World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org

ANTI-OXIDANT AND ANTI-BUTYRYLCHOLINESTERASE ACTIVITY OF AN AQUEOUS EXTRACT OF *MORUS NIGRA*.

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Article Received on 22/06/2015 **Article Revised on 08/07/2015** **Article Accepted on 30/07/2015**

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ABSTRACT

Alzheimer's disease (AD), which is considered to be most common form of dementia leads to neurodegeneration of primarily the cholinergic neurons. The currently available medication based on cholinesterase inhibiting activity can only provide symptomatic relief to the patients undergoing treatment. The present study evaluates the anti-oxidant and anti-butyrylcholinesterase activity of an aqueous extract of *Morus nigra*. Our findings demonstrated that an aqueous extract of *Morus nigra's* fruit significantly inhibited butyrylcholiesterase enzyme and showed maximum inhibition of 54.28±0.33% at the final concentration of 200µg/ml with IC_{50} value of 126.80µg calculated using the percentage inhibition plot. An aqueous extract of *Morus nigra* also possessed significant antioxidant activity of 2511.593±0.123 µmol Fe^{2+} E/g of dried sample and 0.89±0.0005 mmol Trolox E/g of dried sample assessed by Ferric reducing activity of plasma assay and Trolox equivalent antioxidant capacity assay respectively. It is concluded that this plant has potential for alleviating the symptoms associated with AD.

KEYWORDS: Alzheimer's disease, butyrylcholinesterase, acetylcholine, *Morus nigra*.

INTRODUCTION

Alzheimer's disease (AD), regarded as the most common form of dementia is a chronic neurodegenerative disorder leading to degeneration of neurons in thin layer of grey matter, with initial observable deficits beginning in hippocampus and the entorhinal cortex [1]. AD is

characterized by the disruption of the synaptic function which usually starts slowly and worsens as the disease progresses. The old age related disorder is associated with symptoms such as difficulty in remembering recent events (short-term memory loss), confusion, abrupt mood swings and become apathetic and depressed [2]. The characteristic feature of AD is the formation of amyloid fibrils (known as senile plaques or neuritic plaques) from beta amyloid (Aβ) fragments resulting from the proteolytic processing of transmembrane β amyloid precursor protein (APP) by an enzyme β secretase [3][4]. The most distinctive cytopathological change observed during AD is the presence of neurofibrillary tangles in the central nervous system formed as a result of hyperphosphorylation of tau protein (a microtubule associated protein), hampering the cell's cytoskeleton and collapsing the neuron's transport machinery[5]. These neurofibrillary tangles exist in the cytoplasm of neurons predominantly found in the hippocampal region of the cortex which is primarily involved with functions such as memory and learning [6]. The drug development strategies comprising both natural as well as synthetic compounds rely on cholinergic hypothesis which states that decline in the levels of cholinergic neurotransmitter acetylcholine (ACh) by activity of acetylcholinesterase (AChE) as one of the major causes of AD [7]. Extensive work related to neurotransmission during AD has been carried out using AChE, with focus now shifting on to butyrylcholinesterase (BuChE) which could possibly function as a co-regulator during cholinergic transmission [8][9]. BuChE is a non specific hydrolase enzyme catalyzing hydrolysis of ACh, butyrylcholine, succinylcholine, acetylsalicylic acid etc, hence being referred to as "Pseudocholinesterase' [10]. Cholinesterase (ChE) inhibitors serves a crucial role in treating symptoms associated with AD by inhibiting cholinestreases, thus preventing the hydrolysis of substrate ACh and BuCh, thereby upregulating the levels of ACh improving cognitive functions [11]. Oxidative stress is considered to play a significant role in pathogenesis of AD by inducing changes in the membrane like lipid peroxidation, ion-transport and fluidity thus disrupting the structural and functional integrity of the neurons [12]. The major free radicals generated due to mitochondrial oxidative stress leading to damage to the membrane includes hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide radical (O_2^-) [13]. These reactive oxygen species (ROS) arises from the by- products generated during electron transport chain (ETC) of mitochondria resulting in oxidative cell damage and cell death [14]. Currently available medication comprising synthetic drugs are found to be associated with certain limitations and side-effects [15], suggesting the need for discovery of alternative effective drugs with minimal side effects, least toxicity, safety, bioavailability and

maximum potency. Exploiting compounds from natural sources like plants having anti-oxidant and anti-cholinesterase activity for improving cognitive deficits incurred due to AD.

Morus nigra, often referred to as mulberry is a deciduous tree belonging to the family moraceae, which is of economic importance as it is widely used in food and beverage industry in the formulation of jellies, jams and sherbets [16]. The fruit is of high nutritive value and can be consumed directly. Extensive experimental analysis has been carried out on *Morus nigra* concerning a number of clinical disorders. In the present study the fruit of *Morus nigra* was used. In this study, an aqueous extract of plant was screened for anti-butyrylchonesterase activity using Ellman's assay and for anti-oxidant activity using Trolox equivalent antioxidant capacity (TEAC) assay and Ferric reducing ability of plasma (FRAP) assay.

MATERIALS AND METHODS

Chemicals and reagents

BuChE (Sigma-Aldrich) from equine serum, butyrylthiocholine iodide (BTChI) (Sigma-Aldrich), 5, 5- dithiobis [2-nitrobenzoic acid] or DTNB or Ellman's reagent (Sigma-Aldrich), sodium bicarbonate (Sigma, Himedia), acetate buffer, Tripyridyltriazne (TPTZ) (Sigma-Aldrich), ferric chloride (Sigma-Aldrich), ferrous sulphate (Sigma-Aldrich), phosphate buffer solution (PBS), potassium persulphate (Sigma-Aldrich), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (Sigma-Aldrich).

Plant material

The fruit of the plant *Morus nigra* (Voucher no. NK/USBT0171311) was obtained from a local market and authenticated by botanist. The voucher specimens of this plant sample are stored in a herbarium at USBT, GGSIP University, Delhi, India.

Equipments and instruments

96-well plate (Corning Inc. NY), pipettes (Biomate and Thermo labsystem), tips (Tarsons products Ltd. India), centrifuge tubes, eppendorf tubes, eppendorf tube stand, measuring cylinder, conical flask, aluminum foil, tissue paper, cotton plugs, needle, spatula, scissors, butter paper, whatman filter paper No.1, parafilm, ice box, blotting paper, vortex machine (REMI), pestle and mortar, -20^{o} refrigerator, hot air oven, rotatory shaker, centrifuge machine, weighing balance, pH meter (EuTech), spectrophotometer (Spectra Max).

Preparation of an aqueous extract of the plant

20 gm of the fruit was weighed, washed, dried and again weighed in order to remove the dust and other impurities which might interfere with the activity of the plant sample and to remove excess of moisture. The weighed fruit was then crushed using mortar and pestle, which was then transferred into a conical flask containing 200 ml of distilled water [1:10 w/v]. The flask kept on a rotatory shaker for 48 hrs. After 48hrs the solvent was filtered using Whatman filter paper No.1. Solvent was evaporated by keeping the flask in hot air oven for 24 hours at 40ºC. The sample stored in eppendorf tube at-20ºC.

Antioxidant Assays

Ferric reducing ability of plasma (FRAP) assay

The total antioxidant potential of an extract is determined using the FRAP assay which measures the Ferric reducing activity of plasma as a measure of antioxidant power. The antioxidant present in the sample will reduce the ferric (Fe^{3+}) ion to ferrous ion (Fe^{2+}) which then reacts with Tripridyltriazine (TPTZ) to form a blue-colored compound ferrous Tripridyltriazine, detected spectrophotometrically at 593 nm [17]. The FRAP values of sample are obtained in comparison to the absorbance of TPTZ of test samples to that of the ferrous sulphate standard solution. 1mM stock solution of an aqueous extract of *Morus nigra's* fruit was prepared and from that stock different concentrations ranging from 200 µg/ml to 0.39 µg/ml were made. In a flat-bottom 96-well microtitre plate, 20µl sample was added to 180µl FRAP reagent. The reaction for each concentration was carried out in triplicates. A triplicate control reaction was also carried out, in which 20µl of distilled water was added to 180µl FRAP reagent. The mixture was incubated for 10 minutes. The change in absorbance was measured at 593nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader. The same procedure was followed for standard assay of ferrous sulphate solution [18].

Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is based on the scavenging of the 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical which gets converted to blue color on addition of potassium persulfate. The reaction between ABTS cationic radical and the antioxidants in the extract leaves the solution colorless, which is detected spectrophotometrically at 734 nm [19]. The degree of decolorization by an antioxidant is compared to that of trolox, giving the TEAC value. 1mM stock solution of an aqueous extract of *Morus nigra's* fruit was prepared and from that stock different concentrations ranging from 800 µg/ml to 0.78 µg/ml were made. In

a flat-bottom 96-well microtitre plate, 10µl sample solution was added to 190µl TEAC reagent. The reaction for each concentration was carried out in triplicates. A triplicate control reaction was also carried out, in which 10µl of distilled water was added to 190µl TEAC reagent. The mixture was incubated for 30 minutes. The change in absorbance was measured at 734 nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader. The same procedure was followed for standard assay of Trolox reagent [20].

Cholinesterase inhibitory assay

Cholinesterase assay is a colorimetric assay involving reaction where Butyrylthiocholine iodide (BuTChI) is hydrolyzed by Butyrylcholinesterase (BuChE) to produce thiocholine and butyrate. Thiocholine reacts with DTNB to produce yellow colored TNB anion that has absorbance maxima at 412 nm [21]. An estimation of cholinesterase inhibition was carried out in flat-bottom 96- well microtitre plate.1mM stock solution of an aqueous extract of *Morus nigra's* fruit was prepared and from that stock different concentrations ranging from 200 µg/ml to 12.5 µg/ml were made. The reaction mixture consisted of 5 µl of 0.08 U/ml BuChE solution, 200 µl of 0.1 M phosphate buffer, 5 µl of 0.5mM DTNB and 5 µl of the test aqueous extract. The reactants were mixed and pre-incubated for 15 min. The reaction was initiated by adding 5 µl of 0.5mM BTChI. As a control the inhibitor solution was replaced with buffer. To monitor any non-enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared in triplicates. One blank consisted of buffer replacing enzyme and a second blank had buffer replacing substrate. The reaction was carried out in triplicates. Change in absorbance was measured at 412 nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader over a period of 3 min.

RESULTS AND DISCUSSION

Antioxidant assays

Ferric reducing ability of plasma (FRAP) assay

The results demonstrate that an aqueous extract of *Morus nigra's* fruit at concentration ranging from 0.39 µg/mL to 200 µg/mL displayed concentration-dependent anti-oxidant property. The maximum value of equivalent ferrous sulphate concentration was found to be 2511.593±0.123 µmol Fe^{2+} E/g of dried sample for the 200 µg/mL aqueous extract sample concentration (Figure 1).

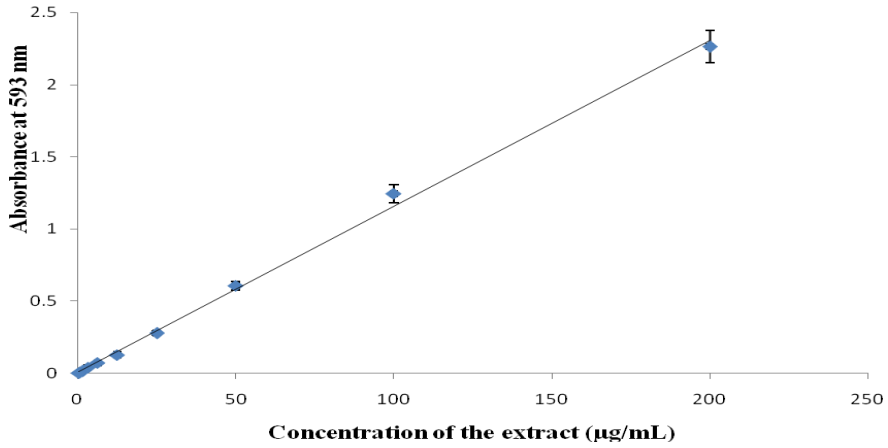

Figure 1: Absorbance values at different concentrations of an aqueous extract of *Morus nigra's fruit* showing concentration-dependent anti-oxidant property in FRAP assay. [The equation of the line is [y = 0.011x + 0.006, R^2 = 0.997].

Table 1. FRAP value of an aqueous extract of *Morus nigra* at different concentrations with their absorbance values.

S.No.	Concentration (µg/ml)	Absorbance Mean value (at 595 nm)	µmol Fe^{2+} E/g of dried sample)
1.	0.39	0.003333	1.592593±0.003
2.	0.78	0.009	7.88888±0.002
3.	1.56	0.018	17.88889±0.001
4.	3.12	0.041	43.4444±0.004
5.	6.25	0.073	79±0.006
6.	12.5	0.129333	141.5926±0.007
7.	25	0.278	306.7778±0.007
8.	50	0.608667	674.1852±0.017
9.	100	1.245333	1381.593±0.343
10.	200	2.262333	2511.593±0.123

Trolox equivalent antioxidant capacity (TEAC) assay

The results demonstrate that an aqueous extract of *Morus nigra's* fruit at concentration ranging from 0.78 µg/mL to 800 µg/mL displayed concentration-dependent anti-oxidant property. The maximum value of equivalent trolox concentration 0.891±0.0005 mmol trolox E/g of dried sample was observed at 200 µg/mL ethanolic extract sample concentration (Figure 2).

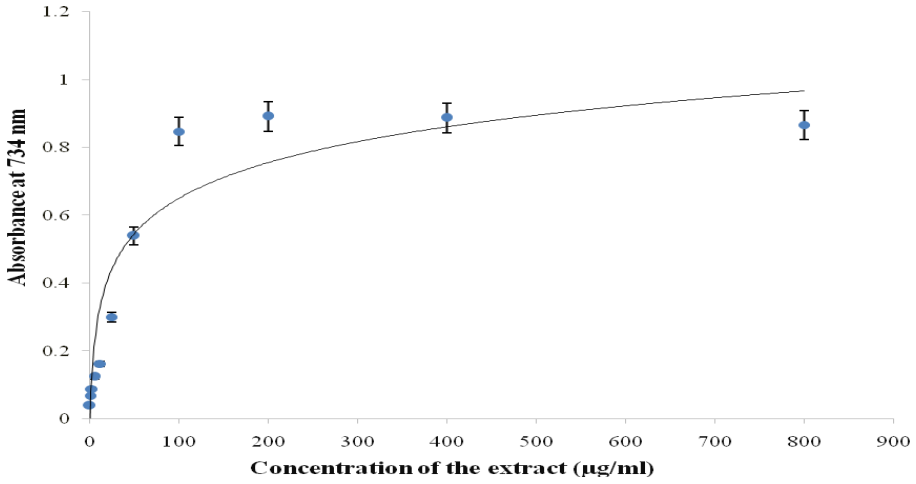

Figure 2. Absorbance values at different concentrations of an aqueous extract of *Morus nigra's* fruit showing concentration-dependent anti-oxidant property in TEAC assay. [The equation of the line is [y = 0.152ln(x) - 0.054, R^2 = 0.893].

Table 2. TEAC value of an aqueous extract of *Morus nigra* at different concentrations with their absorbance values.

S.No.	Concentration (µg/ml)	OD Mean Value (at 734 nm)	mmol Trolox E/g of dried sample)
1.	0.78	0.386667	0.0389±0.0047
2.	1.56	0.375	0.0679±0.0075
3.	3.12	0.367	0.0878±0.0036
4.	6.25	0.352333	0.1242±0.0117
5.	12.5	0.337	0.1623±0.0026
6.	25	0.281667	0.2999±0.0015
7.	50	0.185333	0.5393±0.0070
8.	100	0.061333	0.8475±0.0035
9.	200	0.043667	0.8914±0.0005
10	400	0.045333	0.8873±0.0005
11.	800	0.053667	0.8661±0.0092

Cholinesterase inhibitory assay

The results demonstrated that an aqueous extract of *Morus nigra's* fruit at concentration ranging from 12.5 µg/mL to 200 µg/mL displayed concentration-dependent inhibition of enzyme BuChE. The maximum inhibition of 54.28±0.03% for enzyme BuChE was observed at 200µg/mL final assay concentration. The IC_{50} value calculated from the equation obtained from the concentration versus percentage inhibition curve was 126.80 µg/mL (Figure 3).

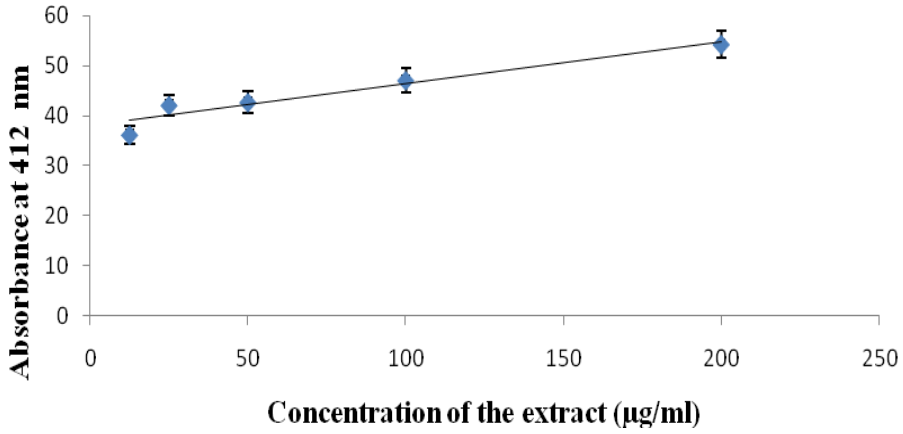

Figure 3. Percentage inhibition of BuChE activity at different concentrations of an aqueous extract of *Morus nigra's* fruit. . [y = 0.084x + 37.90, R^2 = 0.926].

The currently approved synthetic drugs for treating AD like donepezil and tacrine have been reported to be associated with certain side effects like irritation, gastrointestinal disturbances, high production costs and low bioavailability[22][23]. These drugs functioning as cholinesterase inhibitors (ChEI) helps in improving the symptoms underlying AD thereby enhancing the cognitive functions. These limitations along with inability to provide complete cure paves a way for discovery of new ChEI using natural resources like plants or plant derives compounds with minimal side effects, higher bioavailability, and higher potency.

The present study establishes that an aqueous extract of *Morus nigra's* fruit inhibited BuChE in a concentration dependent manner. *Morus nigra* has been reported to possess tyrosinase inhibitory constituents [24], anti-inflammatory properties [25], and antinoceceptive effect [26]. *Morus nigra* have been shown to substantially reduce the oxidative stress markers and improve the lipid profile status of streptozotocin induced diabetic rats [27]. The results of present study are complementary to the previous studies which have established a considerable cholinesterase inhibiting activity from another plant within the same genus [28]. Morus nigra possess high antioxidant activity which makes it a good candidate to combat the free radicals generated during oxidative stress [29] [30]. Another advantage of using *Morus nigra* is that it already have exhibited a plethora of medicinal applications as it possess anti-microbial activity [31], cancer-suppressive effects [32], for treatment of hepatitis and jaundice[33]. Some species of *Morus* genus have been reported to prevent amyloid beta fibril formation and neurotoxicity [34].

CONCLUSION

It is concluded that an aqueous extract of *Morus nigra's* fruit possesses anti-BuChE and antioxidant activity. Further studies are required to identify, isolate and characterize the phytoconstituents from an aqueous extract of *Morus nigra's* fruit to find the novel molecules which might be useful in alleviating the symptoms associated with AD.

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