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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ACETYLCHOLINESTERASE INHIBITION ACTIVITY OF HIMALAYAN BERRY FRUIT: MYRICA ESCULENTA BUCH HAM EX D. DON

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ABSTRACT

Intake of fruits rich in antioxidants is well documented to delay the onset as well as progression of Alzheimer's disease. The objective of this study to evaluate the traditionally important Himalayan berry fruit *Myrica esculenta* Buch Ham ex D. Don extracts for antioxidant and acetyl-cholinesterase inhibition (AChEI) activity as well as screened for phytochemical screening analysis. Total phenolic content (TPC) and total flavonoid contents (TFC) of the fruit extracts were also determined to correlate with antioxidant and AChEI activity. Phytochemical screening revealed the presence of various active phytoconstituents including phenolic and flavonoid compounds. Methanol fruit extract showed the most significant acetylcholinesterase inhibitory activity (IC_{50} value 6.06±2.25 mg/ml) with highest TFC; while antioxidant activity was found to maximum in aqueous extract followed by methanol and chloroform extract. The marked inhibition of acetylcholinesterase and potent antioxidant activity of *M. esculenta* fruit due to presence of antioxidant phenolic compounds including flavonoids revealed that it may be used as a potential natural source of bioactive compounds and could be beneficial to the human health, especially in the effective treatment for AD as well as source of natural antioxidants in food and pharmaceutical industry. This is the first report on such activities of *M. esculenta* fruit extracts.

KEYWORDS: Acetylcholinesterase inhibition activity, Alzheimer's Disease, Antioxidant, Berry fruit, *Myrica esculenta*.

INTRODUCTION

Dementia is a syndrome, usually of chronic or progressive nature that affects memory, thinking, behaviour, judgement capacity and ability to perform daily routine activities.[1] Alzheimer's disease (AD) is one of the major causes of dementia, affecting 5% of men and 6% of women aged above 60 years worldwide.[2] The consistent neuropathological hallmarks of AD include β-amyloid plaques, neurofibrillary tangles composed of the hyperphosphorylated tau protein and loss of neurological function.[3,4] The latter is due to cholinergic deficit in the brains of afflicted individuals resulting in decline of higher cognitive functioning due to reduction in level of the neurotransmitter acetylcholine (ACh) which is believed to be responsible for short term memory and learning.[5,6] Hence, acetylcholinesterase inhibitors (AChEIs) have been proven as one of the potential therapeutic strategy to increase the level of ACh molecule within the synaptic region by inhibiting the action of acetylcholinesterase enzyme responsible for breaking down of ACh into an inactive form, thereby reducing the severity of cognitive loss and slow down the disease progression by restoring deficient cholinergic neurotransmission.[7-9]

AChEIs show beneficial effects on cognitive, functional and behavioral symptoms in AD.[10-14] The currently available selective AChEIs namely tacrine, donepezil and rivastigmine neither allow sufficient modulation of ACh levels to elicit the full therapeutic response nor are free of dose-limiting side effects[6] Some natural products belong to major class of phytoconstituents such as alkaloids,[15] phenolic compounds[16,17] and terpenes[18] have shown their marked potential against AChE. Thus, there is a need for exploring the plant sources for potent, long lasting new AChEIs which not only provide symptomatic relief with higher efficacy, fewer side effects but also halt the progression of AD disease.

Oxidative stress due to over production of free radicals and reactive oxygen species is also a critical important factor in the pathogenesis of various neurodegenerative diseases including stroke, Alzheimer's disease, parkinson's disease, vascular dementia, as well as cancer, diabetes, artherosclerosis *etc.*[19-21] Previous studies have revealed that the inhibition of oxidative damage, responsible for chronic detrimental neurodegeneration, is an important strategy for neuroprotective therapy as well as improving cognitive function and behavioral deficits in patients with mild to moderate AD.[22-23] In the recent past years, some

toxicological studies regarding the use of several available synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole and tert-butyl hydroxyquinone have shown adverse effects such as liver damage and carcinogenesis.[24-27] These reports have urged the researchers to focus their study on exploring natural resources to find out newer and safer natural antioxidants as they inhibit oxidative damage and may consequently prevent aging and neurodegenerative diseases.[28] Studies have already highlighted the potential of berry fruits as vital sources for antioxidants and cholinesterase inhibitors.[29,30]

Myrica esculenta (*M. esculenta*) Buch-Ham ex D. Don belonging to family myricaeae; commonly known as Boxberry, kaiphal and Kathphala mainly found in sub tropical Himalayas, is a well recognised Indian species of genus *Myrica*.[31] Its synonym is *Myrica nagi* (*M. nagi*) Hook. F. non Thunb.[32] It is widely used in folk medicine to treat several ailments such as asthma, cough, chronic bronchitis, ulcers, inflammation, anaemia, fever, diarrhoea, and ear, nose, and throat disorders.[33,34] Due to its multidimensional pharmacological and therapeutic effects, it is a well recognized medicinal plant in the Ayurvedic Pharmacopeia.[35] The plant has been reported to be a rich source of Vitamin C and polyphenols compounds such as tannins, phenols and flavonoids including gallic acid, myricetin, caffeic acid and catechin in abundant amount while traces of chlorogenic acid, transcinnamic acid, *p*-coumaric acid and ellagic acid[36,37] possess several pharmacological activities such as analgesic, anti-allergy, anti-diabetic, antioxidant, anxiolytic, hypotensive, antimicrobial *etc*.[38-42]

Previously published research reports demonstrated that *M. esculenta* fruit exhibited significant antioxidant activities due to presence of phenolic content and thus may be utilized as a natural antioxidant.[43,44] It is well documented that polyphenolic compounds such as anthocyanin, caffeic acid, catechin, quercetin, kaempferol and tannin present in berry fruits show neuroprotective effect in various neurodegenerative disorders and improves motor and cognitive functions[45-47] because of their ability to cross the blood brain barrier and directly scavenging pathological concentrations of reactive oxygen species and chelate transition metal ions.[48,49] Therefore, the objective of this study was to assess the antioxidant and acetylcholinesterase inhibition activity of *M. esculenta* fruit and correlate with the phytochemical profiles, in order to determine its relevance for the treatment of AD.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Acetylcholinesterase Enzyme EE (VI-S type), Acetylthiocholine iodide, 5,5'-dithio-bis-(2-nitrobenzoic acid (DTNB), tacrine, quercetin, gallic acid, ascorbic acid were purchased from Sigma (St Louis MO, USA). Unless otherwise specified, all other chemicals and solvents used in this study were of analytical grade.

Plant material

The fresh fruits from healthy plant of *M.esculenta* selected for our study were collected from the agriculture fields of Sundernagar, Himachal Pradesh, India in May, 2016 and authenticated by S.K Srivastava, Scientist E, Botanical Survey of India, Dehradun (Reference No-BSI/NRC/Tech/2017-18/117209 dated 8/5/2017). A voucher specimen of the plant was deposited in the herbarium of same department for further reference. The collected fruits were dried under shade and then pulverized with mechanical pulverizer for size reduction.

Microscopical evaluation

Preparation of extracts

Air dried coarse powder of *M. esculenta* fruit was firstly defatted with petroleum ether then sequentially extracted with chloroform and methanol by using soxhlet apparatus. Finally, the mark was refluxed at temperature 50°C by using distilled water as solvent to prepare aqueous extract. The colour, consistency and percentage yield of all obtained extracts were calculated and kept in desiccators till for further use.

Phytochemical screening

The prepared extracts of *M. alba* fruit were subjected to various phytochemical tests by adopting standard methodology to determine the nature of phytoconstituents present.[50]

Determination of total phenolic content

Total phenolic content of all prepared extracts was estimated by using Folin Ciocalteu's spectrophotometric method.[51] Quantification of total phenolic content in extracts was done by extrapolation of a calibration curve prepared with the help of standard gallic acid. The methanol was used as blank. The data for total phenolic contents of extracts were expressed as mg of gallic acid equivalent weight (GAE)/ 100 g of dried mass. All the readings were taken in triplicate.

Determination of total flavonoid content

Aluminium chloride colorimetric assay was used for the estimation of total flavonoid content in all prepared extracts[52] by using quercetin as a standard. All determinations were done in triplicate. The standard calibration curve was plotted using quercetin. The data of total flavonoids of tested extracts were expressed as mg of quercetin equivalents/ 100 g of dried extract.

Determination of *in vitro* antioxidant activity

In vitro antioxidant activity was estimated by using DPPH assay according to method of Blois with slight modifications.[53] This method relies on decrease in the absorption of the blue coloured DPPH solution after the addition of an antioxidant measured at 517 nm.

Briefly, 1.5 mL of freshly prepared 0.1 mM DPPH reagent was added to the 1.5 ml of extract/standard at various concentrations. The mixture was shaken and then allowed to stand in dark at room temperature for 30 minutes. Then, absorbance was measured at 517 nm spectrophotometrically using UV- visible spectrophotometer. Any absorbance due to extract colour was removed by including a blank solution containing methanol in place of DPPH solution for each extract. Methanol was used as negative control while ascorbic acid was used as positive control. The assay was executed in triplicate. The scavenging effect of DPPH free radical was calculated by using the following equation:

$$\% \text{ scavenging effect} = \left(1 - \frac{A_{extract/std} - A_{background}}{A_{control}}\right) \times 100$$

Where Aextract/std, Abackground and Acontrol are the absorbance readings of the extract/std, background solution and negative control, respectively at 517 nm.

IC_{50} value *i.e.* the concentration of extract required to scavenge DPPH free radical by 50% was estimated from the curve of scavenging effect (%) plotted against the respective concentrations and reported as means ± standard deviation (SD).

Determination of *in-vitro* acetylcholinesterase inhibition activity

In-vitro acetylcholinesterase inhibition activity of various plant extracts was assessed by modified Ellman's colorimetric method.[54] The acetylcholinesterase enzyme hydrolyses the substrate acetylthiocholine iodide resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm.

In each well of 96-well micro-titre plate, 260 µl of Phosphate buffer (0.1 M, pH 8), 20 µl extract, 10 µl of AChE (0.1 U/ml) and 10 µl of DTNB (10 mM) were added and the reaction mixture was incubated at room temperature for 30 minutes. Then, 10 µl of acetylthiocholine iodide solution (75 mM) was added to test tube and then absorbance was read at 412 nm after 15 minutes. The reaction was quenched by adding 3% sodium lauryl sulphate solution before taking the absorbance. The percentage of inhibition was calculated in comparison to control (extract absent) by using formula:

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

Where, A_{sample} = absorbance of the test extract, $A_{control}$= absorbance of the control.

Tacrine was used as standard cholinesterase inhibitor and concentration of extract providing 50% inhibition (IC_{50}) was calculated by plotting the inhibition degrees against the sample concentrations. The test was run in triplicate, and the IC_{50} values were reported as means ± standard deviation (SD).

Statistical analysis

All the experiments were carried out in triplicate and the data were expressed as the mean ± standard error and subjected to one-way analysis of Variance (ANOVA) followed by post-hoc Tukey's Multiple range test. Differences were considered significant at $p<0.05$.

RESULT AND DISCUSSION

Preparation of plant extracts

To the best of our knowledge, there is no report published on the effect of extraction conditions on the polyphenol content and antioxidant as well as acetylcholinesterase inhibition activity of *M. esculenta* fruit. Therefore, in the present study, sequential exhaustive extraction was employed with a view to separate constituents on the basis of polarity. It was observed that methanol extraction produced maximum yield (34.82% w/w) followed by aqueous extraction such as 30.16% w/w whereas petroleum ether and chloroform extraction yielded lowest 3.65%w/w and 0.24% w/w.

Phytochemical screening

The results of qualitative screening of phytochemical analysis on *M. esculenta* fruit extracts was similar with earlier reports published for *M. esculenta* fruit powder showing the presence of various secondary plant metabolites such as alkaloids, phenols, flavonoids, steroids, terpenoids, saponins, glycosides and tannins.[55] Petroleum ether extract showed only the

presence of steroids and terpenoids. It is most likely that this is the first such comparison report on phytochemical screening of various extracts of *M. esculenta* fruit (Table 1).

Determination of total phenolic content

The total phenolic content for chloroform, methanol and aqueous extracts of *M. esculenta* fruit were estimated by Folin Ciocalteu's method using gallic acid as standard. Methanol extract showed highest total phenolic content 27.64±0.22 mg GAE/g followed by aqueous extract and lowest total phenolic content was reported in chloroform extract (Table 2). The gallic acid solution of concentration (10-80 µg/ml) conformed to Beer's Law at 765 nm with a regression co-efficient (r^2)=0.9994. The plot has a slope (m) = 0.0105 and intercept = 0.0108. The equation of standard curve is y = 0.0105x + 0.0108 (Fig. 1).

Determination of total flavonoid content

The total flavonoid content for chloroform, methanol and aqueous extracts were measured with the aluminium chloride colorimetric assay using quercetin as standard. Highest total flavonoid content was reported in methanol extract 2.93±0.18 mg QE/g while chloroform extract showed lowest amount of total flavonoid content (Table 2). The quercetin solution of concentration (20-160 µg/ml) confirmed to Beer's Law at 415 nm with a regression co-efficient (r^2) = 0.9921. The plot has a slope (m) = 0.004 and intercept= 0.0092. The equation of standard curve is y = 0.004x + 0.0092 (Fig. 2).

***In vitro* antioxidant assay (DPPH radical scavenging assay)**

Accumulating evidence suggests that polyphenols including tannins, flavonoids, lignans and proanthocyanidins have excellent antioxidant activity in both *in vitro* and *in vivo* investigations.[56] The *in vitro* antioxidant activity of tested extracts was assessed by DPPH radical scavenging assay in comparison to standard drug ascorbic acid.

Antioxidant properties of fruit juice and 80% v/v methanol extract of *M. esculenta* had been reported in previous studies[43,44] The result of present study revealed that methanol extract which has the maximum amount of phenolic compounds exhibited the most significant antioxidant activity as compared to other extracts with lowest IC_{50} value 100.17±4.35 µg/ml followed by aqueous fruit extract (Table 3).

***In vitro* acetylcholinesterase inhibition activity (Ellman's assay)**

The critical role of acetylcholinesterase in neural transmission makes them a key target for the treatment of neurodegenerative disorders, including AD. Currently, AChEIs are the standard clinical strategy for the symptomatic relief of AD[57] and also AD patients on high doses of antioxidants were reported to have a slower rate of cognitive deterioration[58]. Dietary approaches, in particular polyphenol-enriched diets have attracted much attention for the prevention and delay of AD progression due to their effectiveness and safety profile. Moreover, these are cheap and easily available.[59,60] In this study, methanol extract showed most significant acetylcholinesterase inhibition activity in dose dependent manner may be due to presence of maximum amount of phenolic and flavonoid compound as compared to other extracts with IC_{50} value 6.06±2.25 mg/ml comparable with standard drug tacrine (Table 3).

Literature shows that phenolic/flavonoids rich fraction and/or isolated phenolic compounds including flavonoids produced marked AChE inhibition.[61-64] Thus, significant antioxidant and acetylcholinesterase inhibition activities of methanol extract of *M. esculenta* fruit in this study suggest that it can be used as good natural source of phenolic compounds, with potential cholinesterase inhibitory and antioxidant properties that may find usefulness in the management of AD. This is the first report of such activities in *M. esculenta.*

TPC and TFC of *M. esculenta* fruit extracts were correlated with antioxidant and acetylcholinesterase inhibition properties (Table 4). A significant negative correlation shows that as the content of constituents (TPC and TFC) increases, there is decrease in the IC_{50} value in DPPH and Ellman's assay. Thus, showing that TPC and TFC are contributing to the antioxidant and acetylcholinesterase inhibitory activities of the plant. A better correlation was observed between the phenolic content and antioxidant activity and acetylcholinesterase inhibition (r^2 = -0.890; -0.91; $p<0.05$) respectively. Thus, it can be predicted that phenolic compounds present in the extracts may be main contributor for antioxidant and acetylcholinesterase inhibition activity.

Graphical abstract

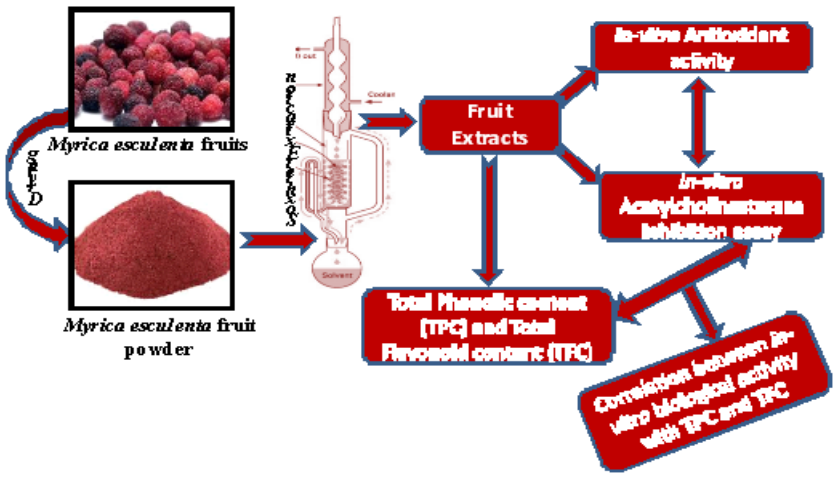

Table 1: Phytochemical screening of different extracts of *M. esculenta* fruits.

Sr. No	Test		PEE	CE	ME	AQE
1.	Alkaloids	Mayer's	-	+	+	+
		Dragendroff's	-	+	+	+
		Wagner	-	+	+	+
		Hager's	-	+	+	+
2.	Glycosides	Keller Killiani	-	-	-	+
		Borntager's Test	-	-	+	-
3.	Proteins/AA	Millon's Reagent	-	-	+	-
		Xanthoprotein	-	-	-	-
4.	Saponins	Froth/Foam Test	-	-	+	+
5.	Carbohydrates	Benedict's	-	-	+	+
		Fehling solution	-	-	-	+
		Molisch's Reagent	-	-	+	+
6.	Sterols	Salkowski Test	+	-	+	+
7.	Terpenoids	Antimony trichloride	+	-	+	+
8.	Flavonoids	Shinoda Test	-	+	+	+
		Alkaline reagent	-	+	+	+
		Lead acetate test	-	+	+	+
		Sulphuric acid test	-	+	+	+
9.	Tannins	Gelatin Test	-	-	+	+
10.	Phenols	$FeCl_3$ Test	-	+	+	+

*** Present= +, Absent= -;** PEE=pet-ether extract; CE=chloroform extract; ME=methanol extract; AQE=aqueous extract

Table 2: Total Phenolic and Flavonoid content of *M. esculenta* fruit extracts.

Extract	Total Phenolic Content (mg GAE/g extract) Mean^n±S.D	Total Flavonoid Content (mg QE/g extract) Mean^n±S.D
Chloroform	12.00±0.39	0.52±1.23
Methanol	27.64±0.23	2.93±0.18
Aqueous	18.31±0.22	0.85±0.26

*n=3

Table 3: Antioxidant and Acetylcholinesterase inhibitory activity of *M. esculenta* fruit extracts.

Sample	Antioxidant activity IC_{50} (µg/ml) Mean^n±S.D	Acetylcholinesterase inhibition activity (IC_{50} mg/ml) Mean^n±S.D
Chloroform	439.86±3.03	18.17±4.29
Methanol	100.17±4.35	6.06±2.25
Aqueous	158.55±3.34	8.63±2.59
Ascorbic Acid	4.22±0.27	----
Tacrine	----	7.35±0.31 µg/ml

*n=3

Table 4: Correlation of the total phenolic and total flavonoid content with antioxidant and acetylcholinesterase inhibitory activities.

Assays	r^2	
	Total Phenolic Content	Total Flavonoid Content
Antioxidant activity	-0.890	-0.470
Acetylcholinesterase inhibition activity	-0.670	-0.753

Data is presented as Pearson Correlation Coefficien

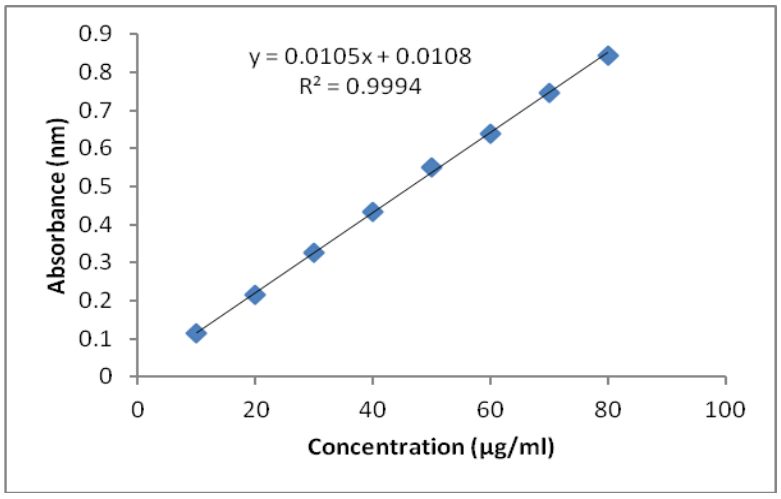

Fig. 1: Standard curve of absorbance against gallic acid concentrations.

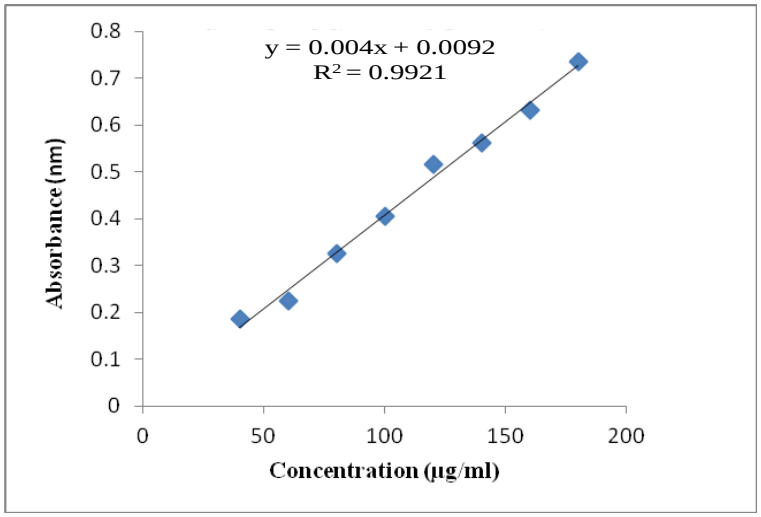

Fig. 2: Standard curve of absorbance against quercetin concentrations.

CONCLUSION

To the best of our knowledge, this is the first report describing the cholinergic inhibitory and antioxidant activities of *M. esculenta* fruit extracts and determinations of their phenolic and flavonoid content. The obtained extracts possess significant and dose-dependent *in vitro* antioxidant and acetylcholinesterase inhibition capacities. The findings of this study revealed that *M. esculenta* fruit extracts could be used as a readily accessible source of natural antioxidants, and as such may be used as crude material in the pharmaceutical industry. Further testing in an animal model of Alzheimer's disease is needed to elaborate the *in vivo* effectiveness of this plant.

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CONFLICT OF INTEREST

Authors declare that they do not have any competing interests.

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