

CHEMICAL COMPOSITION, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF *PHYTOLACCA AMERICANA* L. FRUITS AND LEAVES EXTRACTS

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Abstract

The study aimed to investigate the chemical composition, antimicrobial and antioxidant activity of alcoholic extracts obtained from *Phytolacca americana* leaves and berries. The total phenols, flavonoids (spectrophotometric method, HPLC), carotenoids, betalains, saponins, antioxidant activity (ABTS and DPPH methods) and antimicrobial activity of *P. americana* were assayed. The highest content of phenols (323.80 µg/mL extract), flavonoids (9.27 µg/mL extract) and saponins (1.61mg/mL extract) was obtained for *P. americana* leaves extract. The HPLC analysis performed in this study fills an important knowledge gap regarding the profile of *Phytolacca* phenolic compounds. The *P. americana* extracts exhibited a selective antimicrobial effect against Gram positive and Gram negative bacterial strains and yeasts (*Candida* sp). Minimum inhibitory concentrations ranged from 25 - 200 µL/mL, the fruits extracts proving to be the most active, probably due to higher amount of betacyanins (76.11% from the total betalains content).

Rezumat

Studiul a avut ca scop investigarea compoziției chimice, precum și a activității antimicrobiene și antioxidante a extractelor alcoolice obținute din frunze și fructe de *Phytolacca americana*. Au fost determinate concentrațiile de fenoli totali, flavonoide (metoda spectrofotometrică și HPLC), betalaine, carotenoide, saponine, și activitatea antioxidantă (ABTS și DPPH) și anti-microbiană. Cele mai mari concentrații de fenoli, (323,80 µg/mL extract), flavonoide (9,27 µg/mL extract) și saponine (1,61 mg/mL extract) au fost obținute în cazul extractului de frunze de *P. americana*. Analiza HPLC efectuată în acest studiu completează lipsa de date referitoare la profilul compușilor fenolici ai speciei *P. americana*. Extractele de *P. americana* au prezentat un efect antimicrobian selectiv împotriva tulpinilor bacteriene Gram pozitive și Gram negative și levurice (*Candida* sp). Concentrațiile minime inhibitorii au variat între 25 și 200 µL/mL, extractul din fructe dovedindu-se a fi mai activ, probabil datorită conținutului mai mare de betacianine (76,11% din total de betalaine).

Keywords: *Phytolacca americana* L., antioxidant activity, antimicrobial activity, phenols

Introduction

Phytolacca americana L. (pokeweed) is a perennial herb, native from eastern North and South America which has been used in traditional medicine in its native area for antimicrobial, anti-inflammatory, anti-cancer and stimulator effects [20].

The leaves' triterpenoid saponins with irritant and anti-inflammatory activities, flavonoids, a high

percentage of C and B complex vitamins, and a pokeweed-derived N-glycosidase ribosomal-inactivating protein with anti-viral activity have been described [10]. *Phytolacca* extracts, containing esculentosides, display a range of pharmacological activities, including antibacterial, anti-viral, antifungal, anticancer and antiparasitic activities [2].

The phenols from pokeweed were scarcely investigated, despite their potential health-promoting

properties, mainly as antioxidants, anti-inflammatory, anticancer and antimicrobial agents [8]. The increased antibiotic resistance in the recent years has led to the development of new strategies to fight microbial pathogenesis and development. Therefore, intensive research is needed to evaluate the antimicrobial potential of phenols alone or in mixtures with saponins of the *P. americana* fruits and leaves extracts. On the other side, toxic effects, such as vomiting and abdominal pain [5] have been observed after accidental [5] or repeated administration of pokeweed plant roots [11]. The aim of the study consists of the determination of chemical composition, antimicrobial and antioxidant activities of *P. americana* leaves and fruits extracts.

Materials and Methods

Plant material. Plants were harvested at physiological maturity period (July - September 2018), from the Botanical Garden "Dimitrie Brandza", Bucharest, Romania. The working plant materials were represented by *P. americana* leaves and fruits. The plants were manually sorted and dried at room temperature. The species was identified by the Department of Botany from the Faculty of Biology, University of Bucharest, Romania. A voucher specimen was deposited in the herbarium of the Botanical Garden "Dimitrie Brandza" from the University of Bucharest (No. 400712). The loss on drying for plant raw material was 62.33% and 72.73%, and the content of extractives soluble in extractant (ethanol 70%) were 27.14% and 28.76% (on a dry basis) for fruits and leaves, respectively.

Plant extracts. The extraction was performed by using an ultrasonic bath (Elma Sonic 80H), with frequencies ranging from 20 kHz to 2000 kHz, which increases the permeability of cell walls and causes cell lysis, thereby making possible the extraction of biologically active compounds. 2.2880 g of dried leaves were extracted with 30 mL 70% ethanol [17]. The extract resulted was filtered while the residual was extracted three times and brought to 100 mL with the same solvent over the first extract. The procedure was repeated for fruits when a quantity of 1.1310 g was used. The extracts were stored in tightly closed containers at 4°C. The dry residue (in 70% ethanol) was 0.36% and 0.76% for fruits and leaves, respectively.

Determination of total phenols (TPC). The total phenols content (TPC) was assessed out by Folin-Ciocalteu method [21]. The calibration curve with standard solutions of gallic acid concentrations ranging from 5 to 150 mg/L was traced. The TPC was expressed as mg of gallic acid equivalents (GAE)/mL extract.

Determination of total flavonoids (TFC). It was applied the aluminium chloride method [24]. The total flavonoids content was expressed in mg quercetin/g of product and was calculated using the calibration curve obtained for concentrations of quercetin in the range of 0 - 120 mg/mL.

Determination of total carotenoids. The method was based on xanthophyll ester saponification developed by Ciulei and Istudor [6]. The total carotenoid content from leaves and berries extracts was determined at wavelength of 465 nm, using β -carotene (1 - 4 μ g/mL) for the standard curve. The total carotenoid content was expressed based on β -carotene equivalents (β -carotene; mg/g dry weight).

Determination of total betalains. The content of betaxanthins and betacyanins from the obtained extracts was determined using the Shimadzu UV-1800 type Spectrophotometer, at 538 nm and 480 nm respectively according to the methods described by Ravichandran *et al.* and Stintzing *et al.* [19, 22]. The registered absorbance was used to calculate the betalain concentration for each sample. The betalain content (BC) was calculated as:

$$BC(mg/mL) = \frac{A \times DF \times M \times 1000}{\epsilon \times l}$$

where A = absorbance, DF = the dilution factor and l the pathlength of the cuvette (1 cm), M = the molecular weights (550 g/mol for betacyanins and 308 g/mol for betaxanthins), ϵ = molar extinction coefficients (60000 L/mol x cm for betacyanins and 48000 L/mol x cm for betaxanthins).

Determination of total saponins (TSC). It was used vanillin-sulphuric acid assay [12]. Briefly, 0.5 mL of each samples (reagent blank, extracts, or diosgenin) were incubated at 60°C with 0.5 mL 8% (w/v) vanillin in ethanol (96.9%) and 5 mL of 72% (v/v) sulphuric acid in water. The absorbance of the diosgenin and extracts were measured at 544 nm against the reagent blank, using a UV-VIS spectrophotometer (Jasco V-530, Tokyo, Japan), after cooling for 5 min at room temperature. The TSC was expressed in mg of standard equivalents *per* mL of extract (mg diosgenin/mL).

HPLC analysis. All standards (Table II) were purchased from Sigma-Aldrich (Germany). Stock solutions of all the standards were prepared in methanol. Phenolic compounds were evaluated by reversed phase - high performance liquid chromatography (RP-HPLC) with direct injection. Chromatographic analysis was carried out with a Thermo Finnigan Surveyor Plus equipped with a Surveyor Photodiode Array Detector (PDA), Surveyor autosampler, Surveyor LC Pump (Quaternary gradient) and Chrome Quest Chromatography Work-station. The separation was performed using an Accuacore PFP (2.6 μ m, 100 mm \times 2.1 mm) column at 30°C. The flow rate was 0.4 mL/min. The mobile phase was composed of

solvent (A) 0.1% formic acid in water and solvent (B) 0.1% formic acid in acetonitrile using a gradient elution applied as follows: 0 - 30 min from 98 - 70% A, 30 - 35 min from 70 - 25% A, 35 - 40 min from 25 - 98% A, 40 - 50 min, washing and re-equilibration of the column. The injection volume was 1 μ L, while 280 nm was the detection wavelength [13].

TEAC assay (Trolox Equivalent Antioxidant Capacity). The method is based on the ability of antioxidants to quench the long-lived ABTS^{•+}, a blue-green chromophore with characteristic absorption at 734 nm according to Pellegrini *et al.* [16]. Results were expressed relative to Trolox, in mmol of Trolox per mL extract.

DPPH assay. Sample stock solutions were diluted to final concentrations of 30 - 0.3 mg/mL in 70% ethanol. The positive controls were represented by a standard solution (gallic acid). The EC₅₀ values were calculated by linear regression of plots where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of antioxidant activity [7].

Evaluation of the antimicrobial activity of the studied phytochemical mixtures. For testing the antimicrobial activity there were used reference and clinically isolated microbial strains, belonging to Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*) Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*) bacteria and yeasts (*Candida famata*, *C. utilis*, *C. albicans*).

Qualitative screening of the antimicrobial activity. For assessing the antimicrobial activity, microbial suspensions were prepared from 18 - 24 h cultures grown on solid medium and adjusted to 0.5 McFarland standard. The qualitative antimicrobial activity was determined by disc diffusion method. The stock solutions were the alcoholic plant extracts, as well as the chemical compounds identified by HPLC in the studied extracts and also solvent control,

represented by 70% ethanol. The microbial growth inhibition area at around the solution spot was interpreted as a positive result [25].

Quantitative assessment of the minimal inhibitory concentration (MIC). Quantitative analysis was performed by binary serial microdilution method in liquid medium (broth for bacteria and Sabouraud for yeasts) in 96-well plates using the solvent control (70% ethanol). The concentration range of the working stock solutions for alcoholic extracts was from 0.78 to 400 μ L/mL. At the same time, serial dilutions were made with 70% ethanol in the same working conditions in order to obtain the negative control. Each well was inoculated with 10 μ L microbial suspension adjusted to 0.5 McFarland standard from 20 - 24 h cultures grown. MIC was established both macroscopically, as the last concentration at which no microbial growth was observed, and spectrophotometrically by measuring microbial cultures' absorbance was measured at 620 nm using the spectrophotometer Apollo LB 911 [13].

Influence of *P. americana* extracts on inert surface adherence capacity. The influence on the ability of adherence to the inert substrate were measured after the quantitative analysis protocol of the antimicrobial effect, through the microtiter method, evaluating the biomass, after fixation with cold methanol and crystal violet staining (1% concentration for dye). The absorbance of the biological material resuspended with acetic acid (33%) was determined by reading the absorbance at 490 nm.

Results and Discussion

Chemical Composition and Antioxidant Activity

Table I shows the total phenols, flavonoids, betalains, saponins, carotenoids and the antioxidant activity of *P. americana* fruits and leaves extracts. The leaves extract exhibited a higher phenols content compared with fruits [26].

Table I

Total phenols, flavonoids, betalains, saponins, carotenoids and antioxidant activity of <i>P. americana</i> samples		
Parameters	<i>P. americana</i> leaves extract	<i>P. americana</i> berries extract
Total phenols content (μ g GAE/mL extract)	323.80	144.70
Total flavonoids content (μ g QE/mL extract)	9.27	3.47
% flavonoids content from total phenolic content	2.86	2.40
Total betalains content (μ g/L extract)	45.62	113.77
% betacyanins from total betalains content	39.78	76.11
% betaxanthins from total betalains content	60.22	23.89
Total carotenoids content (μ g β -carotene equivalent/mL extract)	3.48	1.07
Total saponins content (mg diosgenin/mL)	1.61	0.59
TEAC assay (mmol Trolox/mL extract)	2.71	1.06
DPPH assay IC ₅₀ (mg/mL)	2.29	18.17

The leaves extract has a higher amount of flavonoids and representing approximately 2.94% of total phenols, results confirmed by the literature [9].

As expected, betacyanins content was higher in the fruits extract, while the leaves extract had a higher content in betaxanthin. The antioxidant activity

(either TEAC or DPPH assay) was superior for the leaves extract and it is correlated with the total content of phenols, flavonoids and carotenoids, in agreement with the literature [26]. Most of the compounds identified through HPLC were from the leaves extract (Table II, Figure 1) accounting about 30.88% of the total phenol content

quantified by the Folin Ciocâlteu method, the major components being chlorogenic acid, 4-hydroxybenzoic acid, followed by rutin and ellagic acid. According to Proestos *et al.* [18], *P. americana* leaves contain caffeic, p-coumaric, vanillic, 4-hidroxi benzoic acids, and rutin. So far, the phenolic profile of *Phytolacca* fruits was a relatively poorly addressed topic.

Table II

HPLC analysis of phenols from extracts of *P. americana* leaves and fruits

Analytical standards	<i>P. americana</i> leaves extract	<i>P. americana</i> berries extract
	µg compound/mL extract	
Gallic acid	n.d.	0.33
3,4-dihydroxybenzoic acid	n.d.	0.13
4-hydroxybenzoic acid	12.63	1.09
(+) – Catechin	n.d.	0.83
Caffeic acid	0.32	n.d.
Chlorogenic acid	45.95	0.10
Syringic acid	0.16	n.d.
(-) – Epicatechin	0.30	0.39
<i>p</i> -Coumaric acid	1.54	n.d.
Ferulic acid	0.98	0.12
Ellagic acid	4.04	0.15
Rutin	6.94	0.22
Resveratrol	0.11	0.69
Quercetin	0.31	0.21

n.d.– not detected

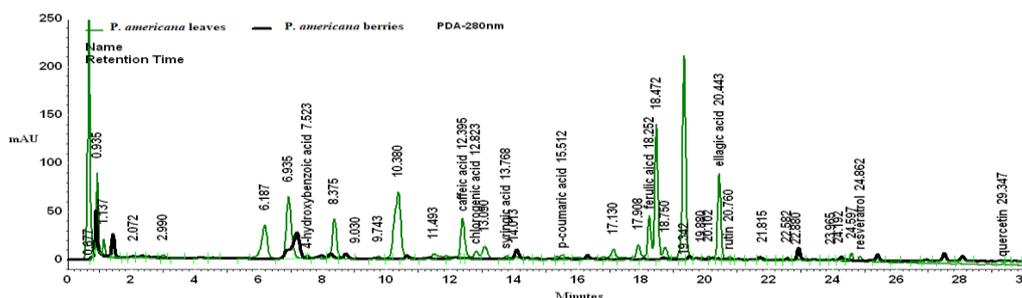


Figure 1.

HPLC chromatograms of *P. americana* leaves (green) and fruits (black) extracts

Antimicrobial Activity

We have included in the study those microbial strains which proved to be sensitive to the extracts studied by the qualitative assay. The MIC assay revealed a broad spectrum of antimicrobial activity against the studied microorganisms. The comparative results of the antimicrobial activity obtained for the vegetative and reproductive organs

extracts and the differences between them and the solvent are shown in Table III.

Figure 2 shows the antibacterial (against *K. pneumoniae* and *E. coli*) and antifungal activity (against *C. famata*) of *P. americana* leaves and fruits extracts as well as of the used solvent (ethanol).

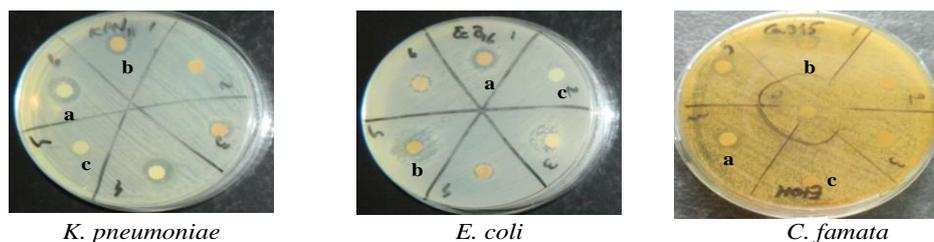


Figure 2.

The occurrence of the antibacterial and antifungal activities of the tested *P. americana* leaves and fruits extracts. a - leaves extracts; b - fruits extracts; c - solvent (ethanol 70%)

Table IIIThe antimicrobial and anti-biofilm activities of *P. americana* leaves and fruits alcoholic extracts vs. solvent control

Microbial strains	<i>P. americana</i> leaves			<i>P. americana</i> fruits			Solvent control (70% ethanol)		
	1	2	3	1	2	3	1	2	3
<i>S. aureus</i> ATCC 6538	-	-	-	++	50	6.25	-	200	100
<i>S. aureus</i> MRSA 1263	-	-	-	++	100	25	-	400	200
<i>B. subtilis</i> 6683	+	100	100	+	50	6.25	-	200	100
<i>B. subtilis</i> 12488	++	25	25	++	200	100	-	400	200
<i>E. coli</i> ATCC 8739	+	100	100	+	100	100	-	200	200
<i>E. coli</i> O ₁₂₆ B ₁₆	+	> 200	200	±	100	50	-	400	200
<i>K. pneumoniae</i> 134202	+	> 200	>200	+	50	25	+	200	100
<i>K. pneumoniae</i> 11	++	200	25	+	> 200	100	+	200	100
<i>P. aeruginosa</i> ATCC 27853	+	50	50	++	100	50	-	200	200
<i>P. aeruginosa</i> 326 sc	+	> 200	100	-	-	-	-	400	400
<i>A. baumannii</i> 77 sc	+	100	50	+	100	12.5	+	200	100
<i>C. utilis</i>	+	50	50	+	50	50	+	200	200
<i>C. famata</i>	+	100	25	+	50	6.25	-	200	100
<i>C. albicans</i> 945	+	> 200	200	+	100	50	+	200	100
<i>C. albicans</i> 393	++	200	50	++	50	6.25	+	200	100
<i>C. albicans</i> ATCC	-	-	-	+	50	12.5	-	400	200

1 - qualitative antimicrobial activity; 2 - MIC; 3 - minimum inhibitory concentration of the microbial ability to adhere to the inert substrate eradication; -: 5 mm; ±: 6 - 8 mm; +: 9 - 13mm; ++: 14 - 20 mm.

The minimum inhibitory concentrations of alcoholic extracts of *P. americana* range from 25 to 200 µL/mL, the fruits extract being more active. The Gram-positive strains proved to be most sensitive to the studied phytochemical mixtures, leading to the conclusion that the outer membrane of Gram-negative bacteria functions as an additional barrier which limits the penetration of the active principles [14] and phenolic acids proved to be more active on the studied microbial strains in comparison with the flavonoids.

The antimicrobial activity of saponins is somewhat controversial. The saponins are detergent-like substances that have antibacterial as well as antifungal potential. However, there are studies that have shown that the saponin from *Quillaja saponaria* bark e.g. (20 - 35% saponin which has quillaic acid, a triterpene of the D¹²-oleanane type, as main aglycone) stimulates the growth of *E. coli* strains due to increased flow of environmental nutrients in cells, at a dose of 12 µg/mL [1]. On the other side, Butassi *et al.* [4] have shown that the dichloromethane extract from berries (PtDEb) showed the best antifungal activity from twelve *Phytolacca tetramera* extracts tested against *C. albicans* and *C. glabrata*. The main active markers were phytolaccagenin and phytolaccoside B, that were also identified in *P. americana*.

The phytochemical mixtures decreased the ability of the tested strains to colonize the inert substratum adherence leading to the inhibition of microbial biofilm development.

The adherence to inert substrate was inhibited for all microbial strains tested at concentrations ranging from 6.25 to 200 µL/mL, the extract

obtained from *P. americana* fruit proving to be the most active on Gram positive strains adherence probably due to the betalains and phenolic acids high content (Table III). The influence of *P. americana* extracts on the microbial adherence capacity is reported for the first time in this study and extends the potential applications of this medicinal herb for the design of novel antimicrobial strategies for topical treatments. In accordance with Borges *et al.* [3], natural phenols present anti-adhesion capacity, thus inhibiting the first step of microbial biofilm formation. Among the tested analytical standards, phenolic acids proved to be the most active. The microbial membrane destabilization and especially the antimicrobial activity of phenolic compounds at relatively low concentrations is most likely caused by a pH decrease, due to hydroxyl groups, thus supporting a possible role for the mutual cation exchange at the level of the cell membrane leading to its destabilization [23]. In addition, the phenolic nucleus could interfere with cellular wall proteins involved in the microbial adhesion [15].

Conclusions

The phytochemical mixture of *P. americana* leaves and fruits proved to be rich in phenols and carotenoids, compounds known for their strong antioxidant capacity. The HPLC analysis performed in our study completes the lack of information about the profile of *Phytolacca* phenolic compounds. The *P. americana* fruits extract proved to be more active compared with the leaves one, in terms of antimicrobial activity, probably due to the content

of betalains, catechins and gallic acid, compounds which are missing in the case of leaves extract. The tested *P. americana* extracts inhibited the adherence of microbial cells and their ability to form biofilms on the inert substratum, these properties being for the first time reported in our paper. Our results reflect the potential of alcoholic extracts to be used as therapeutic agents complementary to antibiotherapy.

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Conflict of interest

The authors declare no conflict of interest.

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