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ARTICLE · MARCH 2015

DOI: 10.1016/j.bfopcu.2015.02.002

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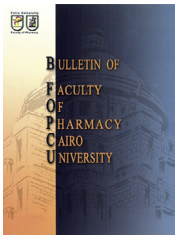

ORIGINAL ARTICLE

Egyptian *Pancratium maritimum* L. flowers as a source of anti-Alzheimer's agents

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Received 18 October 2014; accepted 5 February 2015

KEYWORDS

Pancratium maritimum;
Flower;
Alzheimer;
AChE;
Anti-oxidant;
Cytotoxicity

Abstract Elevation of acetylcholinesterase enzyme (AChE) has been reported to be implicated in the etiology of Alzheimer disease (AD). One of the encouraged strategies to fight AD is the plant-derived inhibitors. Amaryllidaceae species are enriched source of alkaloids. The inhibitory properties of roots and bulbs of *Pancratium maritimum* L. against AChE have been previously reported. In the present study, the flowers of the wild Egyptian *P. maritimum* were subjected to screening assays to evaluate its potency as inhibitor to AChE. Besides, its antioxidant and cytotoxic properties were also addressed. The acetylcholinesterase inhibitory properties of *P. maritimum*; total extract and its alkaloid mixture were examined using Ellman's assay. The direct antioxidant examination was carried out using DPPH assay whereas the indirect was monitored by the ability to protect Hepa1c1c7 cells against the induced cytotoxicity produced by tert-butyl hydroperoxide (TBHP). The cytotoxic effect of the total extract and crude alkaloid mixture was evaluated against the human liver hepatoma cell line (HepG2). *P. maritimum* flowers showed significant inhibitory activity against AChE. The potency of the alkaloid mixture, representing 5.0% of the flowers weight (IC_{50}; 22.02 ± 0.59 μg/ml) was about fourfold of its total extract (IC_{50}; 97.67 ± 4.06 μg/ml). The total extract was able to protect about 33.4% of Hepa1c1c7 against the induced intoxication that carried by TBHP rather than the alkaloid mixture. Weak antioxidant and cytotoxic activities were recorded by both examined samples. Flowers of the Egyptian *P. maritimum* L. could be an enriched source of AChE inhibitors.

1. Introduction

Alzheimer disease (AD) is a neurodegenerative disorder that progressively attacks the cognitive function, mainly of elders and eventually causing death. Medication and extra burden

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Peer review under responsibility of Faculty of Pharmacy, Cairo University.

http://dx.doi.org/10.1016/j.bfopcu.2015.02.002

would be inevitable to take care of such patients particularly when caregivers are not volunteers. Elevation of acetylcholinesterase enzyme (AChE) has been reported as a hall mark in the etiology of the disease encouraging several trials of seeking inhibitors against the accused enzyme.[1]

One of the recommended inhibitors of AChE is the alkaloids-class of phytochemical compounds. Amaryllidaceae species are enriched source of the concerned alkaloids secondary metabolites. *Pancratium maritimum* L. belongs to Amaryllidaceae where the inhibitory properties of its roots and bulbs against AChE were reported and attributed to their contents of alkaloids.[2,3] Galanthamine; the FDA-approved potent medication to reduce the mild and moderate symptoms of AD was reported to present as a predominant alkaloid in *P. maritimum* L. growing in Egypt. The present study aimed to investigate the ability of the wild Egyptian *P. maritimum* flowers to inhibit the AChE. Besides, test of the antioxidant properties, concerning both direct against the free radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the indirect by monitoring its ability to protect Hepa1c1c7 cells against the induced intoxication produced by tert-butyl hydroperoxide (TBHP). Moreover, the cytotoxic effect against human liver hepatoma cell line (HepG2) was also examined.

2. Materials and methods

2.1. Plant material

The fresh flowers, of the wild plant, *P. maritimum*, were collected in August, 2011, from Matrouh Governorate, on the Mediterranean cost, Egypt (Fig. 1). The plant was kindly identified by Dr. M. Gibali, Senior Botanist, Faculty of Sciences, Cairo University. Voucher specimen (BUPD24) is deposited in the Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Egypt.

2.1.1. Preparation of natural samples for the biological study

Fresh flowers of *P. maritimum* (100 g) were exhaustively extracted with 70% EtOH. The combined solvents were evaporated under reduced pressure, to dryness. The obtained extract was weighed and stored in a dark container at −20 °C till biological study. Portion of the extract was taken to prepare the crude alkaloid mixture following the method previously described.[4] The obtained alkaloid mixture was also stored in the dark and at low temperatures till biological study. All chemicals used during the work were of analytical grade.

2.2. Methods

2.2.1. Phytochemical screening of P. maritimum L. flowers

The total 70% ethanol extract (Total extract) of *P. maritimum* flowers was subjected to phytochemical screening for various phytochemicals such as alkaloids, flavonoids, carbohydrates, phenolic compounds, sterols and triterpenoids.[5–8]

2.2.2. Biological evaluation of P. maritimum flowers

2.2.2.1. Acetylcholinesterase inhibition (Ellman's assay). The acetylcholinesterase inhibitory properties of *P. maritimum*, total extract and its alkaloid mixture were examined using Ellman's assay. The assay is based on the reported method by Ali et al.[9] which is modified from the previously published literature.[1,10] Briefly, the total extract and its alkaloid mixture (25 µl of 10 × of final concentrations in DMSO) was dispensed in triplicate onto 96 well microplate and mixed with 200 µl of Ellman's mixture containing 10 mM Tris–HCl, pH 8, 0.1% bovine serum albumin (BSA, fraction V), 1.5 mM acetylthiocholine iodide (ATCI, Sigma–Aldrich, Germany) and 3 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma–Aldrich, Germany). The control wells contained the vehicle (DMSO) where Donepezil hydrochloride (Aricept®, Pfizer) was routinely used as standard inhibitor of AChE. The reaction was started after the addition of enzyme solution (25 µl, 0.1 U/ml). Autohydrolysis of the substrate was corrected by replacing the enzyme with 25 µl of enzyme buffer (10 mM Tris–HCl, pH 8, containing 0.1% BSA). The enzymatic activity was monitored kinetically after 5 min of incubation with the enzyme, every 30 s intervals for 3 min (linear reaction) at 30 °C and wave length 450 nm. The enzyme rate was calculated from the slope of the curve of absorbance change versus time.

Figure 1 *Pancratium maritimum* L. Flowers, The Mediterranean cost, Matrouh Governorate, Egypt.

2.2.2.2. Direct antioxidant assay (radical scavenging). The assay used in the present study was based on a modified procedure[11] which is based essentially on the previously published literature.[12]

Briefly, the total extract and its alkaloid mixture were prepared in DMSO as 10 × stocks from each test concentration. As strategy, radical scavenging activities equal to or higher than 50% at 100 µg/ml in a preliminary screen were further tested and IC_{50} (concentration of the extract/compound producing 50% scavenging of DPPH radicals) determined using nonlinear regression analysis of the dose-% AA relationship Eq. (1).

$$\%\ \text{Antioxidant activity (\%AA)} = 100 \times \frac{[\text{OD540 nm (blank)} - \text{OD540 nm (sample)}]}{\text{OD540 (blank)}} \quad (1)$$

The OD in the equation represents the measured optical density.

Quercetin was tested in the assay as the positive control. The extract and its alkaloid mixture stock solutions (20 µl/well) were dispensed in triplicate onto 96-well plate (flat-bottomed, Greiner bio one, Belgium). The assay was started with the addition of DPPH reagent (0.004% wt/v) in

Please cite this article in press as: Soltan MM et al. Egyptian *Pancratium maritimum* L. flowers as a source of anti-Alzheimer's agents, *Bulletin Facult Pharmacy Cairo Univ* (2015), http://dx.doi.org/10.1016/j.bfopcu.2015.02.002

methanol, 180 µl/well. Appropriate blanks were prepared using the solvent only in addition to the same amount of DPPH reagent to get rid of any inherent solvent activity. Negative controls were also run in parallel to correct for any non-DPPH absorbance by colored extracts at the test wavelength. The plate was immediately shaken for 30 s and incubated in the dark for 30 min. at room temperature. The remaining DPPH was measured in the microplate reader at 540 nm. The percentage of antioxidant activity (%AA) was calculated relative to vehicle control according the the equation.

2.2.2.3. Indirect antioxidant assay in Murine hepatoma cell line Hepa1c1c7). Monolayer culture of Hepa1c1c7 (generously provided by Professor M.S. Denison, University of California, USA) was maintained in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 250 ng/ml Amphotericin B. Cells were grown in 75-cm^2 tissue culture flasks at 37 °C in a 5% CO_2 humidified environment using CO_2 incubator (CERTOMAT® CS-20). Cells were routinely sub-cultured at about 75–85% confluence using Trypsin/EDTA solution (Lonza, Belgium) containing 0.5 g/L Trypsin and 0.2 g/L. (All culture reagents were purchased from Lonza, Belgium).

Hepa1c1c7 cells were seeded onto 24-well culture plates (Greiner Bio-one, Germany) as 0.2×10^4 cells/well. Exponentially growing monolayers (at about 75–85% confluency) were treated with DMSO (vehicle control), total extract or the alkaloid mixture. The preliminary screening was based on dilution of the plant samples stock solution in DMSO (200×) in culture medium to give final concentration of 100 µg/ml of the extract and 10 µg/ml of the fraction. Quercetin as standard antioxidant was examined in parallel at 4 µg/ml. Treated plates were incubated for 24 h prior to the exposure to 125 µM of TBHP as induced dose for oxidative cytotoxicity. The Neutral Red Uptake (NRU) Assay[13] was performed as the endpoint for cell viability measurement to monitor the cytoprotection produced by tested samples.

2.2.2.4. Cytotoxicity assay against Human hepatocellular carcinoma cell line (HepG2). HepG2, (ATCC®) cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM, Lonza, Belgium) supplemented with 10% FBS, 4 mM L-glutamine, 100 U penicillin and 100 µg/ml streptomycin sulfate). Cells were incubated and routinely sub-cultured as carried with Hepa1c1c7 cultures.

HepG2 cells were seeded onto 24-well culture plates (Greiner Bio-one, Germany) as 0.15×10^4 cells/well. Exponentially growing mono layers were treated separately with the total extract and alkaloid mixture as performed with the indirect antioxidant assay. A reference cytotoxic compound; Adriamycin-Doxorubicin (Pfizer) was used as a positive control for cytotoxicity. Treated plates were incubated for 48 h prior to evaluation of the produced cytotoxicity using NRU. At the end of the incubation time, the culture medium was aspirated from the wells and replaced with 0.4 ml Neutral red (Sigma, cat. No. N4638) solution (40 µg/ml). The plates were then incubated for 2 h. Every well was then washed with Dulbecco's phosphate buffered saline (DPBS). Fixative solution (1% (v/v) glacial acetic acid in 50% ethanol) was then added to every well. The plates were shaken for 10 min. to extract the cell-retained dye. Aliquots of the Desorbed dye solution (100 µl) were then transferred onto wells of 96-well plates. Triplicate absorbances were then read on Fluostar Optima microplate reader (BMG LABTECH, Germany) at 540 nm. Cell free blank was used for background correction of the absorbance. The viability was expressed as a percentage relative to the solvent control absorbance value after subtracting the contribution from cell-free blank.

2.2.3. Statistical analysis

Regression analysis was used to determine IC_{50} values using the concentration-activity relationship. All data were represented as the mean value of triplicate absorbance measurements.

3. Results and discussion

3.1. Yield and phytochemical screening

The total extract yielded 7.5 g obtained from 100 g fresh flowers. The prepared crude alkaloid mixture was about 67% of the total extract (two thirds) and 5% of the flowers fresh weight. The phytochemical screening of the total extract revealed the presence of alkaloids and flavonoids as major components, phenolic compounds, carbohydrates and/or glycosides and traces of sterols and/or triterpenes.

3.2. Biological screening

3.2.1. Anti-cholinesterase activity using Ellman's assay

The examined samples; total extract and crude alkaloid mixture, of *P. maritimum* flowers produced 100% inhibition of AChE in a preliminary experiment at 1000 µg/ml (single concentration). Both samples were subjected to further investigation in order to determine their IC_{50} against AChE. The results are represented in Table 1. The IC_{50} value of the alkaloid mixture was reduced about 4.4-fold (22.02 ± 0.59 µg/ml) than the IC_{50} of the total extract (97.67 ± 4.06 µg/ml). Different types of alkaloids were identified in the *P. maritimum* growing in Egypt such as Tazettine, galanthamine and its derivatives, Lycorine, Crinine and its derivatives. Some of them could contribute to the potent inhibition effect of the extract; lycorine, maritidine, lycoramine, and galanthamine[4,14] that could be synergistically interacting to inhibit the accused enzyme, AChE.[3] Galanthamine is the potent agent of Razadyne®. It was approved by FDA[15] to treat mild to moderate symptoms concomitant to Alzheimer's disease. Flowers of *P. maritimum* growing in Egypt contain Galanthamine and N-Demethygalanthamine representing about 33.7% of the alkaloid mixture[4] equivalent to 1.65% of the plant flowers weight, so flowers could be a good source for preparing Galanthamine. The results of AChE inhibitions in the present

Table 1 IC_{50} determination of the total extract and alkaloid mixture of *P. maritimum* flowers as inhibitors to AChE.[a]

Examined sample	IC_{50} of inhibiting AChE (m ± SEM)[b]
Total 70% EtOH extract	97.67 ± 4.06 µg/ml
Crude alkaloid mixture	22.02 ± 0.59 µg/ml
Aricept®	1.14 ± 0.05 µM

[a] The results represent the average of three replicates obtained from three independent experiments.
[b] AChE: acetylcholinesterase enzyme; m: Mean; SEM: standard error of mean.

Table 2 Antioxidant activity and cytotoxicity results of *P. maritimum* flowers.[a]

Examined sample	Anti-oxidant properties		Cytotoxicity properties
	% DPPH scavenging	% Protection of Hepa1c1c7	% Toxicity of HepG2
Total 70% EtOH extract	12.7 (100 μg/ml)	33.4 (100 μg/ml)	52.5 (200 μg/ml)
Crude alkaloid mixture	14.1 (100 μg/ml)	1.4 (5 μg/ml)	29.2 (5 μg/ml)
Quercetin	95.6 (10 μg/ml)	77.2 (4 μg/ml)	–
Doxorubicin	–	–	80.0 (5 μg/ml)

[a] The % protection was calculated by subtracting the % viability recorded by the treated Hepa1c1c7 monolayers with 125 μM TBHP (3.5%) from the % viability recorded from treated monolayers with the total extract, alkaloid mixture or quercetin.

study support, in part the traditional uses of Amaryllidaceae species where some of them are claimed to cure myasthenia, gravis, myopathy and diseases of the nervous system.[3]

3.2.2. Direct and indirect anti-oxidant activity

P. maritimum flowers could not be a promising source of anti-oxidants where ⩽15% radical scavenging was recorded by both examined samples at single concentration (100 μg/ml). However, the total extract was able to protect about 33.4% of Hepa1c1c7 against the induced intoxication that was produced by TBHP (Table 2).

The implication of the oxidative stress in AD pathogenesis was reported.[16] A study that investigated a group of plant species attributed their traditional uses in the remedy of AD symptoms to different properties including antioxidant or AChE inhibitions.[9] Our results emphasize, the ability of the *P. maritimum* against AD toward the inhibition of the AChE rather than the antioxidation activity. However, this protection power of the total extract could be attributed to its contents of flavonoids and/or phenolic compounds.

3.2.3. Cytotoxicity against HepG2

Both of the total extract and alkaloid mixture showed weak cytotoxicity (52.5% at and 29.2%) against HepG2 at single screening concentration; 200 μg/ml and 5 μg/ml, respectively (Table 2). However, we could attribute, in part the produced cytotoxicity to alkaloids such as pancratistatin and/or 3-caffeoylquinic acid methyl ester. Both alkaloids were previously isolated from the flowers[17] and showed a potent cytotoxic effect against Hela cells. However, the cytotoxic results of *P. maritimum* could be considered in future risk assessment of monitoring the concerned plant against AD.

4. Conclusion

Flowers of the Egyptian *P. maritimum* could be an enriched source of AChE inhibitors. To the best of our knowledge, this is the first report that investigated the antioxidant ability of the wild *P. maritimum* L. flowers to protect the induced Hepa1c1c7 cells.

Conflict of interest and author agreement

The authors declare that they have no competing interests as well they certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Acknowledgments

The biological study was supported by the National Research Centre, Egypt.

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