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ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF *OPOPANAX HISPIDUS*(APIACEAE) EXTRACTS

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SUMMARY

The present study was aimed to investigate antioxidant and antimicrobial potential of methanol and ethyl-acetate extracts from dried aerial parts, inflorescences and fruits of *Opopanax hispidus* (Friv.) Griseb., fam. *Apiaceae*. The antioxidant potential was evaluated with the help of two *in vitro* antioxidant models – DPPH and ABTS assays and estimation of total phenolic and flavonoids using spectrophotometric methods. BHA and Vitamin C were used as standard and positive control for above models. Microdilution assay was used to evaluate antimicrobial potential for the most common human gastrointestinal pathogenic microbial strains. The results of DPPH and ABTS assay showed that the highest antioxidant activity have methanol (IC_{50}=1.157 mg/ml) and ethyl-acetate (IC_{50}=3.167 mg/ml) extracts from inflorescences. The highest value of total phenolic (89.95±0.005 mg GA/g) and total flavonoid (24.06 ± 0.004 mg Qu/g) was measured in inflorescences extracts also. Results indicate that both extracts (methanol and ethyl-acetate) of inflorescences have high amount of phenol and flavonoids, which could be responsible for its good antioxidant activity.The most susceptible were *Listeria monocytogenes* and *Escherichia coli* on ethyl-acetate extracts from fruits and inflorescence, respectively. This is the first record of antioxidant and antimicrobial activity of *Opopanax hispidus* from Serbia. It is also worth noting that these results validate the therapeutic use of the plant in traditional medicine.

Key words: *Opopanax hispidus*, Apiaceae, extracts, antioxidant, antimicrobial activity.

INTRODUCTION

The genus *Opopanax*, family Apiaceae, consists of two species, *O. chironium* (L.) Koch, and *O. hispidus* (Friv.) Griseb, widely distributed in Balkan Peninsula and Aegean region (S. Italy and Sicilia) [1]. *O. hispidus* is found at only one locality in Serbia - Rujan Mountain, between Cer hill and the village of Mamince near Kosovska Mitrovica. This species is described in Red data book of flora of Serbia and qualified as critically endangered taxon [2].

The species *O. hispidus* is perennial plant 300 cm high with a stout, solid stem with branches verticillate or subopposite, offten very close below the terminal umbel. Leaves are 2-pinnate, with stellate hairs beneath. Lobes usually are 2-4 cm, ovate-lanceolate and hispid. Flowers are yellow and fruits are broadly elliptical, border wide and thin 7-9 mm [1].

The term "opopanax" is a source of confusion in pharmacognosy, since three different products bear this name. Thus, *O. hispidus* is not to be confused with perfumery's opopanax, a gum-resin obtained from *Commiphoraerytraea* var. *glabrescens* Engler, a tree endemic to the Horn of Africa [3]. This gum resin was probably obtained from a variety of umbelliferous plants including, besides *O. chironium* and *O. hispidum* (Friv.) Griseb., also plants from the genus *Ferula*, *Peucedanum*, *Laserpitium* and *Heracleum* [4,5]. For *O. chironium*has only been known to be used as expectorant and antispasmodic in folk medicine [6]. Recently, in phytochemical study of chloroform extract of the aerial parts of *Opopanax hispidus* a new dihydrofuranocoumarin: 3′-isobutyryl-3′-hydroxymarmesin, together with six known coumarins: oreoselon, peucedanin, officinalin, smirniorin, 4′-acetyl-3′-isobutyryl-3′-hydroxymarmesin and 3′-hydroxyprantschimgin were published [7].In Turkish folk medicine stem of *O. hispidus* is used for treat of infertility (for women) [8].

As we know, this is the first report of antioxidant and antimicrobial activity of methanol and ethyl-acetate extracts from *O. hispidus*, the plant which may possess many pharmacognosy effects.

MATERIAL AND METHODS

Plant material and preparing extracts

The aerial plant parts with flowers and fruits were collected in July 2010 at Rujan Mountain, Southeast Serbia. The plant material was collected and determined as *Opopanax hispidus* (Friv.) Griseb., by the author Ranđelović Vladimir. A voucher specimen (BEOU 16435) has been deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade.

Classical extraction was used for preparing extracts: 10g plant material (aerial parts, inflorescences and fruits separately) was dissolved with 100ml

methanol or ethyl-acetate. After 24h in the dark, extracts were filtrated and solvent was evaporating with vacuum evaporator. The first and last hour extracts were in ultrasonic bath. Yield of dry extracts are shown in Table 1.

Table 1. Yield of dry extracts from *O. hispidus*.
Tabela 1. Prinosi suvih ekstrakata za *O. hispidus*.

Yield (g)	Methanol extract	Ethyl-acetate extract
Aerial parts	0.460	0.084
Inflorescences	1.595	0.302
Fruits	1.674	0.190

Determination of total phenolic content

The total phenolic content of extracts was determined by Folin-Ciocalteu method according to the procedure reported by Singleton et al. (1999) with some modifications [9]. Briefly, 300 µl of methanol and ethyl acetate extracts and 1500 µl of 1:10 Folin-Ciocalteau reagent were mixed and after 6 minutes in the dark were added 1200 µl of sodium carbonate (7.5%). After 2 h in the dark of incubation at room temperature, the absorbance at 740 nm was measured (Shimadzu, UV-Visible PC 1650 spectrophotometer). The total phenolic concentration was calculated from gallic acid (GA) calibration curve (10-100 mg/L). Data were expressed as gallic acid equivalents (GA)/g of extract averaged from 3 measurements.

Determination of flavonoid content

The total flavonoid content was evaluated using aluminium nitrate nonahydrate according to the procedure reported by Woisky and Salatino (1998) with some modifications [10]. The sample for determination was prepared by mixing a 600 µl of methanol and ethyl acetate extracts and 2580 µl of mixture (80% C_2H_5OH, 10% $Al(NO_3)_3$ x 9 H_2O and 1M $C_2H_3KO_2$). After 40 min of incubation at room temperature, the absorbance at 415 nm was measured using Shimadzu, UV-Visible PC 1650 spectrophotometer. The total flavonoid concentration in different extracts was calculated from quercetin hydrate (Qu) calibration curve (10-100 mg/L) and expressed as quercetin equivalents (Qu)/g of dry extract. Measurements were done in triplicates.

Antioxidant activity

Antioxidant activity aerial parts, inflorescences and fruits of plant methanol and ethyl-acetate extracts had been tested by DPPH and ABTS assays. All results were obtained using Shimadzu, UV-Visible PC 1650 spectrophotometer. Data analysis was performed with OriginPro 8.0 software.

DPPH assay **–** For this assay was applied from Blois method [11]. Standard methanol solution contains 0.04 mg/mL DPPH. After shaking, 0.3 mL methanol (concentration 125, 500, 1000, 2000 and 5000 µl/ml) and ethyl-acetate solution of different extracts (concentration 2000, 3000, 5000, 8000 and 9000 µl/ml) and 2.7 mL DPPH radical solution, the reaction mixture had been incubated in the dark for 30min at room temperature. Each sample, vitamin C (AnalaR Normapur, VWR; assay 99.0–100.5%): and BHA [Sigma Chemicals Co.; ≥ 98.0% (sum of isomers, GC): ≤ 5.0% 2-BHA basis (GC)] standards were measured in triplicate. Blank probes were done in the same way using methanol instead of investigated solution (A_0). The decrease of absorption of DPPH solution is calculated by equation:

$$\% \text{ of absorption decrease (on 517 nm)} = (A_0 - A_1) \times 100 / A_0$$

Sample concentrations necessary to decrease the absorbance of DPPH by 50% (IC_{50}) were obtained from the absorption of curves DPPH solution at 517 nm for each compound and standard antioxidant.

ABTS assay – A method of Miller and Rice-Evans with some modifications was used for this assay [12]. Reagans ABTS·+ was mixed with 2.46 mM potassium persulfate and as such a solution was standing in the dark 12-16h at room temperature. After that, 1ml ABTS·+ solution was diluted with 100–110 mL water to give an absorbance of 0.7±0.02 units at 734 nm using spectrophotometer. Methanol ant ethyl-acetate solutions of different extracts (concentration for tested extracts were been 1.5, 2 and 3 mg/mL) or standard solutions (concentration for BHA were been 0.10 mg/mL) 75 µL, were mixed with 3 mL of diluted ABTS solution. After 30 min incubation at 30ºC, the absorbance was measured at 734 nm. Modifications of this method are that the water was used as a blank. ABTS radical scavenging activity in aerial parts, inflorescences or fruits of plant extracts were calculated from the Vitamin C (VitC) calibration curve (0–2 mg/L) and expressed as Vitamin C (VitC)/g of extracts. All measurements were performed in triplicate and were expressed as average of three analyses ± standard deviation.

Antimicrobial activity

Microbial cultures – The antimicrobial activity of all tested samples was evaluated using laboratory control strains obtained from the American Type Culture Collection: Gram (-) bacteria - *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076; Gram (+) bacteria: *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC 25923 and yeast *Candida albicans* ATCC 10231.

Micro-well Dilution Assay **–** The inocula of the microbial strains were prepared from the overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7-10^8 CFU/ml, depending on

genera - consensus standard by the NCCLS) [13]. A series of doubling dilutions of the tested extracts from *O. hispidus* - 100 mg/ml in 30 % ethanol were prepared in a 96/well microtiter plate over the range of 0.1–50.0 mg/ml in inoculated Mueller-Hinton broth. The final volume was 100 µl and the final microbial concentration was 10^6 CFU/ml in each well. The plate was incubated for 24 h at 37 ºC for bacterial and 24 h at 25 ºC for yeast. All experiments were performed in triplicate. Two controls were included - medium with 30 % ethanol (negative control) and medium with Streptomycin, Chloramphenicol and Nystatin (positive control). Microbial growth was determined by adding 20 µl of 0.5 % triphenyl tetrazolium chloride (TTC) aqueous solution [14]. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the samples inhibiting visible growth (red colored pellet on the bottom of the wells after the addition of TTC). To determine MBC/MFC, the broth was taken from each well without visible growth and inoculated in Mueller-Hinton agar (MHA) for 24 h at 37 ºC for bacterial and 24 h at 25 ºC for yeast. Minimal bactericidal/fungicidal concentration (MBC/MFC) was defined as the lowest samples concentration killing 99.9 % of bacterial/fungal cells.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

It has been shown that phenolic compounds represent a class of antioxidant agents which act as free radical terminators [15]. In other hand, flavonoids have antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process [16-17]. The results obtained for total phenolic and flavonoid content of *O. hispidus* extracts are presented in Table 2. The highest value of total phenolic (89.95 ± 0.005 mg GA/g) and total flavonoid (24.06 ± 0.004 mg Qu/g) was measured in inflorescences extracts. These results are in correlation with results for antioxidant activity, which is a confirmation of previous studies.

Antioxidant activity (DPPH and ABTS assay)

It was reported that the antioxidant constituents from natural, plant sources provide protection from damage caused by free radical induced oxidative stress [18-19].

The results of DPPH and ABTS assay showed that the highest antioxidant activity have methanol (IC_{50}=1.157 mg/ml) and ethyl-acetate (IC_{50}=3.167 mg/ml) extracts from inflorescences of *O. hispidus* in compare to aerial parts and fruits extracts. Methanol extracts possessed better antioxidant activity than ethyl-acetate ones.

Table 2. Total phenolic/flavonoid contents and antioxidant capacities by DPPH and ABTS assay of aerial plant parts, inflorescences and fruits from *O. hispidus* extracts.
Tabela 2. Ukupni sadržaj fenola/flavonoida i antioksidativna aktivnost DPPH i ABTS testa ekstrakata iz nadzemnih delova, cvasti i plodova vrste *O.hispidus*.

Opopanax hispidus	**Tested concentrations**	**Total phenolic content** (mg GA/g)	**Total flavonoid content** (mg Qu/g)	**ABTS** mg VitC/g	**DPPH IC_{50}** (mg/mL)
Methanol extracts					
Aerial parts	3 mg/mL	59.68± 0.004	18.60± 0.003	2.15± 0.016	4.280
Inflorescences	1.5 mg/mL	89.95± 0.005	24.06± 0.004	3.14± 0.006	1.157
Fruits	2 mg/mL	78.82± 0.002	21.46± 0.004	2.63± 0.015	2.661
Ethyl-acetate extracts					
Aerial parts	5 mg/mL	45.05± 0.004	70.01± 0.015	1.63± 0.005	7.284
Inflorescences	5 mg/mL	85.10± .004	78.96± 0.004	2.90± 0.004	3.167
Fruits	5 mg/mL	46.36± 0.005	77.28± 0.004	1.78± 0.005	5.011
BHA	0.1 mg/mL	63.31± 0.001	–	2.660± 0.005	0.093
Vitamin C	0.1 mg/mL	40.91± 0.002	–	–	0.054

Values in the table are obtained by calculating the average of three analysis ± standard deviation.

Antimicrobial activity

Methanol extracts acted in the range MIC/MBC = 0.78/>50 mg/mL, while the range of antimicrobial activity for ethyl-acetate extracts were MIC/MBC = 1.56/>50 mg/mL (Table 3). Generally, the most sensitive strains were *L. monocytogenes* for ethyl-acetate extracts from fruits (MIC/MBC = 3.125/6.25 mg/mL) and ethyl-acetate extracts from inflorescences (MIC/MBC = 6.25 mg/mL) for *E. coli.*

So far, the resuts of antimicrobial effects of plant extracts are published for a number of Apiaceae species from Serbian flora in relation to medically significant bacteria and fungi [20-23]. There is no data on those activities on *Opopanax* species extracts.

Table 3. Antimicrobial activity of aerial parts, inflorescences and fruits from *O. hispidus* extracts against pathogenic microbial strains using micro-well dilution assay.
Tabela 3. Antimikrobna aktivnost ekstrakata nadzemnih delova, cvasti i plodova vrste *O. hispidus* na patogene sojevekorišćenjem mikrodilucione metode.

Opopanax hispidus	***Extracts (MIC/MBC(MFC) in mg/mL)***					***Antibiotics***
	Methanol extract (A. p.)	Methanol extract (Inflor.)	Methanol extract (Fruits)	Ethyl-acetate extract (Inflor.)	Ethyl-acetate extract (Fruits)	MIC/MBC (MFC) mg/mL
Gram (-) bacteria						**Streptom.**
E. coli ATCC 25922	3.125/>50	6.25/12.5	3.125/50	6.25/6.25	6.25/50	0.016/0.016
P. aeruginosa ATCC 9027	0.78/>50	1.56/50	0.78/>50	6.25/50	3.125/>50	0.008/0.008
S. enteritidis ATCC 13076	1.56/>50	1.56/50	3.125/>50	12.5/25	12.5/25	0.004/0.004
Gram (+) bacteria						**Chloramph.**
B. cereus ATCC 10876	6.25/>50	3.125/12.5	3.125/>50	1.56/50	1.56/>50	0.004/0.016
L. monocytogenes ATCC15313	6.25/25	6.25/12.5	6.25/25	12.5/12.5	3.125/6.25	0.008/0.016
S. aureus ATCC 25923	1.56/50	1.56/50	1.56/50	6.25/>50	3.125/>50	0.001/0.008
Yeast						**Nystatin**
C. albicans ATCC 10231	6.25/50	12.5/50	6.25/50	12.5/>50	12.5/>50	0.016/0.016

CONCLUSION

From the results obtained in the present study, it can be concluded that methanol extract of *O. hispidus* show strong antioxidant activity by DPPH and ABTS assay when compared with ethyl-acetate extracts. In addition, methanol and ethyl-acetate extract of *O. hispidus* were found to contain noticeable amount of phenol and flavonoids that could have great importance as a therapeutic agent in preventing or slowing oxidative stress related degenerative diseases. Both extracts of different parts of this plant species showed good antimicrobial activity. Therefore, *O. hispidus* can be further harnessed for novel antioxidant/antimicrobial compounds, which is well evidenced by the present study.

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ANTIOKSIDATIVNI I ANTIMIKROBNI POTENCIJAL EKSTRAKATA*OPOPANAX HISPIDUS* (*APIACEAE*)

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IZVOD

Cilj ovog rada je da istraži antioksidativni i antimikrobni potencijal metanolnih i etil-acetatnih ekstrakata osušenih nadzemnih delova, cvasti i plodova vrste *Opopanax hispidus* (Friv.) Griseb, familija *Apiacea*e. Antioksidativni potencijal je određen zahvaljujući dva *in vitro* modela – DPPH i ABTS testa i procenjen je ukupni sadržaj fenola i flavonoida koristeći spektrofotometrijske metode. BHA i vitamin C su korišćeni kao standardi i pozitivne kontrole za oba modela. Mikrodilucionom metodom je utvrđen antimikrobni potencijal protiv najčešćih gastrointestinalnih patogena. Rezultati DPPH i ABTS testa su pokazali da najveću antioksidativnu aktivnost imaju metanolni (IC_{50}= 1.157 mg/ml) i etil-acetatni (IC_{50}= 3.167 mg/ml) ekstrakti cvasti. Najveći sadržaj ukupnih fenola (89.95 ± 0.005 mg GA/g) i ukupnih flavonoida (24.06 ± 0.004 mg Qu/g) su takođe izmereni u cvastima.Rezultati su pokazali da oba ekstrakta (metanolni i etil-acetatni) imaju visok sadržaj fenola i flavonoida, što bi mogao biti uzrok njihovoj dobroj antioksidativnoj aktivnosti. Najosetljiviji bakterijske vrste su *Listeria monocytogenes* i *Escherichia coli* na etil-acetatne ekstrakte plodova i cvasti. Ovo su prvi rezultati antioksidativne i antimikrobne aktivnosti vrste *Opopanax hispidus* iz Srbije. Takođe, treba napomenuti da ovi rezultati potvrđuju terapijsku upotrebljivost biljaka u tradicionalnoj medicini

Ključne reči: *Opopanax hispidus*, Apiaceae, ekstrakti, antioksidativna, antimikrobna aktivnost.