

Original article:

BIOLOGICAL ACTIVITIES OF EXTRACTS FROM CULTIVATED GRANADILLA *PASSIFLORA ALATA*

Sava M. Vasić^{1*}, Olgica D. Stefanović¹, Braho Z. Ličina², Ivana D. Radojević¹, Ljiljana R. Čomic¹

¹ Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Republic of Serbia

² Department of Biomedical Sciences, State University of Novi Pazar, Vuka Karadžića bb, 36300 Novi Pazar, Republic of Serbia

* corresponding author: Tel.: +381 34 336 223; Fax: +381 34 335 040
e-mail: savavasic@gmail.com

ABSTRACT

Research conducted in this study showed the influence of ethanol, acetone and ethyl acetate extracts of the outgrowth of cultivated *Passiflora alata* on microorganisms, as well as the antioxidant activity and the concentrations of total phenols, flavonoids and tannins. *In vitro* antimicrobial activities of extracts were studied on 27 species of microorganisms, of which 17 species of bacteria and 10 species of fungi. The strongest antimicrobial activity was detected on G+ bacteria while the activities on other species were moderate. Ethyl acetate extract showed the strongest effect. The concentrations of total phenols were examined by using Folin-Ciocalteu reagent and the obtained values ranged from 14.04 to 34.22 mg GA/g. By using aluminium chloride method, the concentrations of flavonoids were obtained and the values ranged from 33.19 to 62.30 mg RU/g. In determining the amount of tannins we used the method with butanol-HCl reagent and the obtained value was 5.1 % of dry matter. The efficiency of antioxidation, which we identified through the reduction of DPPH, was in the range from 808.69 to 1107.79 µg/ml for a particular IC₅₀, and AAI values were between 0.07 and 0.10. The best parameters were shown by ethanol extract. All data were statistically analyzed. Overall, extracts showed potential for further investigation and use.

Keywords: *Passiflora alata*, antimicrobial capacity, antioxidant, phenols, flavonoids, tannins

INTRODUCTION

Granadilla, *Passiflora alata* Curtis, belongs to the family *Passifloraceae*, tribe *Passifloreae*. In subtropical and tropical areas, genus *Passiflora* includes more than 400 species. *P. alata* is a perennial plant, an evergreen climber, native of South America. The internode is quadrangular in cross section and the plant is about 10 meters long and branched. The leaves are round to slightly elongated, with the length of 10 to 15 cm and 10 cm wide, sometimes asymmetrically divided, and with one tendril in the base. The flowers are individual, 7 to 10

cm wide, red, fragrant and last for about 24 hours. The fruits are egg-shaped, yellow, 8 to 15 cm long, 90 to 300 g of weight and edible.

Numerous literature data confirm the use of plants of the genus *Passiflora*. Since most species inhabit South America tea products are represented in folk treatment from the time of American Indian civilizations. Today, this plant is an integral part of phytopharmaceutical products worldwide. A study on the effects of compounds from plants of this genus shows a wide range of activities: antimicrobial, antioxidant, cyto-

toxic, anti-inflammatory, antitumor, hemolytic, anti-anxiety, antihypertensive (Ingale and Hivrale, 2010). On the other hand, the occurrence of severe allergies was detected in people employed in processing dry materials from *P. alata* (Giavina-Bianchi et al., 1997). Invasiveness of *P. alata* in southern Brazil has initiated the study of genetic variability of this plant for the purpose of understanding the mechanisms of adaptation to different environmental factors (Koehler-Santos et al., 2006). The richness of biologically active compounds contained in *Passiflora* species were examined and isolated, which was also done on phenols and flavonoids (Pereira et al., 2004; Müller et al., 2005; Rudnicki et al., 2007). Products of different species of this genus have been studied and their effects on microorganisms have given certain results (Bendini et al., 2006; Johnson et al., 2008; Wei et al., 2008; Kannan et al., 2011, Martin et al., 2012). 2S albumins from seeds of *P. edulis* showed specific antifungal activities on pathogenic and nonpathogenic yeasts as well as some additional biochemical properties (Ribeiro et al., 2012). 2S albumins from seeds of *P. alata* showed the effect on *Colletotrichum gloeosporioides*, which indicate their potential for further development of biotechnological products in order to protect fruits from fungal diseases (Ribeiro et al., 2011). Tea of *P. alata* affected a change of blood parameters in rats, reducing total and LDL cholesterol and increasing HDL cholesterol, while it did not affect their condition and behavior (Doyama et al., 2005). Hydro-ethanol leaf extracts of *P. edulis* and *P. alata* were also examined on the rats, which showed a preliminary sedative effect of these extracts (Petry et al., 2001). Aqueous extracts of these two species also showed effects similar to diazepam (De-Paris et al., 2002).

The aims of this study, by examining the properties of *P. alata* from the perspective of a plant grown in a container in a temperate continental climate region, were determining the effects of ethanol, acetone and ethyl acetate extracts, derived from the plant outgrowth, on bacteria and fungi *in*

vitro, followed by the determination of antioxidant activity, total phenols, flavonoids and tannins.

MATERIAL AND METHODS

Chemicals

Organic solvents (ethanol, ethyl acetate and acetone) and concentrated hydrochloric acid (HCl) were purchased from Zorka Pharma (Šabac, Serbia). 2,2-diphenyl-1-picrylhydrazyl (DPPH), chlorogenic acid and Folin-Ciocalteu phenol reagent were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Gallic acid, rutin hydrate and aluminium chloride hexahydrate ($\text{AlCl}_3 \times 6\text{H}_2\text{O}$) were purchased from Acros Organics (New Jersey, USA). Sodium carbonate (Na_2CO_3) was obtained from MP-Hemija (Belgrade, Serbia). Dimethyl sulfoxide (DMSO), *n*-Butanol and ferric ammonium sulfate ($\text{NH}_4\text{Fe}(\text{SO}_4)_2 \times 12\text{H}_2\text{O}$) were purchased from Centrohem (Stara Pazova, Serbia). Resazurin was obtained from Alfa Aesar GmbH & Co. (KG, Karlsruhe, Germany). Nutrient liquid medium, a Mueller–Hinton broth was purchased from Liofilchem (Italy), while a Sabouraud dextrose broth was from Torlak (Belgrade, Serbia). An antibiotic, doxycycline, was purchased from Galenika A.D. (Belgrade, Serbia) and antimycotic, fluconazole, was from Pfizer Inc. (USA).

Plant material

In October 2011 outgrowths of *P. alata* were collected from a plant grown in pot outside, in Kragujevac, central Serbia: (position: 44°22'N, 20°56'E, altitude: 180.00 m, exposition: S, habitat: south facing wall in a garden). Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac. The voucher sample is deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at ambient temperature. The dried plant material was cut up and stored in paper bags until needed.

Preparation of plant extracts

Dried ground plant material was extracted by maceration with ethanol, ethyl acetate and acetone. 18 g of plant material was soaked with 150 ml of solvent for 24 h at room temperature. The resulting extract was then filtered through filter paper (Whatman No.1). The residue from the filtration was extracted again twice using the same procedure. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40 °C. The obtained amounts of crude extracts of *P. alata* were 1.32 g of ethanol extract, 1.81 g of ethyl acetate extract, 0.89 g of acetone extract. The extracts were kept in sterile sample tubes and stored at -20 °C.

Phytochemical analysis

Determination of total phenolic content

The total phenolic content was determined by using Folin-Ciocalteu's method (Wootton-Beard et al., 2011). The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extract (1 mg/ml) and 1.5 ml of 1:10 Folin-Ciocalteu's reagent dissolved in water. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml 6 % Na₂CO₃ solution. After incubation for 90 min at room temperature in darkness, the absorbance of the mixture was read at 725 nm against a blank using spectrophotometer. The blank was prepared with methanol instead of extract solution. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for gallic acid which was used for calibration of standard curve. Total phenol content is reported as gallic acid equivalents by reference to linear equation of the standard curve ($y = 0.008x + 0.0077$, $R^2 = 0.998$). Then the total phenolic content was expressed as a milligram of gallic acid equivalent per gram of extract (mg GAE/g of extract).

Determination of total flavonoid content

The concentrations of flavonoids were determined by using aluminium chloride method (Quettier-Deleu et al., 2000). The

mixture contained 2 ml of methanolic solution of extract (1 mg/ml) and 2 ml of 2 % methanolic AlCl₃·6H₂O solution. The mixture was vigorously shaken, and after 10 min of incubation at room temperature, the absorbance versus a prepared blank was read at 430 nm using spectrophotometer. The samples were prepared in triplicate and the mean value of absorbance was obtained. Rutin was used as a standard for calibration of standard curve. The concentrations of flavonoids were calculated from the linear equation of standard curve ($y = 0.021x + 0.040$, $R^2 = 0.999$). Then the concentrations of flavonoids were expressed as milligram of rutin equivalent per gram of extract (mg of RU/g of extract).

Determination of condensed tannins (proanthocyanidins)

Condensed tannins were determined by using the butanol-HCl method as described by Makkar (2000). The extract was made from 200 mg of prepared plant material in 10 ml of 70 % acetone. After 20 minutes infusion was filtered through Whatman No. 1 filter paper and 0.5 ml of obtained liquid extract was transferred to glass test tube, adding 3 ml of the butanol-HCl reagent (butanol-HCl 95:5 v/v) and 0.1 ml of the ferric reagent (2 % ferric ammonium sulfate in 2N HCl). Covered tubes were heated in a water bath at 97 to 100 °C for 60 min. After cooling, the absorbance was determined at 550 nm. A blank was measured as the absorbance of the unheated mixture. The samples were prepared in triplicate and the mean value of absorbance was obtained. Condensed tannin (% per dry matter) was calculated as leucocyanidin equivalent using the formula developed by Porter et al. (1986):

$$\text{Condensed tannins (\%)} = (A_{550 \text{ nm}} \times 78.26 \times \text{Dilution factor}) / (\% \text{ dry matter})$$

This formula assumes that the effective $E^{1\%,1 \text{ cm. } 550 \text{ nm}}$ of leucocyanidin is 460.

Determination of antioxidant activity

DPPH radicals scavenging capacity assay

The ability of the plant extract to scavenge DPPH free radicals was assessed by using the method described by Takao et al. (1994), along with antioxidant activity index (AAI) by Scherer and Godoy (2009). The stock solution of the plant extract was prepared in methanol to achieve the concentration of 2000 µg/ml. Further, two-fold dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62 µg/ml. Diluted solutions of extract (2 ml each) were mixed with 2 ml of DPPH methanolic solution (80 µg/ml). After 30 min in darkness at room temperature, the absorbance was read in a spectrophotometer at 517 nm. The control samples consisted of 2 ml of methanol added to 2 ml of DPPH solution. Chlorogenic acid was used as a positive control. The experiment was performed in triplicate. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

$$\text{Scavenging activity (\%)} = 100 \times [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}]$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the extract.

The IC_{50} value is the effective concentration at which 50 % of DPPH radicals were scavenged. It was obtained from the graph of scavenging activity (%) versus concentration of samples. Low IC_{50} value indicates strong ability of the extract to act as DPPH scavenger. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated using the following equation:

$$\text{AAI} = \text{final concentration of DPPH } (\mu\text{g/ml}) / \text{IC}_{50} (\mu\text{g/ml}).$$

The estimation of AAI was: if $AAI < 0.5$ – poor antioxidant activity; $AAI > 0.5 - 1$ – moderate antioxidant activity; $AAI > 1 - 2$ – strong antioxidant activity and $AAI > 2$ – very strong antioxidant activity.

Determination of antimicrobial activity

Test microorganisms

Antimicrobial activity of acetone, ethyl acetate and ethanol extract was tested against 27 microorganisms including seventeen strains of bacteria (standard strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Proteus mirabilis* ATCC 12453, and clinical isolates (*Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus aureus* PMFKG-B12, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Sarcina lutea*, *Salmonella enterica*, *Salmonella typhimurium*, *Bacillus subtilis* and *Bacillus cereus*) and ten species of fungi: *Penicillium italicum* PMFKG-F29, *Penicillium digitatum* PMFKG-F30, *Penicillium chrysogenum* PMFKG-F31, *Aspergillus restrictus* PMFKG-F25, *Aspergillus flavus* PMFKG-F24, *Aspergillus fumigatus* PMFKG-F23, *Aspergillus niger* PMFKG-F26, *Candida albicans* ATCC 10231, *Candida albicans* (clinical isolate) and *Rhodotorula sp.* PMFKG-F27. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Bacterial and yeast suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard (Andrews, 2005). Initial bacterial suspensions contain about 10^8 colony forming units (CFU)/ml and yeast suspensions contain 10^6 CFU/ml. 1:100 dilutions of initial suspension were additionally prepared into sterile 0.85 % saline. The suspensions of fungal spores were prepared by gentle stripping of spores from slopes with grow-

ing mycelia. The resulting suspensions were 1:1000 diluted in sterile 0.85 % saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) by using microdilution method with resazurin (Sarker et al., 2007). The 96-well plates were prepared by dispensing 100 μ l of nutrient broth, Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi, into each well. A 100 μ l from the stock solution of tested extracts (concentration of 40 mg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 20 to 0.156 mg/ml. A 10 μ L of diluted bacterial, yeast suspension and suspension of spores was added to each well to give a final concentration of 5×10^5 CFU/mL for bacteria and 5×10^3 CFU/mL for fungi and yeast. Finally, 10 μ L resazurin solution was added to each well inoculated with bacteria and yeast. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h for bacteria, 28 °C for 48 h for the yeast and 28 °C for 72 h for fungi. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. For filamentous fungi, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth.

Minimum microbicidal concentration (MMC) was determined by plating 10 μ l of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration.

Doxycycline and fluconazole, dissolved in nutrient liquid medium, were used as

positive controls. Stock solutions of crude extracts were obtained by dissolving in DMSO and then diluted into Mueller-Hinton broth to achieve a concentration of 10 % DMSO. Solvent control test was performed to study the effects of 10 % DMSO on the growth of microorganism. It was observed that 10 % DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the two-fold serial dilution assay (the working concentration was 5 % and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Data analysis

All data were presented as means \pm standard deviations (mean \pm SD) where appropriate. For comparison between samples, data was analyzed by the Student's *t*-test and the one-way analysis of variance (ANOVA). In all cases *p* values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS package. Correlation analysis between the DPPH scavenging activity and the total phenol and flavonoid content was performed using Microsoft Excel software.

RESULTS AND DISCUSSION

Total phenol content and flavonoid concentrations

Since there are various possibilities of the extraction of biologically active plant compounds, we used different solvents (ethanol, acetone and ethyl acetate) in this study. Using the method with Folin-Ciocalteu reagent, the concentrations of total phenols were examined. The results are shown in Table 1. The highest concentration was found in ethanol extract of *P. alata* (34.22 mg GA/g). In acetone and ethyl acetate extracts, the measured concentrations were nearly half of the previous.

Using the method with aluminium chloride we obtained the concentrations of flavonoids. The results are shown in Table 1.

The highest concentration of flavonoids had ethanol extract (62.30 mg RU/g).

Table 1: Concentrations of total phenols and flavonoids in the extracts of *P. alata*

Type of extract	Total phenolic content ¹ (mg GA/g of extract)	Flavonoid concentration ¹ (mg RU/g of extract)
Ethanol	34.22 ± 0.39	62.30 ± 2.02
Acetone	14.04 ± 0.49	34.49 ± 0.37
Ethyl acetate	14.53 ± 0.57	33.19 ± 0.21

¹ Each value shown is the mean value ± standard deviation.

Martin et al. (2012) examined the activity of methanol and ethanol extract from the fruit peel of *Passiflora* sp. and determined the concentrations of total phenols. They showed that although the material is a plant waste, it can be used as a source of bioactive compounds. Total phenols of ethanol leaf extracts of *P. alata* (171 ± 1.6 lg/mg) and *P. edulis* (92.5 ± 2.2 lg/mg) were also measured by Rudnicki et al. (2007). The determination of flavonoids from the leaf extracts of *P. alata* was studied by Müller et al. (2005). They proved the presence of C-glycosyl flavonoids isovitexin (0.018 mg/ml) and vitexin in traces, along with other unspecified flavonoids. It should be noted that they observed significant difference between the extracts from leaf harvested in summer and winter, which may have a correlation with the results of this study. The total amount of flavonoids in aqueous-ethanol extract was 2.90 % of dry weight at *P. alata*, in the study conducted by Petry et al. (2001).

Determination of condensed tannins (proanthocyanidins)

Tannins are compounds with antiseptic acting and are an integral part of the extracts of *Passiflora* species (Johnson et al., 2008). Particular emphasis is placed on their antiparasitical and antifungal activity. In determining the amount of tannins, a method with buthanol-HCl reagent was used. Table 2 shows the present of tannin

content in 70 % acetone extract. *P. alata* had high tannin content.

Table 2: The concentration of tannins in the extract of *P. Alata*

Type of extract	The amount of tannin (% dry matter)
70 % acetone	5.1 %

Antioxidant activity

The antioxidant activity of different plant extracts of *P. alata* is determined by the use of the methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. DPPH method is used in the study of antioxidant effects of complex biological compounds and of their ability to reduce the free radicals activity. Activity is measured as a decrease in absorbance of the sample relative to the standard solution of DPPH.

The antioxidant activity of three different extracts of *P. alata* is expressed in the form of IC₅₀ values (µg/ml). In addition, activity was determined for the control substance as a standard parameter in the experiment, also expressed in the form of IC₅₀ values. The results are shown in Table 3. Antioxidative efficiency was in the range from 808.69 to 1107.79 µg/ml. The ethanol extract with the IC₅₀ value of 808.69 µg/ml showed the most active reaction. Antioxidant activity index (AAI) is a number that indicates the success of a compound in the effects of antioxidation. The tested extracts showed weak antioxidant activity (AAI < 0.5), while the control substance, as expected, showed a very strong antioxidant activity (AAI > 2). The results are shown in Table 3.

Table 3: Antioxidant (DPPH scavenging) activity of investigated plant extracts and standard substance presented as IC₅₀ values (µg/ml) and appropriate AAI values

Type of extract	IC ₅₀ value (µg/ml) ¹	AAI value
Ethanol	808.69 ± 2.94	0.10
Acetone	1107.79 ± 20.15	0.07
Ethyl acetate	1107.79 ± 17.26	0.07
Chlorogenic acid	11.65 ± 0.52	6.87

¹ Each value shown is the mean value ± standard deviation.

Comparing the percentage of catching DPPH radicals at a concentration of 1 mg/ml of extract with the obtained levels of total phenols, and then flavonoids, we have shown that the antioxidant activity is directly related to the concentrations of these compounds. The value of R² for the linear dependence of antioxidant activity and phenol content was R²=1 and compared with the flavonoids R²=0.9971.

The literature review which was done by Ingale and Hivrale (2010) presents the results of antioxidant activities of extracts of *P. edulis*: petroleum ether extract of leaf

and stem, and the chloroform extract of leaf and stem (values of IC₅₀: 58.88, 54.01, 56.85 and 51.28 µg/ml). Paper also provides the most proven organic compounds in the genus *Passiflora*.

Antimicrobial activity

The results of *in vitro* antibacterial and antifungal activities of ethanol, acetone and ethyl acetate outgrowth extract of *P. alata* are shown in Table 4 and 5. For comparison, the tables also give the results of the activities of doxycycline and fluconazole. The solvent (10 % DMSO) had no effect on the growth of tested microorganisms. Antimicrobial activities of tested extracts were assessed by determining the MIC and MMC values on 27 species of microorganisms.

The values of minimum inhibitory concentrations and minimum microbicidal concentrations obtained in this experiment range from <0.156 to >20 mg/ml. Efficacy of antimicrobial activity depended on both the type of extract and the species of microorganism.

Table 4: Antibacterial activities of ethanol, acetone and ethyl acetate extract of *Passiflora alata* outgrowth against tested bacteria

Species	Ethanol extract		Acetone extract		Ethyl acetate extract		Doxycycline	
	MIC ¹	MMC ²	MIC	MMC	MIC	MMC	MIC	MMC
<i>Salmonella enterica</i>	>20	>20	>20	>20	5	10	15.625	31.25
<i>Proteus mirabilis</i>	20	>20	20	>20	5	5	250	>250
<i>P. mirabilis</i> ATCC 12453	20	20	>20	>20	2.5	5	15.625	62.5
<i>Salmonella typhimurium</i>	>20	>20	>20	>20	5	10	15.625	125
<i>Escherichia coli</i>	>20	>20	>20	>20	5	10	7.81	15.625
<i>E. coli</i> ATCC 25922	>20	>20	>20	>20	5	5	15.625	31.25
<i>Pseudomonas aeruginosa</i>	20	>20	20	>20	2.5	10	250	>250
<i>P. aeruginosa</i> ATCC 27853	20	>20	20	>20	5	10	62.5	125
<i>Enterococcus faecalis</i>	>20	>20	>20	>20	2.5	5	7.81	62.5
<i>Bacillus subtilis</i>	1.25	2.5	2.5	5	0.625	1.25	0.112	1.953
<i>B. subtilis</i> ATCC 6633	2.5	2.5	2.5	5	1.25	1.25	1.953	31.25
<i>Sarcina lutea</i>	2.5	10	5	20	0.625	1.25	<0.448	3.75
<i>E. faecalis</i> ATCC 29212	<0.156	<0.156	<0.156	<0.156	<0.156	0.156	7.81	62.5
<i>Bacillus cereus</i>	1.25	1.25	2.5	2.5	0.625	0.625	0.977	7.81
<i>Staphylococcus aureus</i>	5	10	10	20	1.25	2.5	0.448	7.81
<i>S. aureus</i> PMFKG-B12	1.25	5	2.5	10	0.3125	0.625	/	/
<i>S. aureus</i> ATCC 25923	2.5	10	5	10	0.625	0.625	0.224	3.75

¹Minimum inhibitory concentration (MIC) and ²minimum microbicidal concentration (MMC) values are given as mg/ml for plant extract and µg/ml for antibiotic (doxycycline); / means not determined

Table 5: Antifungal activities of ethanol, acetone and ethyl acetate extract of *Passiflora alata* out-growth against tested fungi

Species	Ethanol extract		Acetone extract		Ethyl acetate extract		Fluconazole	
	MIC ¹	MMC ²	MIC	MMC	MIC	MMC	MIC	MMC
<i>Candida albicans</i> ATCC 10231	5	10	5	5	10	10	31.25	1000
<i>Candida albicans</i>	10	10	5	10	20	20	62.5	1000
<i>Rhodotorula sp.</i>	5	5	2.5	5	2.5	5	62.5	1000
<i>Penicillium digitatum</i>	10	20	10	20	5	10	31.25	31.25
<i>Penicillium chrysogenum</i>	10	20	10	20	10	10	62.5	500
<i>Penicillium italicum</i>	10	20	10	20	5	10	1000	1000
<i>Aspergillus restrictus</i>	20	20	10	20	5	5	500	2000
<i>Aspergillus flavus</i>	20	20	10	20	5	10	1000	1000
<i>Aspergillus fumigatus</i>	20	20	10	20	5	10	500	1000
<i>Aspergillus niger</i>	>20	>20	20	>20	10	10	500	1000

¹Minimum inhibitory concentration (MIC) and ²minimum microbicidal concentration (MMC) values are given as mg/ml for plant extract and µg/ml for antimycotic (fluconazole)

Statistically the weakest effect on all of the tested microorganisms was shown by acetone extract. It was firstly noticeable that the extracts generally acted weaker on G-bacteria than on the other ($p < 0.05$). On all the tested bacteria, ethyl acetate extract stood out by its influence, where the MIC for the G-species had an average value of 5 mg/ml, the MMC the value of 10 mg/ml, while for G+ bacteria the average value was 1 mg/ml. *E. faecalis* ATCC 29212 is the strain where all extracts acted strongly with values below 0.156 mg/ml. Ethyl acetate extract showed a slightly stronger effect on the isolate *S. aureus* PMFKG-B12 (MIC at 0.3125 mg/ml and MMC at 0.625 mg/ml), and the standard strain of *S. aureus* ATCC 25923 (MIC at 0.625 mg/ml and MMC at 0.625 mg/ml). That is also apparent in other G+ bacteria, where for the ethyl acetate extract MIC ranges from 0.156 to 1.25 mg/ml and MMC from 0.156 to 2.5 mg/ml. The weakest impact is generally allocated at clinical strains of *E. coli* and *S. typhimurium*.

When analyzing the effectiveness of extracts in preventing the growth of fungi, that activity was generally weak. In the tested species, the activity on yeast was more expressed than on the filamentous fungi, where all the extracts had approximately the

same efficacy (MIC from 2.5 to 20 mg/ml and MMC from 5 to 20 mg/ml). Ethyl acetate extract stood out with a stronger effect on all the tested fungi compared to other extracts with the noticeable difference ($p < 0.05$). All extracts showed the strongest impact on preventing the development of *Rhodotorula sp.* (MIC about 2.5 mg/ml, MMC at 5 mg/ml). In filamentous fungi the influence of extracts was slightly stronger on the species of the genus *Penicillium*.

The influence of extracts of outgrowth from *P. alata* on the microorganisms has not been investigated enough, although there are numerous phytochemical and biochemical studies of this plant. The effect of extracts of *P. foetida* on pathogenic bacteria isolated from aquatic organisms was studied by Wei et al. (2008). They came to the conclusion that the methanol and aqueous stem extracts are more efficient than the leaf ones and that there is an influence on *Citrobacter freundii* and various species of the genus *Vibrio*. Johnson et al. (2008) extracted *in vitro* derived callus tissue from leaf of *Passiflora edulis* and also leaves, then they observed inhibition of chloroform and methanol extracts on *Staphylococcus aureus* and *Serratia*, and ethanol extract on *Escherichia coli*. Pure isolated component from the methanol leaf extract of *P. ligu-*

laris had stronger effect on *Staphylococcus aureus* and *Proteus vulgaris* than the extract itself or commercial drug ciprofloxacin by Kannan et al. (2011). Leaf extracts of *P. nitida* and stem extracts of *P. palmeri* in addition to the high antioxidant value and high content of phenolic compounds also showed antimicrobial activity, while leaf extracts of *P. foetida* showed similar antimicrobial activity even though they had weak antioxidant value and low content of phenols (Bendini et al., 2006). In correlation with this, we noticed that the ethyl acetate extract of *P. alata* showed stronger antimicrobial activity than the other, although there were fewer values of phenols and flavonoids than in the extract derived with ethanol.

This study indicates that extracts of *P. alata*, cultured under conditions of moderate continental climate, showed a certain level of antimicrobial activity and may be an additional biologic potential in industrial use, but that additional research is needed, especially on the ethyl acetate extract in order to identify the active component.

CONCLUSION

The results of this research suggest that *Passiflora alata* grown in special living conditions also has certain antimicrobial properties as well as biologically active substances. We showed that there was a way of favoring the production of some compounds (tannins) opposite to the other (phenols), suggesting the possibility of controlled cultivation for specific purposes, like for the needs of phytopharmacy and food industries, beyond the natural propagation of this plant species.

Acknowledgements: This investigation was supported by the Ministry of Education and Science of the Republic of Serbia, grant No. 41010 and 173032.

REFERENCES

- Andrews JM. BSAC standardized disc susceptibility testing method (version 4). J Antimicrob Chemother 2005;56:60-76.
- Bendini A, Cerretani L, Pizzolante L, Toschi TG, Guzzo F, Ceoldo S et al. Phenol content related to antioxidant and antimicrobial activities of *Passiflora* spp. extracts. Eur Food Res Technol 2006;223:102-9.
- De-Paris F, Petry RD, Reginatto FH, Gosmann G, Quevedo J, Salgueiro JB et al. Pharmacological study of aqueous extracts of *Passiflora alata* Dryander and *Passiflora edulis* Sims. Acta Farm Bonaerense 2002;21:5-8.
- Doyama JT, Rodrigues HG, Novelli ELB, Cereda E, Vilegas W. Chemical investigation and effects of the tea of *Passiflora alata* on biochemical parameters in rats. J Ethnopharmacol 2005;96:371-4.
- Giavina-Bianchi PF, Castro FF, Machado ML, Duarte AJ. Occupational respiratory allergic disease induced by *Passiflora alata* and *Rhamnus purshiana*. Ann Allergy Asthma Immunol 1997;79:449-54.
- Ingale AG, Hivrale AU. Pharmacological studies of *Passiflora* sp. and their bioactive compounds. Afr J Plant Sci 2010;4:417-26.
- Johnson M, Maridass M, Irudayaraj V. Preliminary phytochemical and anti-bacterial studies on *Passiflora edulis*. Ethnobotanical Leaflets 2008;12:425-32.
- Kannan S, Parimala Devi B, Jayakar B. Antibacterial activity of *Passiflora ligularis*. Int J Chem Sci 2011;9:393-6.
- Koehler-Santos P, Lorenz-Lemke AP, Muschner VC, Bonatto SL, Salzano FM, Freitas LB. Molecular genetic variation in *Passiflora alata* (*Passifloraceae*), an invasive species in southern Brazil. Biol J Linn Soc 2006;88:611-30.

- Makkar HPS. Quantification of tannins in tree foliage: a laboratory manual for the FAO/IAEA Co-ordinated research project on 'use of nuclear and related techniques to develop simple tannins assay for predicting and improving the safety and efficiency of feeding ruminants on the tanniferous tree foliage'. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria 2000.
- Martin JGP, Porto E, Corrêa CB, de Alencar SM, da Gloria EM, Cabral ISR et al. Antimicrobial potential and chemical composition of agro-industrial wastes. *J Nat Prod* 2012;5:27-36.
- Müller SD, Vasconcelos SB, Coelho M, Biavatti MW. LC and UV determination of flavonoids from *Passiflora alata* medicinal extracts and leaves. *J Pharmaceut Biomed* 2005;37:399-403.
- Pereira CAM, Yariwake JH, Lanças FM, Wauters JN, Tits M, Angenot L. A HPTLC densitometric determination of flavonoids from *Passiflora alata*, *P. edulis*, *P. incarnata* and *P. caerulea* and comparison with HPLC method. *Phytochem Anal* 2004;15:241-8.
- Petry RD, Reginatto F, de-Paris F, Gosmann G, Salgueiro JB, Quevedo J et al. Comparative pharmacological study of hydroethanol extracts of *Passiflora alata* and *Passiflora edulis* leaves. *Phytother Res* 2001;15:162-4.
- Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 1986;25:223-30.
- Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M et al. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *J Ethnopharmacol* 2000;72:35-42.
- Ribeiro SM, Almeida RG, Pereira CAA, Moreira JS, Pinto MFS, Oliveira AC et al. Identification of a *Passiflora alata* Curtis dimeric peptide showing identity with 2S albumins. *Peptides* 2011;32:868-74.
- Ribeiro SFF, Taveira GB, Carvalho AO, Dias GB, Da Cunha M, Santa-Catarina C et al. Antifungal and other biological activities of two 2S albumin-homologous proteins against pathogenic fungi. *Protein J* 2012;31:59-67.
- Rudnicki M, de Oliveira MR, da Veiga Pereira T, Reginatto FH, Dal-Pizzol F, Moreira JCF. Antioxidant and antiglycation properties of *Passiflora alata* and *Passiflora edulis* extracts. *Food Chem* 2007;100:719-24.
- Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods* 2007;42:321-4.
- Scherer R, Godoy HT. Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chem* 2009;112:654-8.
- Takao T, Kitatani F, Watanabe N, Yagi A, Sakata K. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci Biotechnol Biochem* 1994;58:1780-3.
- Wei LS, Musa N, Sengm CT, Wee W, Shazili NAM. Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms. *Afr J Biotechnol* 2008;7:2275-8.

Wootton-Beard PC, Moran A, Ryan L. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin-Ciocalteu methods. Food Res Int 2011;44:217-24.