Trop J Nat Prod Res, February 2024; 8(2):6192-6196 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The *In Vitro* Evaluation of Cholinesterase Inhibition and The Antioxidant Effect of *Cupressus arizonica* Greene, *Cupressus lusitanica* Mill. and *Pinus canariensis* C.Sm. Aerial Parts

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ARTICLE INFO

Article history:
Received 30 November 2023
Revised 21 January 2024
Accepted 02 February 2024
Published online 01 March 2024

ABSTRACT

Globally, several medicinal plants have been reported in treating Alzheimer's disease (AD). AD is characterised by decreased acetylcholine-mediated neurotransmission, in which acetylcholinesterase inhibitors have an impact as neuron-protective. The aim is to discover a new therapeutic agent for AD from available natural sources with fewer side effects than other synthetic ones. The current work provides evidence of the preventive and therapeutic properties of three coniferous plants, *Cupressus arizonica* Greene (CA), *Cupressus lusitanica* Mill. (CL) and *Pinus canariensis* C.Sm. (PC). This was achieved by screening their potential in inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Additionally, the antioxidant activity was determined through three different assays; Ferric-reducing antioxidant power**,** radical cation-based 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and oxygen radical absorbance capacity. Phytochemical screening was performed through the determination of their total polyphenolic content (TPC) and total flavonoid content (TFC). The results proved that the best inhibition of AChE was possessed by CL and CA (IC_{50}: 199.7 ± 15.3 and 263.7 ± 17.3 µg/mL, respectively). CA showed a significantly more potent inhibition on BChE (IC_{50}: 74.3 ± 2.1 µg/mL) than CL and PC (136.3 ± 3.8, >500 µg/mL, respectively), in comparison to standard. The extracts showed potent activity in the antioxidant assays (107-1143 µM Trolox eq/mg sample). CA had the highest concentration of TFC, while CL had the highest concentration of TPC. This study revealed significant *in vitro* antioxidant potential, and AChE and BChE inhibitory effects of CA. In conclusion, CA extract could be a promising source of bioactive metabolites for treating neurological diseases.

Keywords: Coniferous, Acetylcholinesterase, Butyrylcholinesterase, Antioxidant

Introduction

Conifers are woody plants that have been used for many decades for their ornamental, economic, and medicinal value. They are characterised by needle-shaped single-veined leaves and unisexual cones with bract scales.[1] Different phytochemical active metabolites have been reported in conifers such as terpenes lignans, alkaloids, polyphenols and phenolic acids.[2,3]

The genus *Cupressus*, family Cupressaceae, is in second place among its family. It is mostly located in the Mediterranean region, the northwest region of Africa, China, and Central and Northern America. The different extracts and essential oils (EOs) of *Cupressus* species have been reported to have anti-bacterial, anti-dermatophytes, antifungal, antioxidant, anti-inflammatory, anti-aging, anti-diabetic, and anti-Alzheimer properties.[4-6]

The genus *Pinus* is the largest one among the Pinaceae family. It is naturally distributed in the northern hemisphere, but people have introduced and grown it all over the world.[7]

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Citation: Kamal RM, Sabry MM, Younis IY, El-Halawany AM, Hifnawy MS. The *In Vitro* Evaluation of Cholinesterase Inhibition and The Antioxidant Effect of *Cupressus arizonica* Greene, *Cupressus lusitanica* Mill. and *Pinus canariensis* C.Sm. Aerial Parts. Trop J Nat Prod Res. 2024; 8(2):6192-6196. http://www.doi.org/10.26538/tjnpr/v8i2.15

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Different organs of Pines and their extracts have been reported to have antimicrobial, antidiabetic, anti-Alzheimer and anti-inflammatory properties.[8,9]

Recently, the EO of *P. pumila* fresh needles has been tested for its acetylcholinesterase (AChE) inhibition effect. It showed a half-inhibitory concentration (IC_{50}) value (0.76 ± 0.04 mg/mL).[10] Furthermore, extracts and oils of different Turkish *Pinus* species have been tested for their antioxidant effect using different methods and they had a potent effect.[11]Different fractions and isolates of *C. macrocarpa* have been evaluated for their AChE inhibition effect with IC_{50} ranging from (88.79-152.58 mg/mL). Additionally, extracts from the needles and shoots of *C. sempervirens* var. *horizantalis* showed the best AChE and BChE inhibitory effect among six other different coniferous plants (IC_{50} 54.84 and 64.29 µg/mL, respectively).[12]

Natural products and their isolated compounds have been investigated for their valuable effect in treating Alzheimer's disease.[13]Alzheimer's disease (AD) is the commonly prevalent type of dementia, accounting for about 60 to 70% of all cases.[14,15] It is marked by memory loss and abnormal behaviour, in addition to thinking difficulties.[16] Even though the disease's pathology is still not completely understood, inhibitors of the cholinesterase enzyme have recently become the most heavily prescribed drug class. This is because a deficiency of the acetylcholine neurotransmitter, either due to deficiency in its production in the brain or impaired activity of acetylcholinesterase, causes the impairment of the cholinergic neurotransmission leading to AD symptoms.[17]

Moreover, there is a strong correlation between oxidative stress caused by neural damage or metal accumulation and the pathogenesis of AD. So, both anticholinesterase and antioxidant capabilities are crucial for a medication suitable for treating AD.[18]

In order to discover a new therapeutic agent for AD from available natural sources with fewer side effects than other synthetic ones,[19] the

current work aims to provide evidence of the preventive and therapeutic properties of three coniferous plants. This has been done through evaluating the cholinesterase inhibitory and antioxidant activity by different methods using the methanol extracts of aerial parts of three coniferous species, *Cupressus arizonica* Greene, *Cupressus lusitanica* Mill. and *Pinus canariensis* C.Sm. The total polyphenolic and flavonoid contents of the three extracts have also been measured.

Materials and Methods

Collection and identification of plant material

Collection of plant material, the aerial parts of *Cupressus arizonica* Greene (CA), *Cupressus lusitanica* Mill. (CL) and *Pinus canariensis* C.Sm. (PC), was conducted in the Orman Botanical Garden (Giza, Greater Cairo, Egypt), in March 2021. Engineer Therese Labib, consultant in the Orman Botanical Garden and National Gene Bank, Ministry of Agriculture, kindly identified them botanically. At the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt, voucher specimens were deposited [Code (3-10-2021III),(3-10-2021II) and (4-10-2021)] for CA, CL, and PC, respectively.

Chemicals, reagents and instruments

The enzymes acetylcholinesterase and butyrylcholinesterase were obtained from Sigma-Aldrich (Electrophorus Electricus. CAT number: 3389, and CAT number: C7512, respectively). The substrates acetylthiocholine iodide and butyrylthiolcholine iodide, together with the indicator 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester), were obtained from the same company. Gallic acid and rutin analytical standards (≥97.0%, ≥94.0% [HPLC], respectively) were obtained from the same company. The reagent Folin-Ciocalteu was obtained from Loba-Chemie (Mumbai, India). All the solvents used were of analytical grade. The microplate reader used was FluoStar Omega from BMG LABTECH.

Extraction and fractionation

The aerial parts of the three plants were air-dried (one kg each), then separately extracted with methanol (5 L) (EL-Nasr Pharmaceutical Chemicals Company [Adwic], Egypt) by cold maceration at 25° C (4 x 6 L). The total methanol extracts were filtered and evaporated under reduced pressure to yield dried residues (475 g for CA, 286 g for CL, and 269 g for PC).

AChE and BChE inhibitory activity assays

The assays were performed following the reported method by Elmann *et al* and Osman *et al*,[20,21] with a few modifications. Briefly, 140 µL of the buffer (Tris–HCl buffer [100 mM and pH 7.5]) was placed in a 96-well plate, then the sample/standard (20 µL) and lastly the enzyme solution [20 µL of (0.02 U/mL)]. After 15 mins of incubation at room temperature, 10 µL of each of the indicator and the substrate (0.4 mM) were added. At room temperature, for 20 mins, the plate was left to incubate in the dark. Finally, at 412 nm, the colour was measured. Standard donepezil HCL served as a positive control.[22,33] IC_{50} values of all the samples were evaluated.

Antioxidant activity assays

Ferric-reducing antioxidant power (FRAP)

The assay was carried out by applying the reported method conducted by Okoli *et al* and Benzie *et al*,[24,25] with some minor changes. Briefly, in a 96-well plate, a freshly prepared sample of 2,4,6-Tripyridyl-s-triazine **(**190 µL**)** was added, then **(**10 µL**)** of the dissolved sample in methanol (2 mg/mL methanol). At room temperature (30 mins), the reaction was incubated. After the completion of the incubation period, at 593 nm the measurement of blue colour was done. Standard Trolox was prepared as a stock solution **(**3 mM in methanol**),** from which several dilutions were prepared (2,000-50 µM).

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation-based assay

The assay was carried out following the Arnao *et al* method,[26] with few changes. Briefly, in a 96-well plate, a sample of ABTS reagent (freshly prepared, 190 µL) was placed, then (10 µL) of the dissolved sample in methanol (10 mg/mL methanol). At room temperature (30 mins), the reaction was incubated in the dark. In the end, at 734 nm, the decrease in the intensity of the reagent was measured. Standard Trolox was prepared as a stock solution (1 mM in methanol), from which the following dilutions were prepared (700-50 µM).

Oxygen radical absorbance capacity (ORAC) assay

The assay was carried out following the reported method by Liang et al,[27] with some minor changes**.** In brief, 10 µL of the dissolved samples in methanol **(**4 mg/mL in methanol**)** and fluoresceine (30 µL, 100 nM) were incubated **(**for 10 mins at 37 °C). Fluorescence measurement was carried out (3 cycles, each of 90 secs). Then**,** to each well a freshly prepared sample of 2,2'-Azobis(2-amidinopropane) dihydrochloride (30 µL, 300 mM) was added Th**e** measurement was continued until reaching 60 mins (40 cycles, each of 90 secs). Standard Trolox was prepared as a stock solution (2 mM in methanol), from which the following dilutions were prepared (1000-50 µM).

Spectrophotometric determination of total phenolic content (TPC) and total flavonoid content (TFC)

Total phenolic content (TPC)

The method was conducted following the reported procedure conducted by Egharevba *et al* and Puspitarini *et al* [28,29] using the Folin-Ciocalteu colorimetric method. Briefly, the total methanol extracts (100 mg) were dissolved, each separately, in a very small quantity of 50% methanol, then transferred to a measuring flask (100 mL), with the volume supplemented by 50% methanol to reach 1 mg/mL. TPC, as gallic acid equivalents (GAE)/ plant dry weight, was expressed, according to a pre-established standard calibration curve.

Total flavonoid content (TFC)

Following the reported procedure conducted by Rahman *et al* and Shraim *et al*, [30,31] TPC was measured. In summary, The total extracts (100 mg) were dissolved, each separately, in a very small quantity of ethanol (95%), then transferred to a measuring flask (100 mL), and the volume was supplemented with ethanol (95%) to reach 1 mg/mL. Next, in a test tube, 1 mL of each methanol extract was transferred and evaporated to dryness, then 0.1M aluminum chloride (5 mL) was added to the residue. Total flavonoids were expressed as rutin equivalents rutin/ plant dry weight, according to a pre-established standard calibration curve.

Statistical analysis

The mean ± standard deviation (SD) was the expression of all the results. Three separate experiments were done (n=3). Statistical analysis was performed using SPSS (Statistical Package for Social Sciences [version 20; IBM]) the statistical analysis was done. Analysis of data was done using ANOVA (a one-way analysis of variance), then followed by Duncan's multiple range tests. "Statistically significant" was considered to P values < 0.05.

Results and Discussion

One of the best known mechanisms for AD is the lack of ACh, a neurotransmitter produced by neurons. AChE is a hydrolase enzyme that breaks down ACh to acetate and choline. Inhibitors of AChE are widely used for the treatment of AD-associated symptoms. They inhibit AChE, protecting cells from injury induced by β-amyloid and restricting the release of cytokines from microglia and monocytes.[32] In the tested bioassay of AChE inhibition, CL was found to be significantly the strongest inhibitor of AChE, with a noteworthy IC_{50} value (199.7 ± 15.3 µg/mL), followed by CA with IC_{50} of (263.7 ± 17.3 µg/mL then PC with IC_{50} > 500 µg/mL); however, these inhibitions were much weaker than donepezil HCL (positive control [IC_{50} = 0.31 ± 0.002 µg/mL]) (Figure 1, Suppl. Table 1).

Butyrylcholinesterase (BChE) is an α-glycoprotein synthesised in the liver. It catalyses the hydrolysis of choline esters.[33,34] Inhibitors of BChE are potentially important candidates for the treatment of AD.[34] Importantly, CA methanol extract showed a significantly more potent inhibition on BChE with IC_{50} (74.3 ± 2.1 µg/mL) than CL and PC (136.3

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

± 3.8, >500 µg/mL, respectively), in comparison to donepezil HCL (0.24 ± 0.002 µg/mL. Interestingly, CA may affect BChE more selectively.

Recent studies have reported that oxidative stress is initiated with the appearance of neurofibrillary tangles, one of the diagnostic pathologies of AD.[35] This inspires us to evaluate the antioxidant effects of the three total extracts through *in vitro* assays, (FRAP, ABTS and ORAC). Their results have been observed to vary according to the method used (Figure 2, Suppl. Table 2). However, the CA extract was the most significantly potent one in the tested assays, except with the ORAC method, where CL was significantly higher.

Additionally, the present study revealed that the methanol extract of CA had a significantly higher TPC (105.41 ± 0.08 µg Rutin/mg Extract) than the methanol extract of CL and PC (33.26 ± 0.01 and 60.35 ± 0.05, respectively). The TFC of CL (75.14 ± 0.05 µg GAE/mg Extract) was higher than CA and PC (37.15 ± 0.01 and 62.83 ± 0.02 µg GAE/mg extract, respectively), while no significant difference between CL and PC was discovered (Figure 3, Suppl. Table 3). These results are comparable with previously reported data for different extracts of the twigs and needles of different Turkish *Pinus* species and *C. sempervirens* var. *horizantalis*.[11,12]

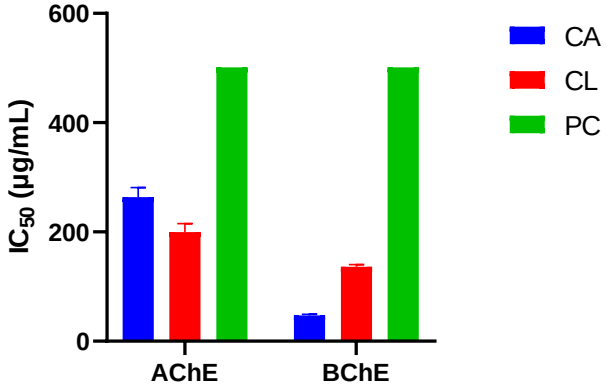

Figure 1: Cholinesterase inhibitory activity of CA, CL and PC extracts. AChE: acetylcholinesterase, BChE: butyrylcholinesterase, CA: *Cupressus arizaonica* Greene, CL: *Cupressus lusitanica* Mill., PC: *Pinus canariensis* C.Sm.

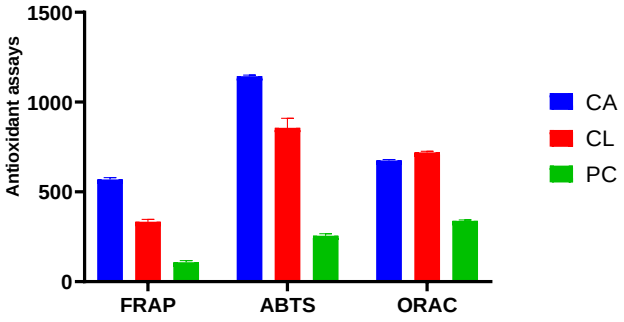

Figure 2: *In vitro* antioxidant activities (FRAP, ABTS and ORAC) of CA, CL and PC extracts. FRAP: Ferric-reducing antioxidant power, ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation-based, ORAC: Oxygen radical absorbance capacity. CA: *Cupressus arizaonica* Greene, CL: *Cupressus lusitanica* Mill., PC: *Pinus canariensis* C.Sm.

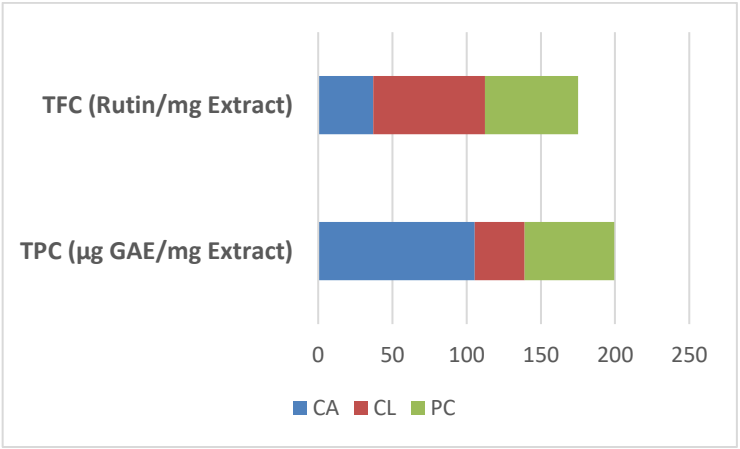

Figure 3: TPC and TFC for the methanol extracts of CA, CL, and CA aerial parts. TPC: Total phenol content, TFC: Total flavonoid content. CA: *Cupressus arizonica* Greene, CL: *Cupressus lusitanica* Mill., PC: *Pinus canariensis* C.Sm.

Suppl. Table 1: Cholinesterase inhibitory activity of the selected plants

Name of plant	Type of extract	[x]AChE (µg/mL)	[y]BChE (µg/mL)
Cupreesus arizonica (CA)	Methanol extract	263.7 ± 17.3[b]	47.3 ± 2.1[b]
Cupressus lusitanica (CL)	Methanol extract	199.7 ± 15.3[c]	136.3 ± 3.8[c]
Pinus canariensis (PC)	Methanol extract	>500[d]	>500[d]
Donepezil HCL		0.31 ± 0.002[a]	0.24 ± 0.002[a]

[x]AChE: acetylcholinesterase, [y]BChE: butyrylcholinesterase, Different letters indicated significant differences at $p < 0.05$.

The results of the bioassays may contribute to the high TPC and TFC of CA (Suppl. Table 3).[36-39] In detail, Romani and colleagues have previously identified bioflavonoids such as cupressuflavone, amentoflavone, robustaflavone, hinokiflavone and methylrobustaflavonee, together with flavonoid glycosides such as quercetin rhamnoside in CA ethanolic extract, using HPLC-diode-array detection.[36] Natchanun and colleagues tested different biflavonoids and found that they could be used as a new type of anti-Alzheimer agent.[39] Furthermore, Sahab Uddin and colleagues have reported that flavonoids represent promising symptomatic anti-Alzheimer agents due to their cholinesterase inhibitory potential.[38]

Moreover, several studies correlate the antioxidant effect of the extracts to their phenolics and flavonoid content. For instance, Sati *et al* [40] showed that the potent antioxidant activity of *Ginkgo biloba* might be due to its quercetin, kaempferol and isorhamnetin glycosides content.

Suppl. Table 2: *In vitro* antioxidant activities (FRAP, ABTS and ORAC)

Name of plant	Type of extract	[x]FRAP (µM Trolox eq/mg sample)	[y]ABTS (µM Trolox eq/mg sample)	[z]ORAC (µM Trolox eq/mg sample)
Cupreesus arizonica (CA)	Methanol extract	568.45 ± 10.9^{b}	1143.38 ± 7.2^{c}	673.98 ± 6.2^{c}
Cupressus lusitanica (CL)	Methanol extract	$333.07 \pm 13.5^{a,b}$	855.79 ± 53.5^{b}	719.52 ± 6.7^{b}
Pinus canariensis (PC)	Methanol extract	107.21 ± 10^{a}	254.79 ± 11.7^{a}	338.08 ± 6^{a}

[x] FRAP: Ferric-reducing antioxidant power, [y]ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation-based, [z] ORAC: Oxygen radical absorbance capacity**.** Different letters indicated significant differences at $p < 0.05$.

Suppl. Table 3: TPC and TFC for the methanol extracts of CA, CL, and CA aerial parts.

Name of plant	Type of extract	[x]TPC (µg GAE/mg Extract)	[y]TFC (Rutin/mg Extract)
Cupreesus arizonica (CA)	Methanol extract	105.41 ± 0.08^{c}	37.15 ± 0.01^{a}
Cupressus lusitanica (CL)	Methanol extract	33.62 ± 0.01^{a}	75.14 ± 0.05^{b}
Pinus canariensis (PC)	Methanol extract	60.35 ± 0.05^{b}	62.83 ± 0.02^{b}

[x]TPC: Total phenol content, [y]TFC: Total flavonoid content. Different letters indicated significant differences at $p < 0.05$.

Conclusion

The study revealed that CA contains a high polyphenolic and flavonoid content and displays promising AChE and BChE inhibition, and antioxidant effects. Interestingly, CA may affect BChE more selectively. We can conclude that CA extract could be a promising candidate for treating neurological diseases. It would be interesting to evaluate the potential of its phytoconstituents in a more in-depth way and evaluate other *Cupressus* species in future studies.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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