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, AlCl₃, and Na₂MoO₄, 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, IC₅₀ 9.83 µg/mL; ABTS, IC₅₀ 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*

**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. 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) and other possible therapeutic applications in the treatment of Parkinson’s disease, senile dementia, and ataxia, among others. Oxidative stress is directly related to neurodegenerative diseases; therefore, the antioxidant potentials of various extracts can be helpful to provide neuroprotection. *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. *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. Previous investigation reported DPPH radical scavenging activity of *A. fruticosa* seeds from Mississippi river basin and *P. americana* berries from Iran. 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 acetylcholinesterase inhibitor activity.
**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), FeCl₃·6H₂O, 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 involving Folin-Ciocalteu 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 AlCl₃ according to the European Pharmacopoeia.

**Determination of total hydroxycinnamic derivatives**

The amount of total phenolic acids was determined following the European Pharmacopoeia method at 505 nm.

**Measurement of antioxidant activity**

**DPPH radical scavenging activity**

Free radical scavenging activity was measured by using DPPH method. 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:

\[
\text{DPPH radical scavenging activity (\%)} = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

where Abs_{control} is the absorbance of DPPH radical in MeOH, Abs_{sample} is the absorbance of DPPH radical solution mixed with sample. IC₅₀ 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) 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:

\[
\text{ABTS radical scavenging activity (\%)} = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

where Abs_{control} is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of an ABTS radical solution mixed with sample. IC₅₀ 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) 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 FeCl₃·6H₂O solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL FeCl₃·6H₂O solution and then warmed at 37°C before using 150 µL 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% (v/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:

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

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 2.03 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.[18] 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 pyrogallol 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
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.

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 Fe²⁺ to Fe³⁺. The Fe³⁺ 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.

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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 activity of A. fruticosa and P. americana showed significant dependence in the case of DPPH, free radical neutralizing properties, FRAP activity, and the total polyphenol contents 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].

**CONCLUSION**

Methanol extracts from leaves and fruits of two invasive 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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REFERENCES

1. Sacan O, Yanardag R. Antioxidant and antiacetylcholinesterase activities of chard (Beta vulgaris L. var. cicla). Food Chem Toxicol 2010;48:1275-80.
2. Kalauni SK, Choudhary MI, Khalid A, Manandhar MD, Shaheen F. Atta-ur-Rahman, et al. New cholinesterase inhibiting steroidal alkaloids from the leaves of Sarcococca coriacea of Nepalese origin. Chem Pharm Bull (Tokyo) 2002;50:1423-6.
3. Atta-Ur-Rahman, Akhtar MN, Choudhary MI, Tsuda Y, Sener B, Khalid A, et al. New steroidal alkaloids from Fritillaria imperialis and their cholinesterase inhibiting activities. Chem Pharm Bull (Tokyo) 2002;50:1013-6.
4. Ahmad VU, Khan A, Farooq U, Kousar F, Khan SS, Nawaz SA, et al. Three new cholinesterase-inhibiting cis-clerodane diterpenoids from Ototestia imbata. Chem Pharm Bull (Tokyo) 2005;53:378-81.
5. Lee SH, Sancheti SA, Bafna MR, Sancheti SS, Seo SY. Acetylcholinesterase inhibitory and antioxidant properties of Rhododendron yedoense var. Poukhanense bark. J Med Plant Res 2011;5:248-54.
6. Sărățeanu V. Assessing the influence of Amorpha fruticosa L. invasive shrub species on some grassland vegetation types from Western Romania. Res J Agr Sci 2010;42:536-40.
7. Weber E, Gut D. Assessing the risk of potentially invasive plant species in central Europe. J Nat Conserv 2004;12:171-9.
8. Aksoy N, Kaplan A, Özkan N, Aslan S. Monitoring invasive alien plants in the Western Black Sea Region of Turkey. In: 2nd International Workshop on Invasive Plants in the Mediterranean Type Regions of the World 2010-08-02/06, Trabzon, Turkey; 2010.
9. Borchardt J, Wyse D, Sheaffer C, Kauppi K, Fulcher R, Ehike N, et al. Antioxidant and antimicrobial activity of seed from plants of the Mississippi river basin. J Med Plants Res 2009;3:707-18.
10. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Bahramian F. In vitro antioxidant activity of Phytolacca americana berries. Pharmacognosy 2009;1:81-6.
11. Determination of tannins in herbal drugs (2.8.14). In: European Pharmacopoeia Council of Europe (COE) - European Directorate for the Quality of Medicines (EDQM). 7th ed. Strasbourg: Council of Europe; 2011; p. 243.
12. Safflower Flower (Carthami flos). In: European Pharmacopoeia Council of Europe (COE) - European Directorate for the Quality of Medicines (EDQM). 7th ed. Strasbourg: Council of Europe; 2011; p. 1229.
13. Rosemary Leaf (Rosmarini folium). In: European Pharmacopoeia Council of Europe (COE) - European Directorate for the Quality of Medicines (EDQM). 7th ed. Strasbourg: Council of Europe; 2011; p. 1227.
14. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958;181:1199-200.
15. Arnao M, Cano A, Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem 2001;73:239-44.
16. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. Anal Biochem 1999;239:70-6.
17. Takao T, Kitafani T, 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.
18. Eillman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
19. Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S-242S.
20. Giovannelli G, Buratti S. Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. Food Chem 2009;112:903-8.