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BIOACTIVITY SCREENING OF *ALNUS RUGOSA LEAVES AND ITS PHYTOCHEMICAL ANALYSIS*

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ABSTRACT

Alnus rugosa is widely used as a traditional remedy, however, no investigations have been carried out as yet to determine biological activity. The aim of the present study was to assess antioxidant, antimicrobial properties and acetylcholinesterase inhibitory capacity of methanol 80% extract of *Alnus rugosa* leaves. The antioxidant activity was determined using 2, 2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) ($ABTS^{\bullet+}$) and DPPH radical scavenging assays whereas antimicrobial activity was assessed by the Disc diffusion assay. The method of Eldeen was used to measure acetylcholinesterase inhibition. The methanol extract of *Alnus rugosa* leaves showed lack of antioxidant, antimicrobial and acetylcholinesterase inhibitory effects. Phytochemical analysis of methanol extract from *Alnus rugosa* leaves showed the presence of triterpenes and/or sterols, flavonoids, tannins and carbohydrates. Under the experimental conditions used, antioxidant, antimicrobial activities and acetylcholinesterase inhibitory properties were not observed for *Alnus rugosa* methanol leaf extract. Further investigations using different solvents are suggested.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent form of dementia, arising as a result of malfunctions of different biochemical pathways. Although the pathogenesis of AD has not been fully elucidated, it is believed to be due to a shortage in the amount of the neuromediator, acetylcholine (ACh). Therefore, the inhibition of acetylcholinesterase (AChE), the key enzyme which hydrolyses ACh to choline and acetic acid, is the widely used treatment option for AD [1]. As the reactive oxygen species have been reported to contribute to cellular ageing

and neuronal damage [2], it is more advantageous for an anti-AD drug candidate to possess antioxidant activity as well as its anticholinesterase effect. There has been huge interest in the development of new drugs in the field of AD. To date most of the drugs approved and licensed for the disease have been AChE inhibitors, such as donepezil and galanthamine [3]. These drugs can cause undesirable side-effects and they have been found to be ineffective in treating severe AD cases.

There are various reports which stress the urgency for development of novel drugs using natural substances as a source of bioactive compounds that may serve as the leads or scaffolds for further chemical elaboration [4, 5]. The increasing incidence of microbial diseases and non-infectious diseases in man together with the associated therapeutic difficulties, has led to the search for new drugs. Bacterial and fungal infections are widespread worldwide. High levels of free radicals tend to cause increased cellular damage and are responsible for oxidative stress that may contribute to cardiovascular and inflammatory diseases, auto-immune deficiency and neurodegenerative diseases. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged.

Alnus rugosa L. (Betulaceae) is a deciduous tree which is found (in which area) and grows to a height of 22 m. It flowers in May, and the seeds ripen in October. In traditional medicine, *A. rugosa* is used as an alterative, anodyne, astringent; cathartic, emetic; febrifuge and tonic [6]. Currently, there are no reports of pharmacological activity from extracts or phytochemicals isolated from *Alnus rugosa.* The aim of the present study was to explore the antioxidant, antimicrobial and acetylcholinesterase inhibitory activities of the methanol extracts of the leaves of *Alnus rugosa* to contribute to the knowledge of this plant's biological activity with the hope that it may represent a potential source of natural compounds for development of a drug for treatment of either AD or as an antimicrobial.

MATERIALS AND METHODS

Reagents

Acetylthiocholine iodide, acetylcholinesterase (type VI-S from electric eel), 2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) (ABTS), bovine serum albumin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dithiobis-2-nitrobenzoic acid, galanthamine, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), magnesium chloride, potassium peroxidisulfate and sodium chloride was purchased from Sigma-Aldrich (St. Louis, USA) and methanol from El-Nasr Company, Cairo, Egypt.

Plant collection and crude extraction

Leaves of *A. rugosa* were collected from the Al-Zohiriya Garden, Giza, Egypt in May 2011. Plant identity was confirmed by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereez

Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of the Orman Botanical Garden, Giza, Egypt. A voucher specimen is deposited in the herbarium of Al-Zohiriya Garden, Giza, Egypt.

For the extract preparation, 450 g of air dried powder of *Alnus rugosa* leaves was extracted with 80% methanol through exhaustive maceration at room temperature. The extract was concentrated under reduced pressure to yield 43 g of crude extract.

Phytochemical investigation

The extract was subjected to phytochemical investigation to identify the presence or absence of sterols and/or triterpenes [7], carbohydrates and saponins [8], flavonoids and alkaloids [9], coumarins [10] and tannins [11]. Also paper chromatography of the extract was done in different solvents systems as butanol-acetic acid-water and 15% acetic acid and water.

Antioxidant assays

2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) radical scavenging assay ($ABTS^{\bullet+}$)

The $ABTS^{\bullet+}$ scavenging activity of the crude extracts was determined according to the method of Re et al. [13]. $ABTS^{\bullet+}$ (7.46 mM) was prepared in distilled water and oxidized using 2.5 mM potassium peroxidisulfate at 4°C for 16 h. The oxidized $ABTS^{\bullet+}$ solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm (Lambda UV/VIS Spectrophotometer, Perkin Elmer). Into a 96-well plate was pipetted: 20 l distilled water (negative control), Trolox (6.25, 12.5, 25, 50, 75 and 100 µg/ml; positive control) or crude extracts (half-log dilutions of 1 mg/ml) followed by 180 l $ABTS^{\bullet+}$ solution. Absorbance was measured at 405 nm after 15 min (Synergy 2, Bio-Tek Instruments, Inc.). The percentage inhibition of $ABTS^{\bullet+}$ was determined relative to the negative control.

DPPH scavenging assay

The DPPH• radical is one of the few stable organic nitrogen radicals, commercially available, which bears a deep purple colour. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH. This antioxidant assay is based on measurement of the loss of DPPH color at 517 nm. The DPPH scavenging activity of the crude extracts was determined according to the method of Gyamfi *et al.* [14]. Into a 96-well plate was pipetted: 20 l distilled water (negative control), Trolox (0.0313, 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) or crude extracts (half-log dilutions of 1 mg/ml) followed by 180 l DPPH solution (240 M). Absorbance was measured after 15 min at 570 nm. The percentage inhibition of DPPH was determined relative to the negative control.

Antimicrobial assay

Microorganisms

Candida albicans (ATCC 90028), *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 9027) were purchased from The American Tissue Culture Collection (ATCC). A clinical isolate of each

strain was obtained from the Department of Microbiology (National Health Laboratory Services, Pretoria, South Africa). Strains were maintained on either Mueller Hinton (fungus) or MacConkey agar (bacteria) at 4°C. Inocula were prepared from 24 h subcultures.

Disc diffusion assay

The disc diffusion assay was carried out as described by Bauer et al. [15] was used to identify extracts with antifungal activity. Sterile filter paper discs (Whatman No 1, 10 mm) were impregnated with 200 l or 300 l of the 10 mg/ml crude plant extract and the discs dried to drive off the solvent. Spread plates were prepared by placing 100 l of the appropriate inoculum (5 x 10^5 CFU/ml) on the agar plates. The extract impregnated filter paper discs were placed on the inoculated plates and incubated at 37°C for 24 h. Amphotericin B was included as positive control for *C. albicans* and ampicillin for *S. aureus* and *P. aeruginosa* (Mast Diagnostics). The negative control was prepared by using the solvent. Antimicrobial activity was expressed as the mean diameter of the zone of inhibition (mm) around the disc.

Acetylcholinesterase inhibitory assay

The acetylcholinesterase inhibitory activity was determined according to the method of Eldeen *et al*. [16]. Three (A-C) 50 mM Tris-hydrochloride buffers (pH 8) were prepared, with buffer B containing an additional 0.1% bovine serum albumin, and buffer C containing an additional 0.1 M sodium chloride and 0.02 M magnesium chloride. Samples were prepared by dilution in buffer A. Into a 96-well plate was pipetted: 25 µl acetylthiocholine iodide (in distilled water), 125 µl 5,5-dithiobis-2-nitrobenzoic acid (3 mM in buffer C), 50 µl buffer B and 25 µl buffer A (negative control), galanthamine (half-log dilutions of 320 µg/ml) or crude extracts (half-log dilutions of 1 mg/ml). Absorbance was measured at 405 nm (four times) to account for spontaneous conversion of the substrate. A further 25 µl acetylcholinesterase (0.2 U/ml in buffer A) was pipetted and the absorbance measured at 405 nm every 45 s (fifteen times). Percentage inhibition of acetylcholinesterase was determined through the rate of the reaction (correcting for spontaneous colour changes) relative to the negative control.

Statistics

Results were expressed as the mean ± standard error of the mean (SEM). All experiments were performed in triplicate on three separate occasions. The concentration able to inhibit 50% of activity (IC_{50}) was determined using non-linear regression (variable, normalized slope). All statistical analysis was done using GraphPad Prism 5.0.

RESULTS AND DISCUSSION

The results of the phytochemical investiagtion indicated the presence of flavonoids, tannins, triterpenes and/or sterols, and carbohydrates (Table 1). Previous phytochemical isolations have indicated the presence of quercetin-3-*O*-α-rhamnoside, isorhamnetin 3-*O*-β-glucoside and isorhamnetin 3-*O*-β-glucoside 7-*O*-α-

rhamnoside in the aerial parts of methanol 80% extract of *A. rugosa (*20). Paper chromatography of the extract in the different systems revealed the presence of different spots with different colours where sparyimg with $AlCl_3$ reagent and the appearance of yellow coloured spots in ultraviolet light indicated the presence of flavonoid compounds as free and combined. Also with $FeCl_3$ reagent, some spots gave a green colour indicated the presence of tannins.

The moderate antioxidant activity displayed by *A. rugosa* (Table 2) could possibly be ascribed to the presence of flavonoids, as these compounds are known free radical scavengers. The antioxidant activity was determined using two colorimetric radical scavenging assays, in which the positive control (Trolox) displayed the highest activity in terms of the IC_{50} ($ABTS^{\bullet+}$ = 2.06; DPPH = 2.62) (Table 2). The extract displayed a 9.5-fold greater affinity for the $ABTS^{\bullet+}$ radical (7.99 µg/ml) than that of DPPH (75.93 µg/ml), but did not reach the potency of Trolox ($ABTS^{\bullet+}$ = 0.48-fold; DPPH = 0.10-fold). Literature describing the antioxidant activity of *A. rugosa* could not be found, although *A. rugosa* has been mentioned to present with moderate free radical scavenging activity [19].

Table 1. Phytochemicals detected in the of *A. rugosa* methanol leaf extract.

(a) Chemical Constituents	**(b) 80% methanol extract**
Carbohydrates and/or glycosides	+
Tannins a. Condensed tannins b. Hydrolysable tannins	 + +
Alkaloids and/or nitrogenous bases	-
Flavonoids	+
Sterols and/or triterpenes	+
Saponins	-
Coumarins	-
(+) denotes the presence of the constituents, (-) denotes the absence of the constituents	

Table 2. **Antioxidant and acetylcholinesterase inhibitory activities of *A. rugosa* methanol leaf extract.**

Plant extract	**IC_{50} (g/ml) ± SEM**		
	Antioxidant activity		**Acetylcholinesterase inhibitory activity**
	$ABTS^{\bullet+}$	**DPPH**	
A. rugosa extract	7.99 ± 1.05	75.93 ± 1.03	98.88 ± 1.11
Trolox	2.06 ± 1.03	2.62 ± 1.04	N/A
Galanthamine	N/A	N/A	1.02 ± 1.09

The positive control (galathamine) displayed the highest acetylcholinesterase inhibitory activity with an IC_{50} of 1.02 µg/ml (Table 2). The extract displayed an IC_{50} of 98.88 µg/ml,

with 50.5% inhibition at 100 µg/ml. Thus very little activity can be attributed to the extract, making it a weak acetylcholinesterase inhibitor. Concerning antimicrobial activity, No zone of inhibition was obtained for the ATCC or clinical strain of any of the microorganisms and so no antimicrobiral activity was present against any of the tested organisms (both ATCC and clinical isolates). In previous studies it has been observed that the 80% methanol extract exhibited antimicrobial activity with an MIC≤0.5 mg/ml for numerous bacterial and fungal species, including *P. aeruginosa* and *S. aureus* [20].

CONCLUSION

Literature pertaining to the selected bioactivities of *A. rugosa* are limited, although antioxidant activity was detected, no acetylcholinesterase inhibitory or antimicrobial activity was observed. Further investigations using different solvents is suggested to determine whether or not potential pharmaceutical leads can be isolated from *A. rugosa*

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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