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Original Article

Evaluation of Two Culinary Plant Species for Anticholinesterase, Antioxidant and Cytotoxic Activity

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a neurodegenerative disorder that markedly affects the cognitive functions. Although drugs are available for symptomatic relief, complete cure is yet to be achieved. Herbals are being explored for their anti-Alzheimer potential and some are excellent obstructers for pathological hallmarks of the disease. In this context we have investigated anticholinesterase, antioxidant and cytotoxic potential of the two culinary plant species *Allium cepa* and *Ocimum basilicum*. **Materials and Methods**: Hydro-methanol extracts of both plants were prepared by maceration. The extracts were standardized with respect to phenolic and flavonoid content. DPPH radical scavenging and ferric reducing assays were employed for antioxidant evaluation. Anticholinesterase prospective was evaluated using Ellman method. The cytotoxic effect of extracts on C6 glioma cells was examined by MTT assay. **Results**: Both the plant extracts displayed significant antioxidant activity in DPPH assay (IC_{50} value of *A. cepa* 30.10±6.96 μg/ml and *O. basilicum* 38.67±1.72μg/ml). Reducing ability was comparatively high for *A. cepa*. In the Ellman assay, *A. cepa* was determined to be a good acetylcholinesterase inhibitor (IC_{50} value: 51.78±1.05 μg/ml). Both the extracts were found to be non-toxic in MTT assay below the concentration of 100 μg/ml. The *A. cepa* extract had higher phenolic and flavonoid content.
Conclusion: *A. cepa* has strong antioxidant and acetylcholinesterase inhibitory effect and can be considered an excellent candidate for developing as a drug for management of AD.
Key words: *Allium cepa, Ocimum basilicum,* Anti-Alzheimer.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with the loss of cognitive functions. It is pathologically characterized by accumulation of extracellular beta amyloid plaques and intracellular neurofibrillary tangles of tau protein.[1] Deterioration of cholinergic neurons in the basal forebrain and subsequent loss of cholinergic neurotransmission in the cerebral cortex are major neurochemical abnormalities linked with AD.[2] Inhibition of the acetylcholinesterase enzyme in order to uphold the levels of acetylcholine is the 'only' approved therapy for treatment of AD.[3] However, there are several side effects associated with these drugs and they have only been able to provide symptomatic relief.[4] Plants are a rich source of numerous phytoconstituents and hence are constantly being explored for anti-Alzheimer potential.[5] In an attempt to search plants with potential for management of AD, the *in vitro* acetylcholinesterase inhibition, antioxidant and cytotoxic potential of two culinary plant species, *Allium cepa* (Amaryllidaceae) and *Ocimum basilicum* (Lamiaceae) has been examined. These plants have neuroprotective effect.[6,7,8,9] However, their anti-Alzheimer potential is not explored comprehensively.

MATERIALS AND METHODS

Plant material

The bulbs of *Allium cepa* var. NHRDF-Red were collected in July 2013 from National Horticulture Research and Development Foundation (NHRDF), Bathinda, Punjab, India. The collected bulbs were authenticated by Mr. H.K. Sharma, Director, NHRDF, Bathinda (NHRDF/SC/BTI/2013-14/458). The leaves of *Ocimum basilicum* were procured in November 2013 from Indian Institute of Integrative Medicine, Jammu and were authenticated by Dr. Sunita Garg, Chief Scientist, Raw Material Herbarium and Museum, Delhi, CSIR-NISCAIR (NISCAIR/RHMD/Consult/2013/2337-117-1).

Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE), 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich. Donepezil was procured from Ind-Swift Ltd Jawahrpur, Punjab. All other chemicals used were of analytical grade.

C6 Glioma cell lines

Rat C6 glioma cell line was obtained from the National Centre for Cell Sciences, Pune, India and maintained on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 U/ml), gentamycin (100 μg/ml), 10% fetal bovine serum at 37°C and humid environment containing 5% CO_2.

Extraction

The outer scales of *A. cepa* bulbs and leaves of *O. basilicum* were shade dried and coarsely powdered. The powdered plant material was defatted with petroleum ether by maceration. The marc was extracted with methanol: distilled water (70: 30) mixture by maceration in a shaking incubator (r.p.m. = 100; Temperature = 37°C). The extracts so obtained were dried under vacuum and the percentage yields were calculated on dry weight basis.

Phytochemical screening and standardization of plant extracts

The prepared hydro-methanol (HM) extracts were subjected to phytochemical screening.[10,11] Total phenolic content (TPC) was estimated by Folin-Ciocalteu procedure.[12] and stated as milligram gallic acid equivalents (mg GAE)/g of extract. Aluminum chloride colorimetric method was used to establish total flavonoid content (TFC).[13] and expressed as milligram quercetin equivalent (mg QE)/g of extract.

In vitro antioxidant activity

DPPH (2, 2- diphenyl-1-picryl hydrazyl) test

Inhibition of DPPH free radical test was carried out by method of Blois.[14] DPPH radical is a compound having a proton free radical with a characteristic absorption which decreases considerably on exposure to proton radical scavengers. The method is based on capability of antioxidant samples to scavenge DPPH, decreasing its primary concentration and changing the color of solution from purple to yellow. The absorbance was read after 30 min incubation period at room temperature against a blank at 517 nm. Ascorbic acid was used as positive control. All readings were taken in triplicate. The percent of inhibition was calculated using following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

Sample concentration exhibiting 50% inhibition (IC_{50}) was calculated from the graph between inhibition percentage and extract concentration.

FRAP (ferric reducing antioxidant power) test

The method reported by Yen and Chen.[15] was used to determine the reducing power of extracts. Due to reductive capability of antioxidant compounds, they cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form. Addition of $FeCl_3$ to the ferrous (Fe^{2+}) form lead to the formation of Prussian blue-colored complex. Reducing power is determined by measuring the formation of Perls' Prussian blue at 700 nm. Different concentrations of HM extracts were mixed with sodium phosphate buffer and potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After that trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Supernatant was mixed with distilled water and ferric chloride solution, and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Acetylcholinesterase inhibition assay

The acetylcholinesterase inhibition assay was determined by Ellman.[16] colorimetric method with some modifications. It is a colorimetric assay based on enzymatic hydrolysis of acetylthiocholine iodide by the enzyme. The basic reaction involved in the process is as follows:

$$\text{acetylthiocholine} \xrightarrow{\text{acetylcholinesterase}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate} \rightarrow \text{yellow color}$$

The anticholinesterase compounds hinder the action of acetylcholinesterase enzyme thus preventing the breakdown of acetylthiocholine. Reduction in the yellow color of reaction mixture indicates preventive effect by the test compounds.

In a total volume of 2 ml, 1500 µl of phosphate buffer 0.1 M (pH 8), 100 µl solution of HM extracts at different concentrations and 100 µl of enzyme solution containing 0.1 U/ml were incubated for 15 min at room temperature. After that, 100 µl solution of acetylthiocholine iodide and 200 µl of DTNB (4 mM) were added and the final mixture was incubated for 30 min at room temperature. Absorbance of the mixture was measured at 405 nm in a UV-Visible spectrophotometer. Donepezil was used as positive control. The percentage inhibition of enzyme activity was calculated by using the following formula:

$$\% \text{ Inhibition} = 1 - (A_{sample} / A_{control}) \times 100$$

Where, A_{sample} = absorbance of the test extracts; $A_{control}$ = absorbance of the control

The concentration of extract providing 50% inhibition (IC_{50}) was obtained by plotting the percentage inhibition against extract concentration.

Cytotoxicity assay

Stock solutions (200 µg/ml) of HM extracts were prepared by reconstituting the dried extracts in 70% methanol and then calculating the percentage yield. The cytotoxic effect of each plant extract was evaluated by tetrazolium- dye, MTT, assay.[17] This colorimetric assay is based on the breakdown of tetrazolium ring of MTT by dehydrogenases in active mitochondria of living cells to estimate the number of viable cells.

In this assay, after adjusting the viable cell count (using trypan blue dye and dilution by DMEM), the C6 glioma cells were seeded in 96-well plates at a density of 40×10^3 cells/well in 100 µl DMEM. Following 24 h of incubation and attachment at 37°C in 5% CO_2, the cells were treated with different concentrations of extracts (200, 100, 50, 25, 12.5, 6.2, 3.1 µg/ml) for a further 24 h under the same conditions. After treatment, the media was replaced with MTT solution (100 µl of 0.5 mg/ml per well) prepared in medium and incubated for 4 h at 37°C in a humidified incubator with 5% CO_2. After removal of the MTT solution by gentle aspiration, the purple formazan crystals formed upon reduction of MTT by succinate dehydrogenase in the mitochondria of viable cells were dissolved by adding 100µl of DMSO per well. The plates were gently shaken for 1 min and absorbance was measured at 490 nm by microtiter plate reader (LabSystems, Finland).

The concentrations at which the cell viability was equivalent to the control cells were considered as non-toxic concentrations. All concentrations were tested in triplicate and the cell viability was calculated by the formula given below.

$$\text{Percentage cell viability} = 1 - \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

RESULTS

The percentage yields of HM extracts on dry weight basis for *A. cepa* var. NHRDF-Red and *O. basilicum* were 5.8 % w/w and 26.7% w/w respectively. The test extracts showed the presence of phenols, flavonoids and triterpenoids; hence were standardized with respect to TPC and TFC. *A. cepa* contains 456.1±11.7 mg GAE/g of extract and 278.4±21.6 mg QE/g of extract whereas *O. basilicum* extract contains 112.1±15.8mg GAE/g of extract and 20.41±8.7mg QE/g of extract.

In vitro antioxidant activity

The prepared HM extracts were evaluated for *in vitro* antioxidant activity using DPPH and FRAP assay. Both the plant extracts exhibited antioxidant activity in both *in vitro* assays.

In DPPH free radical scavenging assay IC_{50} value was calculated for both the plant extracts and compared with the standard (ascorbic acid). The IC_{50} values of ascorbic acid, *A. cepa* extract and *O. basilicum* extract were 6.32±0.27, 30.10±6.96, 38.67±1.72 µg/ml respectively. The present investigation confirmed that HM extracts of both the plants are significant radical scavengers.

In the FRAP assay, a higher absorbance indicates a higher ferric reducing power. With an increase in the concentration of the extracts, there is an increase in absorbance (Figure 1). HM extract of *A. cepa* showed more marked reducing capacity with maximum absorbance at concentration of 320 µg/ml.

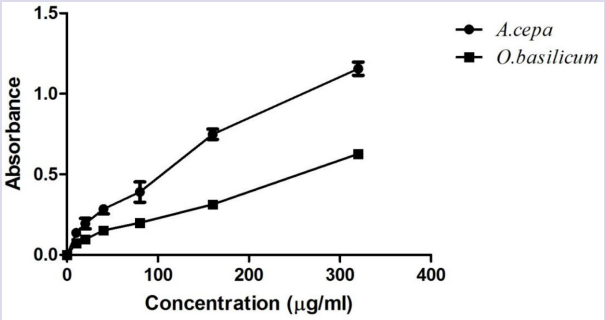
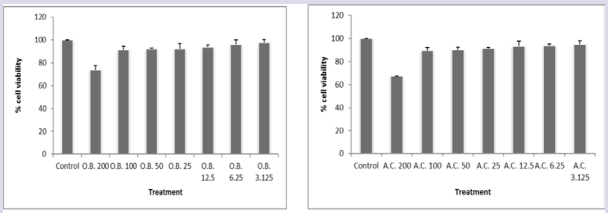


Figure 1: FRAP antioxidant reducing power of HM extracts.

Table 1: IC_{50} value of plant extracts in Ellman assay.

Treatment	IC_{50} (Mean±S.D.)^n (µg/ml)
Donepezil (Standard)	7.06±0.13
Cepa	51.78±1.05
O. basilicum	693.97±3.56

n=3



a) *O. basilicum* HM extracts b) *A. cepa* HM extracts

Figure 2: Percentage cell viability following treatment with HM extracts.

Acetylcholinesterase inhibition assay

The summary of acetylcholinesterase inhibition by plant extracts used in this study is shown in Table 1. With an increase in concentration of extracts, the percentage inhibition increased. Of both the extracts, *A. cepa* extract displayed noteworthy inhibition with IC_{50} value of 51.78±1.05 µg/ml. *A. cepa* extract was found to be an effective cholinesterase inhibitor in comparison to the standard drug.

Cytotoxicity assay

Plant extracts at different concentrations were evaluated for cytotoxicity against C6 glioma cells using MTT assay. The percentage cell viability was calculated for both the extracts. Treatment with the extracts considerably inhibited the proliferation of C6 cells in a concentration-dependent manner as shown in Figure 2 (a and b) MTT assay revealed affected cell viability at higher concentration in comparison to control. Significant toxic effects were observed at concentrations greater than 100µg/ml for both the plant extracts. These results revealed that concentrations below 100µg/ml for each extract are safe to C6 glioma cells and therefore can be selected for further evaluation of neuroprotective experiments.

DISCUSSION

Plants can be an excellent reservoir for drugs for the management of Alzheimer's disease owing to the presence of variety of phytochemicals.[18] Due to multifactorial nature of the disease, different plant constituents present in an extract may act alone on one pathological factor or synergistically to curb a pathway. This may prove beneficial in preventing various pathological events linked to the disease. The loss of cholinergic neurons in cerebral cortex is accepted as the main feature of Alzheimer's disease.[19] The use of acetylcholinesterase inhibitors to enhance the levels of acetylcholine is an important and solitary preventive approach for Alzheimer patients.[20] Free radical production is also a major factor contributing to Alzheimer's disease. Brain of AD patients witnesses changes in the balance of redox transition metals, like Fe and Cu, increase in lipid perxoxidation, increase in protein and DNA oxidation. Studies have also reported that the formation of senile plaques enhance free radical production.[21,22] Reserachers have evaluated *A. sativum* for the management of Alzheimer's disease.[23,24] However, *A. cepa* has not been explored for controlling pathogenic mechanisms related to the disease. Hydro-methanol extract of *O. basilicum* has been reported as acetylcholinesterase inhibitor from our laboratory.[9,25] whereas cytotoxic activity of the extract has not been explored.

CONCLUSION

In our study both plant extracts are potent antioxidants. *A. cepa* extract was found to be a good acetylcholinesterase inhibitor. High phenolic and flavonoid content of *A. cepa* might be responsible for the activity.[26] Cytotoxicity studies on plant extracts revealed that both the extracts were found to be non-toxic to C6 glioma cells at concentrations below 100 µg/ml. This assay is helpful in testing the safety of plant extracts in different cell culture experiments. Both the activities are vital for a drug candidate for the management of Alzheimer's disease hence *A.cepa* is an excellent candidate for drug development.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS USED

AD: Alzheimer's disease; **AChE:** Acetylcholinesterase; **DPPH-2, 2:** Diphenyl-1-picryl hydrazyl) test; **FRAP:** Ferric reducing antioxidant power; **TPC:** Total phenol content; **TFC:** Total flavonoid content.

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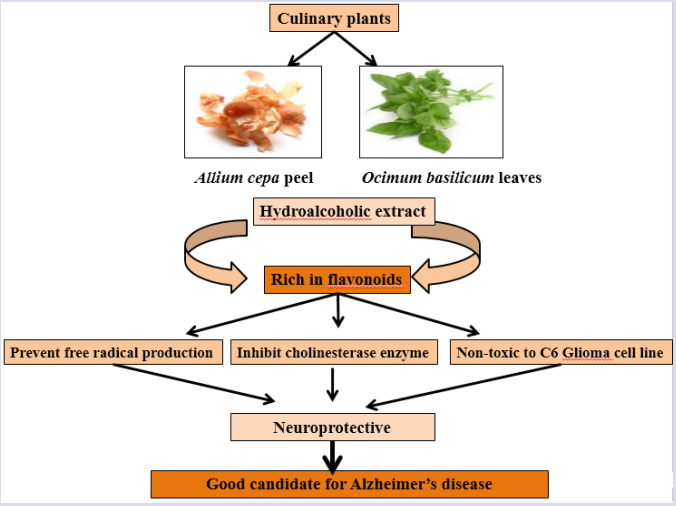
PICTORIAL ABSTRACT



SUMMARY

- In the present investigation anticholinesterase, antioxidant and cytotoxic potential of the two culinary plants- Allium cepa and Ocimum basilicum was investigated. Both the plant extracts displayed significant antioxidant activity and were found to be non-toxic in MTT assay below the concentration of 100µg/ml. The *A. cepa* extract had strong antioxidant and acetylcholinesterase inhibitory effect probably due to higher total phenol and flavonoid content. This can be considered an excellent candidate for developing as a drug for management of AD.