Antioxidant and acetylcholinesterase inhibition properties of *Amorpha fruticosa* L. and *Phytolacca americana* L.

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ABSTRACT

Background: *Amorpha fruticosa* L. and *Phytolacca americana* L. are native plants for North America, but invasive for Central Europe and the Mediterranean areas. Previous investigation reported DPPH radical scavenging activity of *A. fruticosa* seeds from Mississippi river basin and *P. americana* berries from Iran. The aim of the present study was to investigate methanol extracts from leaves and fruits of these invasive species growing in Bulgaria for radical scavenging and acetylcholinesterase inhibitory potential. **Materials and Methods:** Antioxidant activity was investigated using DPPH and ABTS free radicals; FRAP assay and inhibition of lipid peroxidation in linoleic acid system by FTC. Modified Ellman's colorimetric method was carried out to quantify acetylcholinesterase inhibition potential. In addition, the quantities of total polyphenols, flavonoids, and hydroxycinnamic derivatives were determined using Folin-Chiocalteu reagent, $AlCl_3$, and Na_2MoO_4, respectively. **Results:** The highest concentrations of total polyphenols and flavonoids were found in *A. fruticosa* leaves (786.70 ± 1.78 mg/g dry extract and 32.19 ± 0.29 mg/g dry extract, respectively). *A. fruticosa* fruit was found to be the most enriched in total hydroxycinnamic derivatives (153.55 ± 1.11 mg/g dry extract) and demonstrated the highest antioxidant activity: DPPH, IC_{50} 9.83 μg/mL; ABTS, IC_{50} 2.90 μg/mL; FRAP 642.95 ± 3.95 μg TE/mg de, and acetylcholinesterase inhibitory activity, 48.86 ± 0.55% (2 mg/mL). **Conclusions:** *Phytolacca americana* leaves and *Amorpha fruticosa* could be useful in therapy of free radical pathologies and neurodegenerative disorders.

Key words: *Amorpha fruticosa*, antioxidant activity, acetylcholinesterase inhibition, *Phytolacca americana*

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INTRODUCTION

Plants have been used for many years in traditional medicine to treat various diseases and conditions. In the recent decades, there is an increasing interest in finding naturally occurring antioxidants for use in foods, cosmetics, or medicinal materials.[1] The acetylcholinesterase enzyme (AChE) is an attractive target for the rational drug design and for the discovery of mechanism-based inhibitors because of its role in the hydrolysis of the neurotransmitter acetylcholine (ACh). AChE inhibitors are the most effective approach to treat the cognitive symptoms of Alzheimer disease (AD)[2,3] and other possible therapeutic applications in the treatment of Parkinson's disease, senile dementia, and ataxia, among others.[4] Oxidative stress is directly related to neurodegenerative diseases; therefore, the antioxidant potentials of various extracts can be helpful to provide neuroprotection.[5] *Amorpha fruticosa* L. (Indigo bush, Fabaceae) was used in Europe as ornamental, and it became invasive shrub native from North America with great ecologic plasticity being found in different ecological conditions.[6] *Phytolacca americana* (Pokeweed, Phytolaccaceae) is a perennial plant native to North America, but it is invasive alien plant common to the Black Sea and the Mediterranean areas.[7,8] Previous investigation reported DPPH radical scavenging activity of *A. fruticosa* seeds from Mississippi river basin[9] and *P. americana* berries from Iran.[10] In order to discover new natural sources of natural compounds for treatment of neurodegenerative disorders, methanol extracts from *A. fruticosa* and *P. americana* were investigated for antioxidant and actylcholinesterase inhibitor activity.

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MATERIALS AND METHODS

Chemicals and reagents
2,2'-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazine-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$, sodium acetate, potassium persulphate, acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, acetylthiocholine iodide (AChI) were purchased from Sigma-Aldrich. All the others chemicals including the solvents were of analytical grade.

Instrumental
Shimatzu 1203 UV-VIS spectrophotometer (Japan) was used. All determinations were performed in triplicate (n=3).

Plant material
Leaves and fruits of studied species were collected from areas around Sozopol, Bulgaria (*A. fruticosa*) and Botanical garden - Sofia, Bulgaria (*P. americana*) on September 2011. The voucher specimens were confirmed by Assoc. Prof. Ekaterina Kozhuharova and deposited in the Herbarium at the Institute of Botany (SOM), Institute of Biodiversity and Ecosystem Research (IBER), Bulgarian Academy of Science (BAS), Sofia, Bulgaria (№ 168514 - *A. fruticosa*; № 168514 - *P. americana*).

Plant extraction
The plant materials (4 g) were subjected to an ultrasound extraction with 50 mL 80 v/v methanol for 30 min. This was repeated twice with fresh solvent each time, followed by filtration. Filtered extracts were mixed and evaporated under vacuum until dryness.

Determination of total polyphenols content
The determination of total polyphenols was performed according to the European Pharmacopoeia[11] involving Folin-Chiocalteu reagent and pyrogallol as standard. The analyzes were carried out at 760 nm.

Determination of total flavonoids content
The content of the flavonoids was spectrophotometrically determined at 430 nm by creating a complex with $AlCl_3$ according to the European Pharmacopoeia.[12]

Determination of total hydroxycinnamic derivatives
The amount of total phenolic acids was determined following the European Pharmacopoeia method[13] at 505 nm.

Measurement of antioxidant activity

DPPH radical scavenging activity
Free radical scavenging activity was measured by using DPPH method.[14] Different concentrations (1 mL) of dry extracts in MeOH were added to 1 mL methanolic solution of DPPH (2 mg/mL). The absorbance was measured at 517 nm after 30 min. Results were evaluated as percentage scavenging of radical:

$$\text{DPPH radical scavenging activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

where $Abs_{control}$ is the absorbance of DPPH radical in MeOH, Abs_{sample} is the absorbance of DPPH radical solution mixed with sample. IC_{50} value (concentration of sample where absorbance of DPPH decreases 50% with respect to absorbance of blank) of the sample was determined. BHT was used as positive control.

ABTS radical scavenging assay
For ABTS assay, the procedure followed the method of Arnao *et al.* (2001)[15] with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 2 mL ABTS solution with 50 mL methanol to obtain an absorbance of 0.305±0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. Different concentrations (1 mL) of dry extracts were allowed to react with 2 mL of the ABTS solution, and the absorbance was taken at 734 nm after 5 min. The ABTS scavenging capacity of the compound was calculated as

$$\text{ABTS radical scavengin activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

where $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of an ABTS radical solution mixed with sample. IC_{50} value (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) of the sample was determined. BHT was used as positive control.

Ferric reducing/antioxidant power (FRAP)
The FRAP assay was done according to the method described by Benzie and Strain (1996)[16] with some modifications. The stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 ml $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37°C before using 150 μLl of extract in MeOH was allowed to react with 2.8 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were

then taken at 593 nm. Results are expressed in mM Trolox equivalent (TE/g de). BHT was used as positive control.

Determination of antioxidant activity in linoleic acid system by the FTC method

The antioxidant activity of studied extracts against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Takao *et al.*, with some modifications.[17] The reaction solution, containing 0.2 mL of extract (1 mg/mL dry weight in MeOH), 0.2 mL of linoleic acid emulsions (25 mg/mL in 99% ethanol), and 0.4 mL of 50 mM phosphate buffer (pH 7.4), was incubated in the dark at 40°C. A 0.1 mL aliquot of the reaction solution was then added to 3 mL of 70% (v/v) ethanol and 0.2 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.2 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. BHT (1 mg/mL) was used as positive control.

AChE inhibition assay

The enzyme inhibition activities for AChE was evaluated according to the spectrophotometric method previously reported by Ellman *et al.* (1961)[18] with minor modifications. In this method, to a 1 cm path length glass cell, 1500 μL phosphate buffer (pH 8), 200 μL AChE solution (0.3 U/mL), 200 μL test sample (2 mg/mL), and 1000 μL DTNB (3 mM) were mixed and incubated at 37°C for 15 min. Then, 200 μL ATCI (15 mM) were added in the reaction mixture. The samples were incubated for another 10 min at room temperature, and the reactivity was terminated by addition of 200 μL galantamine hydrobromide (1 mg/mL). Absorbance of the produced yellow 5-thio-2-nitrobenzoate anion was measured at a wavelength of 412 nm. A control mixture was performed without addition of extract. Results were expressed as the average of triplicates. The enzyme inhibition (%) was calculated from the rate of absorbance change with time using the following equation:

$$\text{AChE inhibition (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

where $Abs_{control}$ is the absorbance of the control and Abs_{sample} is the absorbance of the sample.

RESULTS AND DISCUSSION

The amount of total polyphenols, measured by Folin-Ciocalteu method, was expressed as pyrogallol equivalent and ranged from 174.76±0.74 mg/g dry extract (de) (in *P. americana* fruit) to 786.70±1.78 mg/g de (in *A. fruticosa* leaves) [Table 1]. The highest level of total polyphenols was found in *A. fruticosa* leaves, followed by *A. fruticosa* fruit. The total flavonoids content in the extracts was expressed as mg hyperoside equivalent and varied from 15.88 ± 0.12 mg/g de (in *P. americana* fruit) to 32.19 ± 0.29 mg/g de (in *A. fruticosa* leaves). The results demonstrated that flavonoids generally were showing higher content in leaves than in fruits. The least quantity of total polyphenols and flavonoids was found in *P. americana* fruit. The content of total hydroxycinnamic derivatives was expressed as rosmarinic acid equivalent and was found in *A. fruticosa* fruits (153.55 ± 1.11 mg/g de), leaves (132.25 ± 5.49 mg/g de) and *P. americana* leaves (23.63 ± 0.39 mg/g de) [Table 1].

Radical scavenging activity, ferric reducing antioxidant power and AChE inhibitory potential of studied invasive plants were compared with those of BHT and are presented in Table 2. Methanol extract from *A. fruticosa* fruit demonstrated the highest antioxidant (DPPH-, ABTS-, FRAP) and acetylcholinesterase inhibitor activity. The DPPH- and ABTS- radical scavenging activity decreased in order: *A. fruticosa* fruit (IC_{50} 9.83 μg/mL and 2.90 μg/mL) > *A. fruticosa* leaves (IC_{50} 11.23 μg/mL and 2.93 μg/mL) > BHT (IC_{50} 64.76 μg/mL and 17.70 μg/mL) > *P. americana* leaves (IC_{50} 88.79 μg/mL and 18.43 μg/mL) > *P. americana* fruit (IC_{50} 412.06 μg/mL and 112.49 μg/mL, respectively). Previous investigation revealed significant DPPH radical scavenging of *Phytolacca* berries growing native in Iran with IC_{50} 62.0 ± 2.1 μg/mL.[4] Differences between quoted and current data are probably due to lower content of flavonoids in our sample and differences in the methods used.

In contrast to *A. fruticosa*, *P. americana* fruit did not manifest any FRAP and acetylcholinesterase inhibition activity. FRAP and acetylcholinesterase inhibition properties was

Table 1: Contents of total polyphenols, flavonoids, and hydroxycinnamic derivatives in *A. fruticosa* and *P. americana*

Sample	Total polyphenols (mg pyrogalol equivalent/g de)	Total flavonoids (mg hyperoside equivalent/g de)	Total hydroxycinnamic derivatives (mg rosmarinic acid equivalent/g de)
A. fruticosa – leaves	786.70 ± 1.78	32.19 ± 0.29	132.25 ± 5.49
A. fruticosa – fruit	782.63 ± 0.69	20.09 ± 0.14	153.55 ± 1.11
P. americana – leaves	215.79 ± 0.54	31.19 ± 1.29	23.63 ± 0.39
P. americana – fruit	174.76 ± 0.74	15.88 ± 0.12	Nd

Nd: Not determined. Results are represented as means ± standard deviation, *n* = 3

Table 2: DPPH, ABTS-radical scavenging, FRAP and AChE inhibitory activities of *A. fruticosa* and *P. americana*

Sample	DPPH IC_{50} (μg/mL)	ABTS IC_{50} (μg/mL)	FRAP (μg TE/mg de)	AchE % (0.17 mg/mL)
A. fruticosa – leaves	11.23	2.93	508.81 ± 1.75	25.43 ± 0.16
A. fruticosa – fruit	9.83	2.90	642.95 ± 3.95	48.86 ± 0.55
P. americana – leaves	88.79	18.43	Nd	Nd
P. americana – fruit	412. 06	112.49	Nd	Nd
BHT	64.76	17.70	30.50 ± 0.24	–

Nd: Not determined. Results are represented as means±standard deviation, *n* = 3

Table 3: Antioxidant activity of *A. fruticosa* and *P. americana* in linoleic acid system

Sample	Absorption at 500 nm				
	1st day	2nd day	3rd day	4th day	5th day
Control	0.81 ± 0.01	1.01 ± 0.09	1.32 ± 0.02	1.88 ± 0.03	3.56 ± 0.15
A. fruticosa leaves	0.81 ± 0.02	0.88 ± 0.04	0.97 ± 0.03	1.09 ± 0.01	1.17 ± 0.02
A. fruticosa fruit	0.82 ± 0.01	0.94 ± 0.02	0.96 ± 0.05	0.97 ± 0.01	1.07 ± 0.02
P. americana leaves	0.90 ± 0.10	0.96 ± 0.02	1.04 ± 0.01	1.11 ± 0.02	1.15 ± 0.01
P. americana fruit	0.82 ± 0.01	0.94 ± 0.01	0.95 ± 0.02	1.03 ± 0.03	1.08 ± 0.01
BHT	0.83 ± 0.01	0.96 ± 0.02	1.05 ± 0.14	1.02 ± 0.01	1.06 ± 0.01

Results are represented as means ± standard deviation, *n* = 3

demonstrated by *A. fruticosa* fruit (642.95 ± 3.95 μg TE/mg de; 48.86 ± 0.55%) and leaves (508.81 ± 1.75; 25.43 ± 0.16). The results obtained revealed the potential importance of the presence of hydroxycinnamic derivatives for the antioxidant and AChE inhibitory properties of plants. FRAP activity of *A. fruticosa* was found to be higher compared to the control BHT (30.50±0.24 μg TE/mg de), nevertheless the species demonstrated lower acetylcholinesterase inhibition activity than the positive control *Galanthamine hydrobromide* with an IC_{50} of 0.15 μg/mL.

In the present study, the inhibition of lipid peroxidation of the extracts (1 mg/mL) was determined in linoleic acid system using the FTC method [Table 3]. During linoleic acid peroxidation, peroxides were formed and these compounds oxidized Fe^{2+} to Fe^{3+}. The Fe^{3+} ion formed a complex with SCN^-, which had a maximum absorbance at 500 nm. Thus, a high absorbance value was an indication of high peroxide formation during the emulsion incubation. The presence of antioxidants in the mixture minimizes the oxidation of linoleic acid and reduces absorption, respectively. The highest significant diminution was demonstrated by *A. fruticosa* fruit followed by *P. americana* fruit. However, the antioxidant activity of studied species was slightly less effective than that of BHT, all of them inhibited lipid peroxidation compared to the control.

Generally, it is known that total polyphenols (a wide class of components including phenolic acids, catechins, flavonols, and anthocyanins) are highly correlated with antioxidant activity.[19,20] The analysis of the correlation between the total phenolic compounds, flavonoids and hydroxycinnamic derivatives content and antioxidant activities showed significant dependence in the case of DPPH, free radical neutralizing properties, FRAP activity, and the total phenolic compounds and hydroxycinnamic derivatives (r = –0.999, r = 0.998; r = –0.990, r = 0.986, respectively). It should be also noted that positive correlations were between inhibition of acetylcholinesterase activity and content of total polyphenols (r = 0.91) and total hydroxycinnamic derivatives (r = 0.946) [Table 4].

Table 4: Correlation between total phenolics, flavonoids, and hydroxycinnamic derivatives content and antioxidant activity of *A. fruticosa* and *P. americana*

Test	Total polyphenols	Total flavonoids	Total hydroxycinnamic derivatives
DPPH	−0.999	0.448	−0.990
ABTS	−0.720	−0.689	−0.767
FRAP	0.998	0.547	0.986
AChE	0.910	−0.079	0.946

CONCLUSION

Methanol extracts from leaves and fruits of two invasive for Europe species were investigated for antioxidant and acetylcholinesterase inhibitory activity. *A. fruticosa* fruit was found to be the most potent and could be useful in therapy of free radical pathologies.

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