snowy winters and cool summers, however in lower zones (below 1300 m), especially on the southern and western slopes, the climate is moderately continental due to the influence of the sub-Mediterranean climate.

The vegetation of the Vranica Mountain area is a unique mosaic of mountain meadows, pastures, and high-altitude forests (fir, spruce, beech, and green alder). Vranica Mountain is also known for its abundance of blueberries and cranberries. Furthermore, a considerable number of Balkan endemic plants occur in habitats of Vranica Mountain such as *Ranunculus crenatus* W. et K., *Crepis aurea* (L.) Cass., *Festuca panciciana* (Hack) Richt. and *Scabiosa leucopylla* Borbas.

Gentiana lutea L. (yellow gentian) is also occurring in habitats of this area, but, unfortunately, very rarely and only in small populations. Although yellow gentian is an endangered plant species, its population on the Vranica mountain is in constant decline, primarily because unregulated and inappropriate harvesting. Therefore, in this study, very small root fragments from three individuals of yellow gentian were sampled.

Collection and processing of root samples

Root samples of three individuals of yellow gentian were collected from the same locality at mountain Vranica (mountain meadow bellow Ločika peak, 1950 m above sea level, latitude: 44° 56' 20" N, longitude: 17° 44' 27" E) in September 2021. The sampled individuals of yellow gentian were not particularly close to each other, but they were scattered within the study area. Since the yellow gentian is an endangered plant, fragments from older roots were cut with a knife very carefully. The collected fresh roots were oven-dried at 40 °C (3 days) to avoid degradation of their chemical compounds. Thereafter, dried root samples were ground to a fine powder using an electric blender and stored at 4 °C until further use.

Preparation of root extracts

1 g of dried root powder was extracted with 30 ml of 60% aqueous ethanol solution (room temperature for 24 h). Subsequently, the extract was filtered through coarse filter paper into the volumetric flask and diluted to the 50 ml mark with extracting solution. The extract thus obtained was used for the determination of total phenolic content, total flavonoid content and total antioxidant capacity.

Total phenolic content

Total phenolic content of different root extracts was determined by the Folin-Ciocalteu method (Ough & Amerine, 1988). Briefly, 0.1 ml of prepared

extract was mixed with 6 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent (diluted in distilled water 1:2, v/v before use). After 10 min, 1.5 ml of saturated Na_2CO_3 (20% w/v) solution was added. The mixture was made up to 10 ml with distilled water, heated in a water bath at 40 °C for 30 min, and then cooled in an ice-bath. The absorbance of this prepared sample solution was read at 765 nm. A standard solution of gallic acid was used to prepare a calibration curve (0-500 mg l^{-1}, R2 =0.999). Results were expressed as mg gallic acid equivalents (GAE) g^{-1} dry weight.

Total flavonoid content

Total flavonoid content of different root extracts was determined by the aluminum chloride colorimetric assay (Zhishen *et al*., 1999). Briefly, 1 ml of extract solution was diluted with 4 ml of distilled water. To this solution, 0.3 ml of 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ and 2 ml of 1 M NaOH was added. Then, the mixture was made up to 10 ml with distilled water and incubated at room temperature for 1 h, after which the absorbance was read at 510 nm. A standard solution of catechin was used to prepare a calibration curve (0-100 mg l^{-1}, R2 = 0.997). Results were expressed as mg catechin equivalents (C) g^{-1} dry weight.

Ferric reducing antioxidant power (FRAP) assay

Total antioxidant capacity of different root extracts was determined using the ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). Briefly, 80 µl extract was diluted with 240 µl distilled and then 2080 µl of fresh FRAP reagent was added. The FRAP reagent was prepared immediately before use by mixing acetate buffer (300 mM, pH=3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ in a volume ratio of 10:1:1. Thereafter, the mixture was heated at 37 °C for 15 min in a water bath, after which the absorbance was read at 595 nm. A standard solution of $FeSO_4 \times 7\ H_2O$ was used to prepare a calibration curve (0-2000 µmol l^{-1}, R2 = 0.996). Results were expressed as µmol Fe^{2+} g^{-1} dry weight. Amersham ultrospec 2100 spectrophotometer (Biochrom, USA) was used for all spectrophotometric measures.

Bacterial strains

Two Gram positive bacterial strains *Enterococcus faecalis* and *Staphylococcus epidermidis*, as well as two Gram negative bacterial strains *Escherichia coli* and *Klebsiella pneumoniae* have been isolated from food samples and food environment. After isolation of bacterial strains on nutrient agar plates, strains were identified and characterized by microscopic analysis and biochemical tests; Gram-straining, catalase, coagulase, citrate, oxidase, urease, indole, etc. All bacterial strains, used in the study, were cultured on nutrient agar (Liofilchem, Italy) and incubated at 37 °C for 18-24 h.

Preparation of bacterial inoculum

Direct colony suspension method was used in preparing the inoculum from colonies grown during 18 to 24 h. Briefly, three to five morphologically similar colonies from fresh overnight grown cultures were transferred with a loop into 5 ml of physiological solution (0.85% NaCl, w/v) in a capped test tube, and mixed thoroughly using a vortex for 1 min. The suspension was then adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard (CLSI, 2012).

Antimicrobial activity evaluation

Evaluation of the antimicrobial activity of gentian roots was conducted according to the agar dilution method (CLSI, 2012). The aim of agar dilution methods is to determine the lowest concentration of the assayed antimicrobial agent (minimum inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. This procedure involved the incorporation of different concentrations of the antimicrobial agent into a nutrient agar medium (ranged from 3 μg ml^{-1} to 24 μg ml^{-1}), followed by the inoculation of a defined microbial inoculum on the surface of the agar plate. The plates were then incubated at 37 °C for 48 h.

Classification of the bacterial strains into susceptible (S), intermediate resistant (IR) and resistant (R) was based on specific ability of each strain to grow on the whole surface area of Petri plates. Cut-off values to differentiate among resistant and susceptible groups were defined on the basis of the growth distribution of the population after incubation at 37 °C. The determination of biofilm forming categories was done per standardized formulas for manual calculation of biofilm forming categories (weak, moderate, strong) according to the CLSI (2012).

Statistical analysis

All assays were performed in triplicates, and measurement data were expressed as the mean ± standard deviation. The Pearson correlation coefficients were calculated in order to identify the relationship between the phenolic/flavonoids and total antioxidant capacity. Microsoft Excel 2010 package for Windows (Office 2010, Redmond, WA, USA) was the software used for the statistical analysis.

RESULTS AND DISCUSSION

Plants with bioactive properties play an important role in health promotion and disease prevention. Antioxidant capacity is without a doubt one of the basic bioactive properties of plants due to the presence of different types of phenolic compounds and other antioxidants (Pinto *et al*., 2021). Even though yellow gentian roots have long been traditionally used, their antioxidant capacity is still insufficiently explored, particularly in the contexts of yellow gentian native to Bosnia and Herzegovina.

In the present study we therefore evaluated the antioxidant capacity of yellow gentian roots from natural habitats of Vranica Mountain. Total phenolic and flavonoid contents were also evaluated. The evaluation of both phenolic and flavonoid contents is relevant because the antioxidant capacity of plant has been mainly attributed to their presence in plants (Yu *et al*., 2021).

The quantification of total phenolic contents (TPC), total flavonoid contents (TFC) and total antioxidant capacity (TAC) in root samples of yellow gentian roots are shown in Table 1.

Table 1. Total phenolics (TPC), total flavonoids (TFC), and total antioxidant capacity (TAC) of yellow gentian roots

Individuals	TPC (mg g^{-1} DW)	TFC (mg g^{-1} DW)	TAC (µmol Fe^{2+} g^{-1} DW)	TFC/TPC ratio
No. 1	8.90 ± 0.67	1.37 ± 0.11	25.54 ± 4.62	0.15 ± 0.03
No. 2	9.05 ± 1.25	1.41 ± 0.27	24.84 ± 4.45	0.16 ± 0.02
No. 3	9.35 ± 1.01	1.55 ± 0.32	27.88 ± 3.01	0.17 ± 0.03

The obtained experimental values for TPC and TFC of yellow gentian root samples are in good agreement with those findings reported in literature. The values found in the literature for the TPC and TFC in dried yellow gentian roots were in the range of 0.96 to 17.46 mg g^{-1} and 0.48 to 2.66 mg g^{-1}, respectively (Nastasijević *et al*., 2012; Azman *et al*., 2014; Drobyk *et al*., 2015; Mudrić *et al*. 2020). Interestingly, the TFC/TPC ratios in the studied root samples of yellow gentian were in the range of 0.15 to 0.17, indicating low percentage rate of TFC vs. TPC. Huang *et al*. (2013) also reported the low TFC/TPC ratios in six different *Gentiana* spp. root samples.

In this study, there were no significant differences in TPC, TFC and TAC in the root samples among the three individuals of yellow gentian studied. These results were expected since all individuals of yellow gentian studied were sampled from the same locality.

The correlation of TPC/TFC with TAC in yellow gentian roots samples is shown in Figure 2.

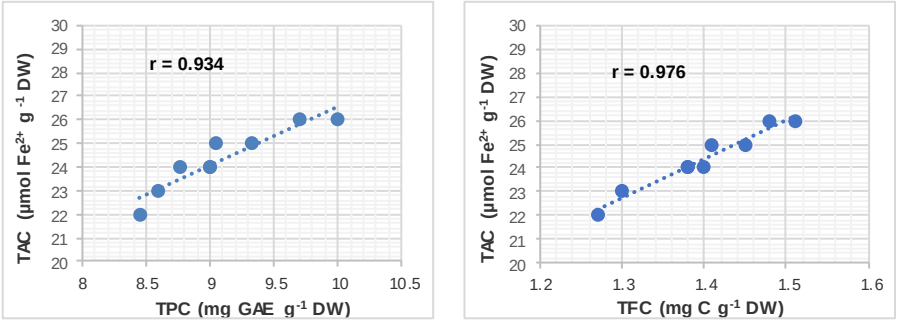

Figure 2. Correlation between TPC/TFC and TAC of the samples

A strong positive linear relationship was found between TPC/TFC and TAC of the samples, indicating that phenolic compounds, especially flavonoids, are highly responsible for the antioxidant activity of yellow gentian root extracts. Numerous studies have also revealed that phenolic compounds play an important role in the antioxidant activity of plants (Stagos, 2019; Khan *et al*., 2020; Kiani *et al*., 2021). Mucha *et al*. (2021) reported that phenolic compounds have high antioxidant activity mainly because of their ability to donate a hydrogen anion, i.e., an unpaired electron to free radicals, which interrupts the cycle of their new creation. The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions responsible for the generation of free radicals (Tungmunnithum *et al*., 2018).

The antimicrobial activities of yellow gentian root powder were also evaluated in this study (Table 2). This activity was tested against two Gram positive bacterial strains: *Enterococcus faecalis* and *Staphylococcus epidermidis*, and two Gram negative bacterial strains: *Escherichia coli* and *Klebsiella pneumoniae*. Gram-positive and Gram-negative bacteria tested in this study were categorized under opportunistic pathogens which can cause severe nosocomial infections. Hence, they are difficult to treat because of multiple drug resistance to the various classes of antibiotics (Cepas *et al*., 2019; Senobar Tahaei *et al*., 2021).

Table 2. Antimicrobial and antibiofilm activity of yellow gentiana root powder against tested bacteria

MIC (minimum inhibitory concentration)	*Staphylococcus epidermidis*		*Enterococcus faecalis*		*Escherichia coli*		*Klebsiella pneumoniae*	
	growth	biofilm	growth	biofilm	growth	biofilm	growth	biofilm
3μg ml^{-1}	IR*	weak	IR	moderate	IR	moderate	R	strong
6μg ml^{-1}	IR	weak	IR	moderate	IR	moderate	R	strong
9μg ml^{-1}	IR	weak	R	strong	S	weak	R	strong
12μg ml^{-1}	IR	weak	R	strong	S	weak	R	strong
*R - resistant, IR - intermediate resistant, S - susceptible								

The strain *Staphylococcus epidermidis* was intermediate resistant (IR) to all minimum inhibitory concentrations (MICs) of gentian root powder used in this study (ranged from 3 to 24 μg ml^{-1}) and all MICs inhibited biofilm formation capability of tested *Staphylococcus epidermidis*.

The strain of *Enterococcus faecalis* showed intermediate resistance to MIC>3μg ml^{-1} and MIC>6μg ml^{-1} and high-level resistance to MIC>12μg ml^{-1} and MIC>24μg ml^{-1} of gentian root powder, due to strong biofilm formation. Numerous studies have revealed that the natural products with or without antimicrobial activity can stimulate bacterial biofilm formation (Singh *et al*. 2002; Bleich *et al*. 2015; Yu *et al*. 2017), and the results obtained in this study support this hypothesis. The bacteria present inside a biofilm are protected against the action of the antimicrobial drugs, thus permitting their survival (Pinheiro *et al*., 2014). In study of Hassan *et al*. (2011) several relationships were

found between the ability to form biofilm and antimicrobial resistance, being different for each species.

In the present study, the strain *Escherichia coli* showed moderate biofilm production capability at MIC>3 μg ml^{-1} and MIC>6 μg ml^{-1} of gentian root powder in agar media, while MIC>12 μl ml^{-1} and MIC>24 μl ml^{-1} inhibited the growth of *Escherichia coli* strain as well as biofilm formation.

Gentian root did not show any antibacterial effect to the strain *Klebsiella pneumoniae*, indicating the high-level resistance of studied *Klebsiella pneumoniae* strain to all MICs of yellow gentian root used in this study.

CONCLUSIONS

In sum, the results of this study revealed that the extracts of gentian roots contained relatively high amounts of phenolic compounds and high antioxidant activity rates. The antioxidant activity of the root extracts was found to be positively associated with the total phenolic and flavonoid contents.

The antimicrobial assays indicated that the yellow gentian roots were more effective against *Staphylococcus epidermidis* and *Escherichia coli* than against *Enterococcus faecalis* and *Klebsiella pneumonia*.

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