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Dedicated to Professor Victor-Emanuel Sahini on the occasion of his 85th anniversary

IN VITRO ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF *TRAPA NATANS* L. AQUATIC PLANT FROM DANUBE DELTA AREA

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The aim of this work was to quantify the total polyphenols and to evaluate the antioxidant and antibacterial activity of ethanolic extracts from the all component parts of water chestnuts – that could serve as agents for the improvement of human health. The total polyphenols of the samples were determined by UV-Vis spectrometry. The antioxidant activity was determined through the antioxidant capacity of liposoluble substance (ACL) method. The analytical method was chosen because it is sensitive, reproducible and rapid. The antioxidant activity for ethanol extracts varies as follows: fruit pericarp > aerial part > aquatic part >> fruit pulp. There was evaluated the antibacterial activity of the ethanolic extracts towards the gram positive *Staphylococcus aureus*, gram negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* bacteria. The results may help in the discovery of new classes of antibiotics and therefore can be successfully used in the pharmaceutical industry.

INTRODUCTION

Besides food, the plants have always been used as remedies in the treatment of certain affections due to their curative properties in maintaining people's health. In the last years, the interest of the scientific world towards the medicinal plants has grown, as a consequence of the pharmacological action of various constituents.[1-4] From the functional point of view, the polyphenolic compounds represent the largest and the most important group in the plants' secondary metabolites.[5] It is well known the role of phenolic compounds as natural antioxidants and free radical scavengers.[1] *Trapa natans* L. (water chestnut) is a sort of plant belonging to the annual submerse aquatic plants, which forms the phytocenoses characteristic of the lakes in the countries with tropical and subtropical climate.[6,7] In Roumania it is seldom met in the Danube Delta area. The therapeutic value of the entire plant and especially of its fruit, is recognized by the folklore medicine in the treatment of various diseases.[8] Fruit are used as nervous tonic,[9] in the treatment of burns, rheumatism,[10] arteriosclerosis,[11] and gums inflammation.[12] Only few data are reported about the antioxidant activity of the extracts obtained from the *Trapa taiwanensis* Nakai species fruit pericarp[13, 14] and the fruit pulp,[12] and from the fruit of *Trapa natans* L. species.[15] Also, several methods for the determination of antioxidant capacity have been reported: oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), trolox equivalent antioxidant capacity (scavenging of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (TEAC),

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ferric-ion reducing antioxidant parameter (FRAP), trolox equivalent antioxidant capacity (scavenging of 2,2-diphenyl-1-picrylhydrazyl radical) (DPPH), cupric reducing antioxidant capacity (CUPRAC), Folin–Ciocalteu reducing capacity (FC assay).[16,17] The chemiluminescence (CL) detection was applied for a fast and sensitive assay of antioxidants in more complex systems.[18,19] Several studies have been performed to demonstrate the antibacterial effect of ethanolic extracts prepared from hulls belonging to *Trapa natans* L. species and from the entire plant belonging to *Trapa bispinosa* species.[20-22]

In order to evaluate the therapeutic properties of *Trapa natans* L. species from the Danube Delta area we report the total polyphenols, the antioxidant activity of different parts of the plant and the evaluation of antibacterial activity of ethanolic extracts against the most spread bacteria: gram positive *Staphylococcus aureus*, gram negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The analytical method proposed in our study presents several advantages: is rapid, sensitive, and reproducible and does not require high temperatures to generate radicals. Given that drug development relies increasingly on isolation of active compounds from natural sources, the water chestnuts studies may represent potential sources for the discovery of new therapeutic agents. In nowadays many synthetic antibiotics became resistant to bacterial pathogens leads. Therefore the need for novel antibacterial therapeutics is a matter of great importance.

EXPERIMENTAL

Reagents

The Folin-Ciocalteu phenol reagent, sodium carbonate, and ethanol were purchased from Merck, Germany. The ACL (Antioxidant Capacity of Liposoluble substance) kit and Trolox ((S)-(2)-6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Analytik Jena AG, Jena, Germany. The bacterial strains studied are *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Proteus vulgaris* ATCC 13315 from Cantacuzino Institute. All microorganisms were maintained at the temperature of 4°C on nutrient agar slants.

Material sampling and preparation

The fresh water chestnuts from *Trapa natans* L. species were collected in August 2009 from the Sinoe Lake, in the Danube Delta, Roumania. The taxonomic identities of these plants were confirmed in the Department of General, Vegetal and Animal Biology, Faculty of Pharmacy, Ovidius University of Constanța, Roumania. The fresh water chestnuts were manually removed and separated into four samples: aquatic part, aerial part, fruit pulp and fruit pericarp. The samples were air-dried, each sample was powdered, stored at the temperature of -15°C and protected from light until further analysis.

Preparation of extracts

10 g of dry samples were extracted with 50% aqueous ethanol (4 x 25 mL) in the dark, at room temperature, for 10 days. Afterwards, each extract was filtered through Whatman no. 4 paper. All samples were re-dissolved in ethanol at a concentration of 10^2 mg/mL and stored at the temperature of -15°C prior to the analysis of polyphenols and antioxidant activity.

Determination of total polyphenol content

The total polyphenols content of the samples was measured spectrophotometrically with Folin-Ciocalteu reagent, according to the method described by Bucur.[27] 1 mL of each sample (10^2 mg/mL) was mixed with 1 mL of diluted Folin-Ciocalteu reagent (diluted 1:1) and double distilled water up to 25 mL solution (solution A). After 5 minute incubation at the room temperature, the sample solutions were prepared by diluting solution A with the solution of 20% sodium carbonate (w/v). The blank solution was prepared in the same conditions with double distilled water, but without analyt. After 40 minutes incubation at the temperature of 22°C, the absorbance was measured and converted to total polyphenols contents according to the calibration curves of gallic acid (GAE) and the results were expressed as gallic acid equivalents in milligrams per gram of extracts (mg GAE/g samples dry plant material). The absorbance was measured at 725 nm. The total polyphenolic content was the average of three replicated measurement.

Chemiluminescent assay

The antioxidant capacity of lipid-soluble substances method was carried out with the procedure described by Popov and Lewin[24,25] by using the standard protocol.[26] The free radicals (superoxide anion radicals) are produced by optical excitation (irradiation) of a photosensitizer substance. The free radicals are visualized with the chemiluminescent detection reagent luminol. These radicals are partially eliminated from the sample by reaction with the antioxidants present in the sample. In the measuring cell the remaining radicals cause the luminescence of the detector substance luminol and thereby the antioxidant capacity of the sample is determined. The extracts of *Trapa natans* L. were measured in the Photochem (Analytik Jena AG, Jena, Germany) system with the kits of ACL where the luminol plays a double role as the photosensitizer and the radical detecting agent.

The lipophilic antioxidants were measured using the ACL kits. In short, 2.3 mL reagent 1 (methanol), 200 µL reagent 2 (buffer solution), 25 µL reagent 3 (photosensitizer), and 10 µL of the sample solution were mixed and measured. In the ACL kit, the antioxidant potential was then determined by means of the area under the curve at different concentrations and expressed as µmol Trolox/g used as a standard for obtaining a calibration curve. Three replicates were made for each test sample.

The antibacterial activity

The antibacterial activity was determined by the disc diffusion method Kirby-Bauer as per National Committee for Clinical Laboratory Standards recommendations.[31-33] The Mueller-Hinton agar plates were inoculated with microbial

suspensions using ATCC strains bacteria. Sterile filter paper discs were impregnated with each extract dilutions (0.15; 0.09; 0.05 and 0.03 µg/ml) and placed on the plates. Paper disc loaded with 100 µg/ disc alchool ethilic 50% served as negative controller. The inhibition area around the discs was measured after standard incubation and compared with positive controls (vancomycin, penicillin G and linezolid for *Staphylococcus aureus*, amoxicillin, gentamicin, imipenem and cefoperazone for *Escherichia coli*, imipenem and cefoperazone for *Proteus vulgaris*, ceftazidime and piperacillin for *Pseudomonas aeruginosa*).

RESULTS AND DISCUSSION

Evaluation of total polyphenols content of *Trapa natans* L. extracts

The total polyphenols were estimated as gallic acid equivalents (GAE) and they are expressed as mg GAE / g of vegetal extract. The total phenol contents of the extract are presented in Table 1. Ethanolic extract of leaves has the highest content of polyphenols, followed by ethanolic extracts of hulls and of roots.

The polyphenols were outlined in all analyzed extracts obtained from the aquatic part, the aerial part, the fruit pulp and fruit pericarp of the genus *Trapa natans* L., the extracts obtained through maceration.

Trolox equivalent antioxidant capacity determination

The antioxidant activity of extracts obtained from the component parts of *Trapa natans* L. species is presented in Table 2. The sample of the antioxidant was added in well before the injection of the chemiluminescent mixture to study the preventive antioxidant effect of extracts.

The highest antioxidant activity was presented in the extracts obtained from the fruit pericarp, followed by those obtained from the aerial and aquatic parts. The extracts obtained from the fruit pulp presented the lowest content of polyphenols and the lowest value of antioxidant capacity. The relationship between the antioxidant activity and the content of total polyphenols does not show a linear dependency of these parameters, which determines us to state that the antioxidant activity depends on the content of total polyphenols, but the antioxidant activity is not influenced by the polyphenolic compounds only. It is about the results regarding the fruit pericarp, where the highest values of the antioxidant activity were obtained, but not the highest values of total polyphenols.

The antibacterial activity

Not all bacterial stalks were resistant to the action of ethanolic extracts obtained from the aquatic and aerial parts, from the fruit pulp and pericarp. The solvent used for the extraction as negative witness (the 50 % ethylic alcohol) did not present a toxic effect on the bacterial stalks. Only the samples obtained from the fruit pericarp presented an antibacterial effect on the *Staphylococcus aureus* bacteria and on the *Escherichia coli* bacteria, but not on the *Proteus vulgaris*. The antibacterial activity is presented in Table 3.

Table 1

Quantification of total polyphenols in *Trapa natans* L. extracts

Studied extract obtained from	$\overline{X} \pm SD$ (mg GAE/g of extract)
aquatic part	2.765 ± 0.077
aerial part	4.902 ± 0.761
fruit pulp	0.499 ± 0.110
fruit pericarp	2.992 ± 0.294

Table 2

The antioxidant activity from the component parts of *Trapa natans* L.

Studied extract obtained from	Inhibition	Antioxidant activity (µM trolox/ g of extract)
aquatic part	0.59 ± 0.01	104.39 ± 0.01
aerial part	0.43 ± 0.05	308.40 ± 0.051
fruit pulp	0.73 ± 0.01	63.75 ± 0.01
fruit pericarp	0.59 ± 0.01	450.40 ± 0.01

Table 3

The antibacterial activity of the extracts obtained from the component parts of *Trapa natans* L. species

Tested samples	Concentration	The diameter of inhibition area [mm]			
		Staphylococcus aureus	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*
aquatic part	0.15 [μg/mL]	-	-	-	-
	0.09 [μg/mL]	-	-	-	-
	0.05 [μg/mL]	-	-	-	-
	0.03 [μg/mL]	-	-	-	-
aerial part	0.15 [μg/mL]	-	-	-	-
	0.09 [μg/mL]	-	-	-	-
	0.05 [μg/mL]	-	-	-	-
	0.03 [μg/mL]	-	-	-	-
fruit pulp	0.15 [μg/mL]	-	-	-	-
	0.09 [μg/mL]	-	-	-	-
	0.05 [μg/mL]	-	-	-	-
	0.03 [μg/mL]	-	-	-	-
fruit pericarp	0.15 [μg/mL]	14.4 ± 0.07	14.2 ± 0.11	-	-
	0.09 [μg/mL]	13.2 ± 0.02	14.0 ± 0.04	-	-
	0.05 [μg/mL]	11.3 ± 0.32	-	-	-
	0.03 [μg/mL]	10.0 ± 0.01	-	-	-
Alchool ethilic 50%	100 μg/disk	-	-	-	-
Vancomycin	30 μg/disk	16.5 ± 0.05	-	-	-
Penicillin G	10 unit/disk	31.2 ± 0.65	-	-	-
Linezolid	30 μg/disk	27.1 ± 0.36	-	-	-
Amoxicillin	25 μg/disk	-	21.1 ± 0.01	-	
Gentamicin	120 μg/disk	-	23.5 ± 0.17	-	-
Imipenem	10 μg/disk	-	27.7 ± 0.02	24.2 ± 0.01	-
Cefoperazone	75 μg/disk	-	22.8 ± 0.01	23.1 ± 0.02	-
Ceftazidime	30 μg/disk	-	-	-	10.1 ± 0.11
Piperacillin	100 μg/disk	-	-	-	26.2 ± 0.02

The responsible for the antibacterial activity of the extracts obtained from the *Trapa natans* L. species are the condensed tannins, quoted within the literature,[7,15,30] which present anti-diarrhoea, anti-mycotic and anti-septic effects.[21] These secondary metabolites usually manifest a specificity of organ or tissue; they can only be activated during the stress periods, caused by the attack of some micro-organisms or by the environment poor in nutrients. The secondary metabolism products have a special significance in the plants' life, significance which derives from the fact that they represent a real chemical barrier against the attack of pathogen agents. The polyphenols are considered to be the major antioxidant compounds in plants,[31] although they are not the only ones. The antioxidant activity of phenolic acids and flavanoids are reported to be mainly due to their redox properties,[32] which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. The data obtained for the evaluation of antibacterial and antioxidant activity and the content of polyphenols, of the ethanolic extracts obtained from the aquatic and aerial parts, from the fruit pulp and fruit pericarp, confirm the data from literature existent until now, and the use of *Trapa natans* L. species in the folklore medicine due to the antioxidant and antibacterial activity.

There is a direct relationship between antioxidant activity and antibacterial effect of the pericarp fruit of water chestnuts. Our results reveal the great potential of plants for the therapeutic treatment and may be associated with antibiotics, to control resistant bacteria, which are becoming a threat to human health.

CONCLUSIONS

We have demonstrated that the aerial part has the highest quantity of polyphenols, followed by the fruit pericarp and the aquatic part. The studies in the domain of antioxidants have intensified in the last years, especially due to the discovery of

beneficial effects of antioxidants to the human health. The researches have shown that the incidence of diseases caused by certain aggressor factors decreases when the consumption of food rich in antioxidants increases. There was determined the antioxidant activity of extracts obtained from the component parts of *Trapa natans* L. specie through the method of chemiluminescence produced through the reaction of radical species with luminol-photochemical excitable compound. The system is extremely efficient from the point of view of its cost. Additionally, it can be used for hydrophilic and lipophilic compounds, at multiple pH and temperature values.

The extracts had a high quantity of polyphenols suggesting that polyphenols may play an important role in extracts antibacterial and antioxidant activity. The antibacterial properties of *Trapa natans* L. do not compare with the usual antibiotics, as in time the microorganisms become resistant to these antibiotics. Recently, the researchers' attention has been directed towards discovering natural sources having antimicrobial and antioxidant effects, beneficial to health. The results of the present study indicate that *Trapa natans* L. extracts can be used as easily accessible source of natural antioxidants and antimicrobials. The studies for developing antimicrobials and antioxidants compounds are beneficial, as they have therapeutic potential and they can serve the purpose without any side effects.

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