

## AQUEOUS EXTRACT OF WILD CYCLAMEN TUBERS (*CYCLAMEN PURPURASCENS* L.) - A POTENTIAL SOURCE OF NATURAL ANTIOXIDANTS AND ANTIMICROBIAL AGENTS

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**Abstract:** Wild cyclamen tubers (*Cyclamen purpurascens* Mill.) (mountain Kukavica, Southeast Serbia) were used as material for extraction in this study. Aqueous extract was obtained by reflux extraction on boiling temperature with hydromodulus 1:20 m/v during 180 minutes. The total phenolic content was determined spectrophotometrically by the method of Folin-Ciocalteu, and the total flavonoids content by method with  $AlCl_3$ . The antioxidant activity of extract was investigated spectrophotometrically by DPPH and ABTS test. Disc-diffusion method was used for antimicrobial activity investigation on the following pathogenic microorganisms: *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The content of total phenols was 8.27 mg GAE/g dry extract while the total flavonoid content was 11.51 mg RE/g dry extract. The extract concentrations required to neutralize 50% of the initial concentration of DPPH radicals ( $EC_{50}$ ) after 20 minutes incubation and immediately after adding DPPH radical solution were 0.413 and 2.0 mg/ml, respectively, while concentrations of extract required to neutralize 50% of the initial ABTS radicals concentration is 0.743 mg/ml. The extract showed the highest antimicrobial activity on bacteria *Staphylococcus aureus*. The presented results indicate that cyclamen tubers extract is a potential source of natural antioxidants and antimicrobial agents.

**Key words:** *Cyclamen purpurascens* L., wild cyclamen tubers, antioxidant activity, antimicrobial activity.

### INTRODUCTION

A large number of plant species are source of secondary metabolites with diverse biological and pharmacological properties. Numerous studies have shown that many of their metabolites possess antioxidant and antimicrobial activity and could protect humans from free radicals and pathogens (Sengul et al., 2009). Antioxidant and antimicrobial activity of plant extracts is often caused by the presence of phenolic compounds in medicinal plants so their use as a substitute for synthetic antioxidants is increasing (Moure et al., 2001). For this reason, it is necessary to characterize different types of medicinal herbs in order to use them as antioxidant and antimicrobial agents in food, beverages and medical preparations (Sengul et al., 2009). Addition of natural plant products rich in bioactive compounds in the food products results in exhibition of functional besides nutritional properties (Moure et al., 2001). Therefore, investigations of biological and pharmacological activities as well as chemical characterization of unexplored plant material are of great scientific and practical interest.

Cyclamen (*Cyclamen purpurascens* Mill.; Syn. *Cyclamen europaeum* L.), Alpine, European or purple cyclamen is a species of flowering plant in the genus *Cyclamen* of the family Primulaceae (Speroni et al., 2007). The genus *Cyclamen* comprises about 21 species that grow wildly or cultivated in Europe, Asia, Africa and around the Mediterranean (Metin et al., 2013). There are some investigations of the chemical composition of several *Cyclamen* species that contain some triterpene saponins, glycosides (Calis et al., 1997) and phenolic components (Metin et al., 2013; Sarikurkcü, 2011).

Extracts of *Cyclamen* spp. tubers showed cytotoxic, spermicidal and antimicrobial activities *in vitro* (Speroni et al., 2007). Analgesic, anti-inflammatory, antimicrobial and antioxidative activities of some

*Cyclamen* species such as *C. repandum*, *C. mirabile* and *C. graecum* have been reported as well (Speroni et al., 2007; Calis et al., 1997; Okmen et al., 2014; Metin et al., 2013).

There are no data related to the research on chemical composition as well as antioxidative and antimicrobial activity of aqueous extract obtained from wild cyclamen originated from Southeastern Serbia in the available literature. Therefore, the aim of the present paper was to extract total extractive matter from cyclamen tubers, originated from Southeastern Serbia, with water; to determine total phenols and total flavonoids content in the extract obtained, as well as to determine its antioxidant and antimicrobial activity.

## MATERIALS AND METHODS

### PLANT MATERIAL

Cyclamen tubers (fresh plant material) (*Cyclamen purpurascens* Mill.) (mountain Kukavica, Southeast Serbia) were used for extraction in this study. The plant material was milled before extraction in a laboratory disintegrator (laboratory electric mill "BRAUN AROMATIC KSM2").

### REAGENTS

Ethanol (Centrochem, Stara Pazova, Serbia), Folin-Ciocalteu's reagent, gallic acid, rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, aluminum (III) chloride hexahydrate, potassium acetate, sodium acetate trihydrate, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, butylated hydroxy-toluene (BHT) (Sigma Chemical Company, St. Louis, USA). All other chemicals were analytical-grade (p.a.).

### REFLUX EXTRACTION

Plant material (15 g) was extracted by reflux extraction with 300 ml of water (hydromodule, 1:20 m/v) at its boiling point during 180 minutes. The obtained extract was separated by filtering under a weak vacuum at 40°C. Finally, it was dried in the vacuum dryer at 40°C till constant mass and the content of total extractive matter (TEM, dry extract) was calculated on the basis of dry residue content.

### TOTAL PHENOLICS CONTENT

The total phenolics (TP) content, in the cyclamen tubers aqueous extract, was determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton et al., 1999) by previously described procedure (gallic acid has been used as a standard compound) (Stanojević et al., 2009).

### TOTAL FLAVONOIDS CONTENT

The total flavonoids (TF) content, in the cyclamen tubers aqueous extract, was determined according to aluminium chloride method (Lin and Tang, 2007) by previously described procedure (rutin has been used as a standard compound) (Stanojević et al., 2009).

### ANTIOXIDANT ASSAYS

#### DPPH-test

Ethanol solution of DPPH radicals (1 ml,  $3 \times 10^{-4}$  mol/l) was added in 2.5 ml of extract with different concentrations (0.0195-2.5 mg/ml). The procedure has been done in two probes. Absorbance at 517 nm of the first probe was measured immediately, while absorbance of the second probe was measured after 20 min of incubation at room temperature in the dark. The absorbance of pure ethanolic solution of DPPH

radical, diluted in an adequate proportion (1 ml of DPPH radical,  $3 \times 10^{-4}$  mol/l, diluted with 2.5 ml of ethanol - "control") was also measured, as well as of the extract without DPPH radical added (2.5 ml of extract diluted with 1 ml of ethanol - "blank"). Free radical scavenging capacity is calculated according to Eq. 1 (Stanojević et al., 2015.):

$$\text{The extent of DPPH radicals neutralization (\%)} = 100 - \left[ (A_U - A_B) \times \frac{100}{A_C} \right] \dots \dots \dots (1)$$

where:  $A_u$  is absorbance of the "sample",  $A_B$  is absorbance of the "blank" and  $A_C$  is absorbance of the "control". DPPH test of standard synthetic antioxidant (BHT) has been also performed.

### ABTS-test

The improved ABTS decolorization test, which could be applied to lipophilic as well as hydrophilic components, was used in this study (Re et al., 1999; Arnao, 2000). ABTS radical cation (ABTS<sup>•+</sup>) is formed in the reaction of ABTS ( $7 \cdot 10^{-3}$  mol/l) and 2.4 mM potassium persulfate (1:1 v/v) solutions during 12-18 h, at +4°C, in the dark (stock solution). After radical formation, working solution of ABTS was made by diluting the stock with ethanol until absorbance value of 0.7 at 734 nm was reached. In 0.1 ml of extract (0.0195-2.5 mg/ml) 1.8 ml of ABTS working solution and 2.1 ml of ethanol were added, and the absorbance of thus obtained reaction mixture was measured at 734 nm ( $A_U$ ) after 6 minutes of incubation in the dark at room temperature. The absorbance was determined for the diluted ABTS working solution (in 1.8 ml of working ABTS solution 2.2 ml of ethanol was added,  $A_K$ ) as well as for the extract before the treatment with ABTS radical (in 0.1 ml of extract 3.9 ml of ethanol was added,  $A_B$ ). Ethanol was used as a blank. The percent of ABTS radical neutralization was calculated according to the equation used for the capacity of DPPH radical neutralization (Dudonne et al., 2009). ABTS test of standard synthetic antioxidant (BHT) has been also performed.

All experiments were carried out in three replications and the results represent the mean value  $\pm$  standard deviation. The obtained data were analyzed by Microsoft Excel 2007 and Origin 7 trial.

### ANTIMICROBIAL ACTIVITY

*Microorganisms and substrates.* Ten microorganisms were selected to determine the antimicrobial activity: (Gram-positive bacterial strains) *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 25923), (Gram-negative bacterial strains) *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603), and (fungal strain) *Candida albicans* (ATCC 10259). Microorganisms are from the collection of the Microbiology Laboratory, Faculty of Technology, Leskovac.

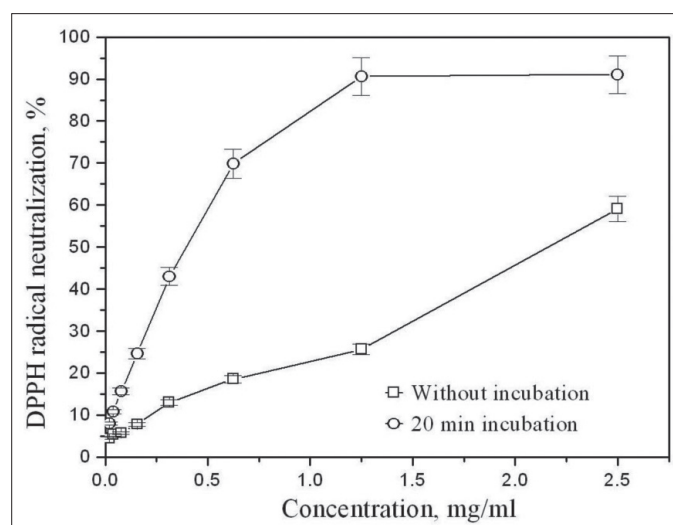
*Disc-diffusion method.* The agar disc-diffusion method was used for testing the antimicrobial activity of extract obtained (Kiehlbauch et al., 2000). The mediums were sterilized for 15 minutes in an autoclave at 121°C under 110 kPa. Bacterial and yeast suspensions were prepared by direct colony method. The colonies were taken directly from the plate and suspended in 5 ml of sterile 0.85% saline. The turbidity of the initial suspension was adjusted by comparing it with 0.5 McFarland's turbidity (Andrews, 2005). After adjusting to the turbidity of the standard, the bacterium suspension contained about  $10^8$  colony forming units (CFU)/ml and the suspension of fungus contained  $10^6$  CFU/ml. Ten-fold dilutions of the initial suspension were additionally prepared into sterile 0.85% saline. Bacterial cell suspensions were inoculated to the trypton soya agar plates (Torlak, Belgrade, Serbia) and the yeast suspension to the Sabouraud maltose agar plates (Torlak, Belgrade, Serbia).

For screening, sterilised filter paper disks (9 mm dia., Schleicher&Schuell) were placed on the surface of inoculated mediums and impregnated with 100 µl of the extract in concentration of 60 mg/ml (dissolved in DMSO). The plates were incubated for 24 hours at 37°C for bacteria, and 48 hours at 25°C for fungi. After incubation, the inhibition zone diameters were measured and expressed in mm. The presence of the inhibition zone indicates the activity of the tested samples against bacteria or fungi. Standardized discs of Ampicillin (10 µg/disc), Cefalexin (30 µg/disc) (Bio Rad), Penicillin 6 µg (10 IU) (BD Sensi-Disc GmbH Heidelberg, Germany) and Nystatin (100 U/disc) (Bioanalyse) were used as reference standards. DMSO was used as negative control.

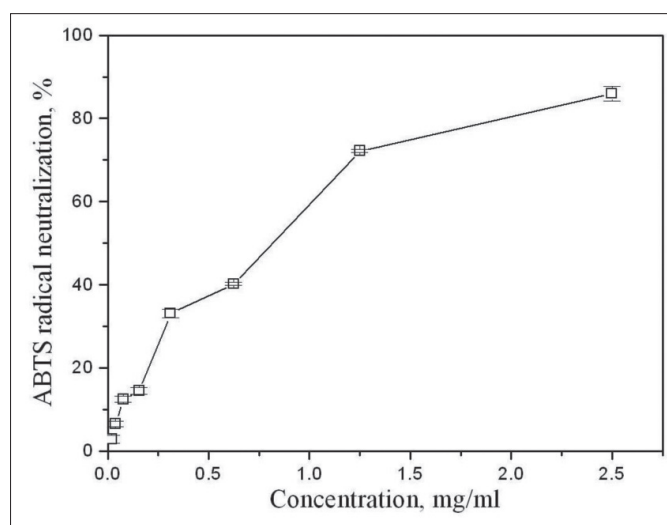
## RESULTS AND DISCUSSION

The yield of TEM obtained by maceration from wild cyclamen tubers was  $10.0 \pm 0.452$  g/100g of plant material.

Cyclamen tubers aqueous extract was subjected to examination of their possible antioxidant activity. Two different systems, DPPH and ABTS test, were used for this purpose. The percentage of DPPH radical and ABTS radical scavenging activity in the function of extract concentration is presented on Figure 1 and Figure 2, respectively, while TP content, TF content and  $EC_{50}$  values, for extract and standard antioxidant, are shown in Table 1.



**Figure 1.** The capacity of DPPH radicals neutralization by wild cyclamen tubers aqueous extracts



**Figure 2.** The capacity of ABTS radicals neutralization by wild cyclamen tubers aqueous extracts

**Table 1.** The yield of TEM, TP content, TF content and  $EC_{50}$  values of wild cyclamen tubers aqueous extract

<b>Total phenolics, mg GAE/g b.m.</b>	$8.27 \pm 0.132$	
<b>Total flavonoids, mg RE/g b.m.</b>	$11.51 \pm 0.254$	
<b><math>EC_{50}</math>, mg/ml DPPH test</b>		
Extract	Without incubation	$2.0 \pm 0.075$
	20 min incubation	$0.413 \pm 0.051$
BHT	20 min incubation	$0.021 \pm 0.001$
<b><math>EC_{50}</math>, mg/ml (ABTS test)</b>		
Extract	$0.743 \pm 0.003$	
BHT	$0.081 \pm 0.001$	

Antioxidant activity determined by DPPH and ABTS test, which is also observed in previous investigations, is probably due to the content of phenolic compounds (Metin et al., 2013; Sarikurkcu, 2011). Many

investigations reported high linear correlation between antioxidant activity of different plant extracts and the content of total phenolics and flavonoids in the extracts (Stanojević et al., 2013; Karabegović et al., 2014).

The aqueous extract obtained from cyclamen tubers showed weaker antioxidant activity in comparison to synthetic antioxidant BHT. Although BHT is one of the most used antioxidants, it exhibits harmful effects on humans (Ito et al., 1986). Therefore, according to the results obtained in this study, the obtained extract could be used as an alternative to BHT with potential application in pharmaceutical, cosmetic and food products.

Antioxidant activity of extracts mostly originates from phenolic compounds, such as phenols and flavonoids, and it is generally ascribed to the presence of hydroxyl groups in their structure (Konczak et al., 2004; Koşar et al., 2004). However, antioxidant activity of cyclamen extract is probably the result of synergistic effect derived from all biomolecules, with or without antioxidant activity, isolated from plant material and present therein. The data about studying the antioxidant activity in such way were not found in the available literature.

Extracts from different parts (leaves and tubers) of *C. graecum* (Sarikurku, 2011), *C. mirabile* (Metin et al., 2013) from Turkey and *C. persicum* from Palestine (Jaradat et al., 2015) have shown antioxidant activity, which is in accordance with our results.

The aqueous extract of cyclamen tubers showed antimicrobial activity on all investigated microorganisms, except on *K. pneumoniae*. The extract showed the highest antimicrobial activity on bacteria *S. aureus* and fungus *C. albicans*. Additionally, the inhibition was stronger comparing with antifungal medication Nystatin – 25 mm vs. 17 mm, respectively (Table 2).

**Table 2.** Antimicrobial activity of wild cyclamen tubers aqueous extract

Microorganism	Inhibition zone, mm				
	Extract	Antibiotic			
		Pen	Amp	Cef	Nys
<i>Candida albicans</i>	25.0	n.t.	n.t.	n.t.	17.0
<i>Bacillus cereus</i>	14.0	13.0	n.t.	n.t.	n.t.
<i>Enterococcus faecalis</i>	13.0	n.t.	n.t.	n.t.	n.t.
<i>Pseudomonas aeruginosa</i>	15.0	15.67	n.t.	n.t.	n.t.
<i>Klebsiela pneumoniae</i>	-	11.67	n.t.	13	n.t.
<i>Staphilococcus aureus</i>	34.0	32.33	36.7	26.0	n.t.

Pen-penicillin; Amp-Ampicilin; Cef-Cefalexin; Nys-Nystatin; n.e.- no effect; n.t. – no treated

*C. albicans* is a fungus that makes normal human microflora in gastrointestinal and genitourinary tract. It is an opportunistic pathogen that attacks patients with weakened immune systems (for example after antibiotic therapy, cancer chemotherapy or HIV infections), so candidiasis are very frequent infections. Commercial antifungal agents used in therapy today, cause harmful effects on human health, and it is necessary to develop new, more effective natural antifungal agents (Molero et al., 1998; Kumar et al., 2012; Berman et al., 2002). Considering that *C. albicans* is very persistent microorganism that causes urinary infection so obtained extract could be used not only in the preventive but also for therapeutic proposes.

One of the most important facts obtained in this study is that the extract examined exhibited stronger effect on *S. aureus* in comparison to widely used antibiotics penicillin and cefalexin (Table 2). *S. aureus* is Gram positive bacteria causing numerous infections and intoxications, from smaller infections of the skin (pimples, ulcers, wound infections, etc.) to severe diseases such as sepsis, inflammation of the lungs, meningitis, endocarditis, etc. (Sing and Prakahsh, 2008). This microorganism is also one of the most frequent mastitis agents in herds of dairy cows, causing severe health and economical problems (Milanov et al.,

2010). Milk contaminated with *S. aureus*, as well as products formed from such milk could cause various infections, both by the bacterium itself and by its enterotoxins (Samaržija et al., 2007). The observed effect of aqueous extract from cyclamen tubers on this bacterium suggests its potential application as a natural antimicrobial agent in milk products. This result is in accordance with studies of Okmen and coworkers who determined the effect of *Cyclamen mirabile* tubers against *S. aureus* and five coagulase-negative staphylococci (Okmen et al., 2014).

The aqueous extract of wild cyclamen tubers showed stronger effect on *B. cereus* in comparison to antibiotic penicillin. *Bacillus* species are most frequently the cause of alimentary toxic infections in humans. These infections are associated with the consumption of various food products rich in starch and proteins such as rice, meat and meat products, deserts and other canned foods. These species are very often present as contaminants of foods of animal and plant origin, because of their ability to survive various physical and chemical conditions by virtue of resistant spores. In addition to alimentary infections, *Bacillus* causes a number of other diseases as well, such as septic meningitis, cellulitis, gangrene as well as numerous eye infections (Kotironta et al., 2000). The result obtained in the present study could be used for making the phytopharmaceutical formulations with an aqueous extract of cyclamen tubers as a natural antimicrobial agent.

The antimicrobial activity of wild cyclamen tubers aqueous extract is probably the consequence of common effects of all components present in the extract. However, it is difficult to compare the data with literature because the variables that influence the results are the extract composition and antimicrobial test method used. The results obtained by different authors are widely different.

## CONCLUSIONS

In conclusion, the results indicate that aqueous extract of cyclamen tubers possess antioxidant activity *in vitro*, determined by DPPH and ABTS tests. Also, it was concluded that extract showed antimicrobial activity on all investigated microorganisms, except on *Klebsiella pneumoniae*. The extract showed the highest antimicrobial activity on bacteria *Staphylococcus aureus* and fungus *Candida albicans*. Presented results showed that the obtained cyclamen tubers extract is a source of natural antioxidants and antimicrobial agents, e.g. natural products with potential application in the food and pharmaceutical industry as safe alternative to synthetic additives. However, the components responsible for antioxidant and antimicrobial activity of the extracts are not identified. Future investigation will be aimed at isolating and identifying the substances responsible for these biological effects.

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

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