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In vitro and *in vivo* Assessment of Cholinesterase Inhibitory Activity of the Bark Extracts of *Pterocarpus santalinus* L. for the Treatment of Alzheimer's Disease

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***Abstract*—**Alzheimer's disease (AD) (a progressive neurodegenerative disorder) is mostly predominant cause of dementia in the elderly. Prolonging the function of acetylcholine by inhibiting both acetylcholinesterase and butyrylcholinesterase is most effective treatment therapy of AD. Traditionally *Pterocarpus santalinus* L. is widely known for its medicinal use. In this study, *in vitro* acetylcholinesterase inhibitory activity was investigated and methanolic extract of the plant showed significant activity. To confirm this activity (*in vivo*), learning and memory enhancing effects were tested in mice. For the test, memory impairment was induced by scopolamine (cholinergic muscarinic receptor antagonist). Anti-amnesic effect of the extract was investigated by the passive avoidance task in mice. The study also includes brain acetylcholinesterase activity. Results proved that scopolamine induced cognitive dysfunction was significantly decreased by administration of the extract solution, in the passive avoidance task and inhibited brain acetylcholinesterase activity. These results suggest that bark extract of *Pterocarpus santalinus* can be better option for further studies on AD via their acetylcholinesterase inhibitory actions.

Keywords*—Pterocarpus santalinus*, cholinesterase inhibitor, passive avoidance, Alzheimer's disease.

I. Introduction

AD, an irreversible neurological disorder, is characterized by cognitive impairment, memory loss, behavioral disturbances and inconvenience in daily activities. Neuropathological occurrence of AD symptoms includes deficiency in acetylcholine, formation of senile plaques and neurofibrillary tangles. Cholinergic deficiency ,occurs due to the degeneration of cholinergic neurons in the basal forebrain part. Reduced cholinergic activity is found as one of the major hallmarks of AD [1]-[3]. There is a correlation between decreased level of acetylcholine (ACh) and cognitive impairment in AD patients. As acetylcholinesterase (AChE) is causing ACh degradation, inhibition of this enzyme is found as a superior way to decrease the severity of AD. Currently, available cholinesterase inhibitors are tacrine, physostigmine, donepezil, rivastigmine, galantamine etc. [4]. However, these inhibitors are effective only in mild to moderate AD and do not reverse the AD progression. Moreover, some of them are reported to have adverse effects such as hepatotoxicity and gastrointestinal disturbances [5]. Therefore, developing a safe, cost effective and active cholinesterase inhibitor form the natural sources has been gaining more attention in recent times.

P. santalinus is a medium sized deciduous plant from the family of Fabaceae. The plant is widely distributed through the tropical regions of the world, especially in India, Sri Lanka, Bangladesh, Taiwan and China. Many important bioactive phytochemical compounds have been isolated and identified from different parts of *P. santalinus*. Generally, bioactive molecules occur in small amounts and have more subtle effects compare to nutrients. These bioactive compounds influence various cellular activities which modify the risk of different disease. A wide range of biological activities and potential health benefits of *P. santalinus* already have been reported in several journals, including antioxidative, antidiabetic, antimicrobial, anticancer, and anti-inflammatory properties, and protective effects on the liver, gastric mucosa, and nervous system [6]-[9]. The major bioactive components present in the bark of *P. santalinus* are santalin A and B, savinin, calocedrin, pterolinus K and L, and pterostilbenes. These bioactive compounds have several potentially important health benefits: such as antioxidants, enzyme inhibitors and inducers, inhibitors of receptor activities, and inducers and inhibitors of gene expression, among other actions [10], [11].

In this study, the hypothesis that the methanolic extract of bark of *P. santalinus* L. can inhibit the activity of the enzyme AChE was tested. Besides this, its ability to enhance memory in scopolamine-induced amnesic mice was also investigated.

II. Materials

A. Drugs and Chemicals

Acetylthiocholine iodide (ATCI), AChE (Electric-eel), dimethyl sulfoxide (DMSO) and 5,5′-dithiobis(2-nitro) benzoic acid (DTNB), physostigmine and scopolamine were collected from Sigma (Japan). Buffers, solvents and other used chemicals were of analytical grade.

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B. Plant Material

Whole plant material of *P. santalinus* (L.) was collected from the Chandpur and Rajshahi, Bangladesh in September, 2017 by the authors, and plant sample was identified by an expert taxonomist. Specimen is stored by the authors in the Department of Pharmacy, East West University, Dhaka, Bangladesh.

C. Extraction of Plant Material

Freshly collected bark from the plant was dried in shade and powdered (500 gram total weight) and finally extracted exhaustively with methanol by cold extraction method. The methanolic extract was then filtered through filter paper and evaporated to dry under reduced pressure in a rotavapor at 45 °C to obtain the crude methanol extract (21.29 g).

D. Animals

As animal model, Swiss Albino Mice of 20–25 g were used for *in vivo* testing (cholinesterase inhibitory activity and Passive shock avoidance test). They are kept in plastic cages in groups of five mice per cage under a controlled environment of 12/12-h lig0ht–dark cycle, at constant temperature 20 ± 2 ^{0}C and humidity of 50±5% and allowed free access to food and water. On the day of the experiment, mice were brought to the experimental room and waited for 60 min before the start of the experiment. This is to adjust the mice habituation to the environmental conditions. Handling and experimentation were conducted in the laboratory, by following international ethical guidelines concerning the care and use of laboratory animals.

E. Animal Treatments

Experimental mice were divided into 6 groups, each group contains 6 animals. The first group, termed as control, received DMSO (1 ml/100 g, i.p.) for seven consecutive days. By intraperitoneal route, each mice of this group were injected with saline on every one hour after the last dose and 30 min before training sessions. The second experimental group received DMSO (1 ml/100 g, i.p.) for seven days. One hour after the last dose and 30 min before training session, mice received scopolamine dissolved in saline (1 mg/kg, i.p.) [12]. Groups 3 to 5 received different dose (100, 200, 400 mg/kg i.p.) for seven days. One hour after the last dose and 30 min before training session, mice received scopolamine dissolved in saline (1 mg/kg, i.p.). Retention of the mice (memory) was recorded after 24 h. Physostigmine, an established anticholinesterase agent (standard), was dissolved in normal saline and administered (0.2 mg/kg, i.p) to positive control group (group 6) followed by scopolamine. Animals that exhibited antiamnestic effects were then sacrificed. Brains were dissected out from the mice and stored at -80 °C till AChE activity estimation.

III. Methods

A. In vitro Cholinesterase Inhibition Assay [13]

With some slight modification Ellman's colorimetric method was applied to run *in vitro* AChE inhibitory assay, where ATCI was used as a substrate. AChE hydrolysis rate was monitored spectrophotometrically. Extract of the plant or standard (various concentrations) was mixed with an enzyme solution (200 µL) and incubated at 37 °C for 30 min. After that, Ellman's reaction mixture which contains 400 µl of 0.35 mM ATCI, 200 µl of 0.7 mM DTNB is mixed in an extraction buffer (50 mM Tris-HCl buffer, 50 mM Magnesium Chloride, 50 mM Sodium Chloride, 1% Triton X-100, pH 8.0) to adjust it to 3 ml of final test tube volume. After incubating the sample and the standard at 37 ^{0}C for 30 min, absorbance was taken. Physostigmine was used as a standard compound. The concentrations of extract that inhibited the hydrolysis of substrate (acetylthiocholine) by 50% (IC_{50}) were determined. Inhibition of the enzyme is monitored by increasing sample concentrations with constant enzyme concentration. After completing the reaction, IC_{50} values were then calculated using a software program (Graph Pad Prism, version 5.01, Inc., 2007, San Diego California USA). The concentration of the compounds which caused 50% inhibition of the AChE activity (IC_{50}) was calculated via nonlinear regression analysis.

B. Passive Shock Avoidance (Step-Through) Paradigm [14]

For passive shock avoidance test, a step-through passive avoidance apparatus for mice was used (Ugo Basile). Each mouse was gone through to two individual sessions, firstly a training session and secondly a test session. The apparatus contains two different compartments, light and dark.

Training session: In the training session, each mouse was trained by gently placing it in the light compartment. When the mouse stepped through the dark compartment putting all its paws on the grid floor, the door automatically closed and an electric shock of 1 mA was delivered for 1 s. Mice that failed to step through within a cutoff time of 90 s were not used.

Test session: After 24 hours of training, every mouse was introduced to the light compartment and the latency to step through to the dark compartment of the chamber was recorded as a passive avoidance behavior indicating memory acquisition of the mice, with an upper cut-off time of 300 second. In the test session no electric shock was delivered to the mice.

C. Estimation of Whole Brain AChE Activity [13]

The estimation of whole brain AChE activity is carried out on the basis of Ellman's colorimetric method with slight modifications. After the behavioral testing, mice were decapitated and brains were dissected out immediately and placed in ice-cold buffer saline. The whole brain was weighed and homogenized in 0.1 M phosphate buffer pH 8 (10%w/v). Homogenized brain tissue was centrifuged for 10 min. 0.4 ml aliquot of the supernatant was added to the other reagents and processed as described before.

D. Statistical Analysis

Every *in vitro* study was repeated three times. IC50 value was determined by response curve and expressed as mean ± standard deviation. Other data of the test were expressed as

mean ± standard error and analyzed by one-way ANOVA followed by Tukey test. Probability values of less than 0.05 were considered statistically significant.

IV. Results

A. In vitro Cholinesterase Inhibition Assay and Determination of IC50

Methanolic extract of the bark of the plant gave significant *in vitro* AChE inhibitory activity against AChE. IC50 of the extract found 0.592±0.067µg/ml whereas IC50 of the standard physostigmine was 0.046 ± 0.017µg/ml.

TABLE I
IN VITRO CHOLINESTERASE INHIBITION ASSAY

Sample	IC 50 (µg/ml) against AChE
Physostigmine	0.046 ± 0.017
P. santalinus extract	0.592 ± 0.067

B. Step-Through Latencies of Mice in Passive Avoidance Paradigm

In the study scopolamine-induced mice showed significant decline in latency time as compared to the control group ($p< 0.001$) of mice. Treatment of mice with *P. santalinus* provides significantly delayed latency times in retention trials in comparison to scopolamine-treated mice ($p< 0.05$, respectively) (Fig. 1). The latency times of mice increases increased extract concentration.

C. Brain AChE Activity

Scopolamine treated mice showed significant high AChE activity in comparison the control group ($p<0.001$) (Fig. 2). Group of mice treated with 400 mg/kg of *P. santalinus* significantly inhibited AChE activity in comparison to scopolamine-treated mice ($p<0.01$). Physostigmine was used as a standard.

V. Discussion

AChE is one of the key enzymes in the cholinergic nervous system. To reverse cholinergic deficiency in AD patients several therapies were designed mostly based on AChE inhibition. These elements enhance cholinergic transmission into the brain with modest and transient therapeutic effects. Several other studies suggested that cholinesterase inhibitors could act on multiple therapeutic targets of AD progression, such as prevention of the formation of beta-amyloid plaques, antioxidant activity and modulation of amyloid precursor protein processing [15]-[17]. However, there is scarcity of a lead compound which is lower in toxicity and side effects as well as higher in central nervous system (CNS) penetration. Many plants form different geographical parts from the world have been examined by bioassay-guided approaches for the identification of a unique inhibitors and different classes of plant-derived natural products have been considered as new AChE inhibitors potentially useful for AD treatment [18]-[20].

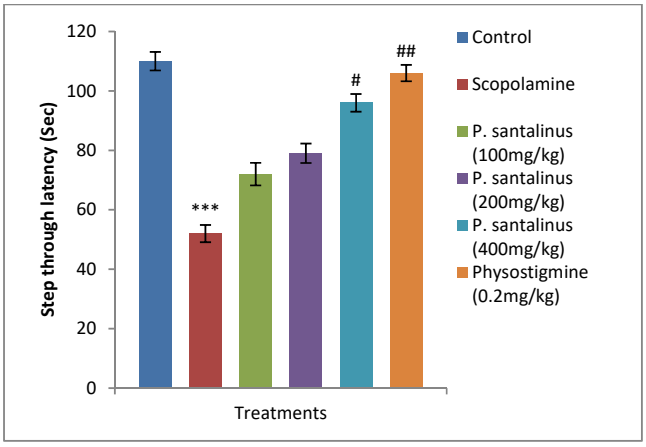

Fig. 1 Effects of *P. santalinus* extract on scopolamine-induced amnesia of a step-through passive avoidance task in mice. The extract was intraperitoneally administered for 7 days. Scopolamine (1 mg/kg, i.p.) was injected 1 h following treatments of day 7, followed 30 min later by the training session. Step through latency values are presented as means ± S.E.M. (n = 6). #p<0.05, ##p< 0.01, compared to scopolamine-treated group, ***p<0.001 compared to control group (one-way ANOVA followed by Tukey test)

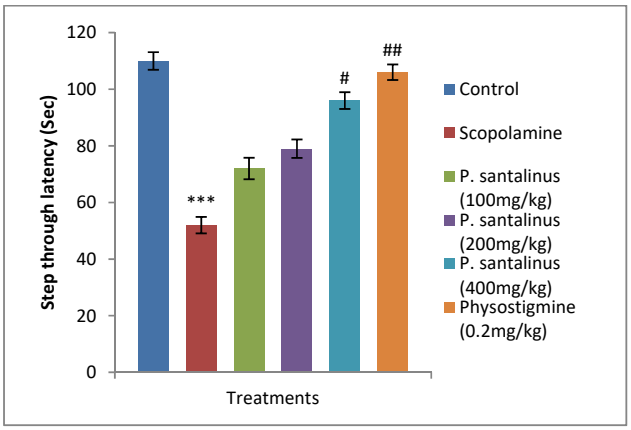

Fig. 2 Inhibitory effects of *P. santalinus* extracts against brain AChE activity in scopolamine treated mice. Inhibition value of AChE (%) was calculated and compared to that of control group. Data represent the mean ±S.E.M (n=6) and were analyzed by one-way ANOVA followed by Tukey test. #p<0.05, ##p< 0.01 compared to scopolamine-treated group, ***p< 0.001 compared to control group

This study showed that the methanolic bark extract of the *P. santalinus* exhibited cholinesterase inhibitory activity in both *in vitro* and *in vivo* conditions. In this study, passive avoidance step through model was used to evaluate memory retention of mice. The results prove that 400 mg/kg of green *P. santalinus* for seven days increased memory retention. This extract increased stepdown latency of mice significantly. The *in vivo* AChE activity has been shown to be increased within and around amnesic brain of scopolamine induced amnesia. In case of scopolamine induced amnesia, activity of AChE is increased because of increased calcium influx followed by oxidative stress in the cell. In this study, the AChE activity in the brain was increased in mice treated with Scopolamine

when compared with the normal. Increase in AChE level was attenuated by bark extract of *P. santalinus*. Bark of *P. santalinus* is a rich source of different types of terpenoids, triterpenes, sesquiterpenes, and related phenolic compounds such as β-sitosterol; lupeol; epicatechin; lignans and pterostilbenes.[11] They possess high therapeutic potentials like anticancer, antioxidant, anti-inflammatory activities and hepatoprotective activities either independently or synergistically. [12] Recent findings reveal that terpenoids have potential neuroprotective effects against ischemic and glutamatergic neurotoxicity, 6-hydroxydopamine toxicity and oxidative stress [21]-[23].These terpenoids, on the other hand, due to their small molecular size and lipophilicity, readily cross the blood-brain barrier and are effective in the treatment of AD.

VI. Conclusion

In conclusion, the findings of this study suggested that imply memory enhancing property of *P. santalinus* in mice, Hence, its bark extract could be useful in conditions associated with neurodegenerative disorders of Alzheimer's type.

References

[1] Nagori, B. P., & Solanki, R. (2011). Role of medicinal plants in wound healing. *Research Journal of Medicinal Plant*, *5*(4), 392-405.

[2] Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., ... & Herrup, K. (2015). Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, *14*(4), 388-405.

[3] Weiner, M. W., Veitch, D. P., Aisen, P. S., Beckett, L. A., Cairns, N. J., Cedarbaum, J., ... & Luthman, J. (2015). 2014 Update of the Alzheimer's Disease Neuroimaging Initiative: a review of papers published since its inception. *Alzheimer's & dementia*, *11*(6), e1-e120.

[4] Dubois, B., Padovani, A., Scheltens, P., Rossi, A., & Dell'Agnello, G. (2016). Timely diagnosis for Alzheimer's disease: a literature review on benefits and challenges. *Journal of Alzheimer's disease*, *49*(3), 617-631.

[5] Ali, T. B., Schleret, T. R., Reilly, B. M., Chen, W. Y., & Abagyan, R. (2015). Adverse effects of cholinesterase inhibitors in dementia, according to the pharmacovigilance databases of the United-States and Canada. *PLoS One*, *10*(12), e0144337.

[6] Arunakumara, K. K. I. U., Walpola, B. C., Subasinghe, S., & Yoon, M. H. (2011). Pterocarpus santalinus Linn. f. (Rath handun): A review of its botany, uses, phytochemistry and pharmacology. *Journal of the Korean Society for Applied Biological Chemistry*, *54*(4), 495-500.

[7] Rao, B. K., Giri, R., Kesavulu, M. M., & Apparao, C. H. (2001). Effect of oral administration of bark extracts of Pterocarpus santalinus L. on blood glucose level in experimental animals. *Journal of Ethnopharmacology*, *74*(1), 69-74.

[8] Bulle, S., Reddyvari, H., Nallanchakravarthula, V., & Vaddi, D. R. (2016). Therapeutic potential of Pterocarpus santalinus L.: An update. *Pharmacognosy reviews*, *10*(19), 43.

[9] Wu, S. F., Chang, F. R., Wang, S. Y., Hwang, T. L., Lee, C. L., Chen, S. L., ... & Wu, Y. C. (2011). Anti-inflammatory and cytotoxic neoflavonoids and benzofurans from Pterocarpus santalinus. *Journal of natural products*, *74*(5), 989-996.

[10] Kondeti, V. K., Badri, K. R., Maddirala, D. R., Thur, S. K. M., Fatima, S. S., Kasetti, R. B., & Rao, C. A. (2010). Effect of Pterocarpus santalinus bark, on blood glucose, serum lipids, plasma insulin and hepatic carbohydrate metabolic enzymes in streptozotocin-induced diabetic rats. *Food and Chemical Toxicology*, *48*(5), 1281-1287.

[11] Wu, S. F., Hwang, T. L., Chen, S. L., Wu, C. C., Ohkoshi, E., Lee, K. H., ... & Wu, Y. C. (2011). Bioactive components from the heartwood of Pterocarpus santalinus. *Bioorganic & medicinal chemistry letters*, *21*(18), 5630-5632.

[12] Rush, D. K. (1988). Scopolamine amnesia of passive avoidance: a deficit of information acquisition. *Behavioral and Neural Biology*, *50*(3), 255-274.

[13] Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2), 88-95.

[14] Jarvik, M. E., & Kopp, R. (1967). An improved one-trial passive avoidance learning situation. *Psychological reports*, *21*(1), 221-224.

[15] Craig, L. A., Hong, N. S., & McDonald, R. J. (2011). Revisiting the cholinergic hypothesis in the development of Alzheimer's disease. *Neuroscience & Biobehavioral Reviews*, *35*(6), 1397-1409.

[16] Castello, M. A., & Soriano, S. (2014). On the origin of Alzheimer's disease. Trials and tribulations of the amyloid hypothesis. *Ageing research reviews*, *13*, 10-12.

[17] Chang, Y. T., Chang, W. N., Tsai, N. W., Huang, C. C., Kung, C. T., Su, Y. J., ... & Lu, C. H. (2014). The roles of biomarkers of oxidative stress and antioxidant in Alzheimer's disease: a systematic review. *BioMed research international*, *2014*.

[18] Mathew, M., & Subramanian, S. (2014). *In vitro* screening for anti-cholinesterase and antioxidant activity of methanolic extracts of ayurvedic medicinal plants used for cognitive disorders. *PloS one*, *9*(1), e86804.

[19] Biswas, K., Azad, A. K., Sultana, T., Khan, F., Hossain, S., Alam, S., ... & Khatun, Y. (2017). Assessment of in-vitro cholinesterase inhibitory and thrombolytic potential of bark and seed extracts of Tamarindus indica (L.) relevant to the treatment of Alzheimer's disease and clotting disorders. *Journal of intercultural ethnopharmacology*, *6*(1), 115.

[20] Biswas, K., Islam, A., Sharmin, T., & Biswas, P. K. (2015). In-vitro cholinesterase inhibitory activity of dry fruit extract of Phyllanthus emblica relevant to the treatment of Alzheimer's disease. *J Phytopharmacol*, *4*, 5-8.

[21] Chang, H. J., Kim, H. J., & Chun, H. S. (2007). Quantitative structure–activity relationship (QSAR) for neuroprotective activity of terpenoids. *Life sciences*, *80*(9), 835-841.

[22] Grassmann, J., Hippeli, S., & Elstner, E. F. (2002). Plant's defence and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiology and Biochemistry*, *40*(6-8), 471-478.

[23] Yoo, K. Y., & Park, S. Y. (2012). Terpenoids as potential anti-Alzheimer's disease therapeutics. *Molecules*, *17*(3), 3524-3538.