IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 5 Ver. V (Sep – Oct 2018), PP 21-25
www.iosrjournals.org

Screening of indigenous plants for acetylcholinesterase inhibition by TLC bioautographic method

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Abstract: *Acetylcholinesterase inhibition is considered as the most suitable therapeutic strategy for Alzheimer's disease. As synthetic drugs lack selective activity, efficacy and bioavailability, many traditional remedies have been explored for improving cognitive function. In the present study, indigenous plants namely Mandookaparni, Liquorice, Gaduchi, Long pepper and Ukshi were screened for acetylcholinesterase inhibition. Methanolic extracts of selected plants were prepared by soxhlet extraction and subjected to preliminary solvent-solvent extraction using dichloromethane and ethyl acetate. All the fractions were screened for acetylcholinesterase inhibition by a modified thin layer chromatography bioautographic method. Ethyl acetate fractions of Mandookaparni and Liquorice exhibited inhibition at Rf 0.74 and 0.76, respectively. Aqueous methanol fraction of Gaduchi showed significant inhibition at Rf 0.61 and 0.8 while this was at Rf 0.68 for Long pepper. The presence of active constituents like flavonoids, saponins and alkaloids seem to be responsible for the inhibitory activity observed in the present study. The selected indigenous plants could be important in the management of AD and identification of active constituents and further evaluation of their clinical efficacy and potential toxicity in larger trials is very much required.*

Keywords: *Acetylcholinesterase, active constituents, bioautography, Indian Systems of Medicine, inhibition.*

Date of Submission: 12-10-2018 Date of acceptance: 28-10-2018

I. Introduction

Elderly people commonly suffer from Alzheimer's disease (AD) which is the most common cause of senile dementia. The disease has the potential of creating financial burden to both caregivers and national economies as the elderly population increases steadily in coming decades. The parts of brain which are associated with higher mental function viz. neocortex and hippocampus are damaged because of the accumulation of β-amyloid precursor protein outside the cells. There is also formation of neurofibrillary tangles. This is because of the hyperphosphorylation of the microtubule-associated protein tau which forms insoluble aggregates filling the entire intracellular space of a neuron. Moreover, degeneration of neuronal synapses and pyramidal neurons is seen with a significant reduction in the levels of the neurotransmitter acetylcholine (ACh) [1]. This leads to gross and progressive damage of cognitive function and the patients suffer from loss of memory, aggression, depression and wandering.

Ach is degraded into choline and acetyl group by acetylcholinesterase (AChE) after it serves its function in the synapses in the central and peripheral nervous systems [2]. Increasing the concentration and thereby the duration of action of synaptic ACh using AChE inhibitors is considered as the most suitable therapeutic strategy for AD. AChE inhibitors like galanthamine (an alkaloid from *Galanthus woronowii*), rivatigmine (a carbamate), tacrine (an acridine-based rapidly reversible cholinesterase inhibitor) and donezepil (a piperidine cholinesterase inhibitor) are the drugs available for the management of cognitive dysfunction and memory loss associated with AD [2]. But these drugs lack selective activity, efficacy and bioavailability and produce adverse peripheral cholinergic side effects such as nausea, vomiting, diarrhea, dizziness and hepatotoxicity [3]. Plants are an attractive source for finding better AChE inhitors with fewer side effects. Indian Systems of Medicine viz. Ayurveda, Siddha and Unani-Tibb is known for rich usage of plants for improvement of cognition. Several plants like *Bacopa monniera, Centella asiatica, Convolvulous pluricauli, Emblica officinalis, Evolvulous alsinoides* and *Withania somnifera* have been widely used to treat cognitive disorders [4, 5]. In this context, it was planned to screen indigenous plants viz. Mandookaparni, Liquorice, Gaduchi, Long pepper and Ukshi for acetylcholinesterase inhibition.

II. Materials and Methods

Plant material Collection

Mandookaparni (Centella, aerial parts of *Centella asiatica* (L.) Urban), Liquorice (roots and rhizomes of *Glycyrrhiza glabra* Linn.), Gaduchi (leaf and stem of *Tinospora cordifolia* (Thunb.) Miers.) and Long pepper (fruits of *Piper longum* Linn.) were purchased from the local Ayurvedic shop. Ukshi (leaf and stem of *Calycopteris floribunda*) was collected from areas around Pune of Maharashtra and identified by Dr. R.B. Deshmukh, Head, Dept. of Botany, Agricultural Development Trust's SP Mahila Mahavidyalaya, Shardanagar, Baramati.

Extraction of Plant materials and Phytochemical investigation

The plant materials were shade dried if needed, coarsely powdered (40 mesh size) and extracted with methanol using a soxhlet extractor. The extracts were concentrated under vacuum using a rotary evaporator and subjected to phytochemical investigation.

Fractionation by solvent-solvent extraction

The methanolic extracts were subjected to preliminary fractionation by solvent-solvent extraction so that the components are separated into groups of compounds sharing similar physicochemical characteristics**.** The extract (5 gm) was suspended in 100 ml 80% methanol and extracted successively with 50 ml of dichloromethane and ethyl acetate three times. All the three fractions were concentrated under vacuum using a rotary evaporator.

Thin layer chromatography

The fractions were dissolved in respective solvents viz. dichloromethane, ethyl acetate and methanol to a concentration of 10 mg/ml, of which 5 µl were spotted on TLC plate (Silica gel 60 F_{254} Merck) and developed using different solvent systems [6]. The developed TLC plates were visualized under ultraviolet (UV) light (254 nm and 365 nm) and sprayed with appropriate visualizing agents.

Thin layer chromatography (TLC) bioautographic assay

The fractions were screened for acetylcholinesterase inhibition by a modified TLC bioautographic method according to Yang et al. [7]. Fractions were spotted on TLC plates and developed with suitable solvent systems. The enzyme solution and 1-naphthyl acetate solution were sprayed to TLC plates subsequently. After each solution was sprayed, TLC plate was blown quickly with cold wind from a hair dryer until no free liquid flowing on it. The TLC plate was put in a plastic box containing a little water in a small cup for humidity. The plate was then incubated at 37^{o} C for 20 min. The solution of Fast Blue B salt was then sprayed onto the TLC plates. The inhibited acetylcholinesterase spots appeared white and other parts purple.

III. Results and Discussion

Extraction of Plant materials and Phytochemical investigation

The different extracts were all semi-solid and the yields varied between 12 to 16.8 %. The results of phytochemical investigation are presented in Table 1. The different fractions were again subjected to phytochemical screening. In general, flavonoids were found to be concentrated in ethyl acetate fractions followed by presence in aqueous methanol fractions. Saponins were detected in both ethyl acetate and aqueous methanol fractions of Mandookaparni and Liquorice. Alkaloids were seen in both dichloromethane and aqueous methanol fractions of Long pepper while they were found in aqueous methanol fraction of Gaduchi.

TLC Bioautographic assay

The AChE inhibitory activity was performed after the development of suitable mobile phase solvent system for each fraction. Ethyl acetate fraction of Mandookaparni and Liquorice showed significant AChE inhibition (Fig. 1). Ethyl acetate fraction of Mandookaparni developed with the solvent system chloroform: methanol: acetic acid (9:0.5:0.5) exhibited inhibition at Rf 0.74. In case of ethyl acetate fraction of Liquorice developed with ethyl acetate: formic acid: water (9:1:1), significant inhibition was seen at Rf 0.76.

Aqueous methanol fraction of Gaduchi and Long pepper showed AChE inhibition (Fig. 2). Aqueous methanol fraction of Gaduchi developed with the solvent system chloroform: methanol: diethyl amine (10:4:0.5) exhibited significant inhibition at Rf 0.61 and 0.8. In case of aqueous methanol fraction of Long pepper developed with chloroform: methanol: ammonia (10:4:1), inhibition was seen at Rf 0.68.

Acetylcholinesterase inhibitors have therapeutic importance in the treatment of Alzheimer's disease and other neurodegenerative diseases. Acetylcholine in the central nervous system plays an important role in regulating cognitive functions. Degeneration of cholinergic neurons in hippocampal area is commonly seen in AD. The therapeutic strategy of utilizing AChE inhibitors for enhancement of central cholinergic activity is usually employed in the management of AD [8].

In the present study, ethyl acetate fractions of Mandookaparni and Liquorice showed significant AChE inhibition in a TLC bioautographic assay. The whole plant of *C. asiatica* was shown to be beneficial in

improving memory and the general mental ability of mentally retarded children [9, 10]. In pharmacological studies, the plant extract was reported to improve the maze learning in rats [11]. Hydrogen peroxide-induced cell death and free radical concentrations were shown to be reduced by asiaticoside derivatives. These were also found to inhibit beta-amyloid cell death *in vitro* [12]. In the present study, ethyl acetate fraction of Mandookaparni was found to contain saponins and flavonoids and the observed inhibition could be due to these active constituents. Liquorice was shown to significantly enhance memory learning in dementia disorder by Dhingra et al [13]. Its antioxidant property could be responsible for improving the memory by decreasing damage to brain by free radicals. Ethyl acetate fraction of Liquorice in the present study was found to contain flavonoids which are known antioxidants.

Also, aqueous methanol fractions of Gaduchi and Long pepper exhibited inhibition in the present study. *T. cordifolia* was shown to overcome cyclosporine induced memory deficit possibly by immunostimulation and increasing the synthesis of acetylcholine [14]. This central action could supplement the effect of choline which is an important constituent of *T. cordifolia*. Methanolic stem extract was found to be a potent inhibitor of acetylcholinesterase in a TLC bioautographic study [15]. Many alkaloids have been shown to posses AChE inhibition activity [4] and in the present study, aqueous methanol fraction of Gaduchi showed the presence of alkaloids. A phenolic active constituent isolated from *Piper betel*, hydroxychavicol, was found to significantly improve cognitive function in experimentally induced AD in rat [16]. Also, methanolic extracts of stems of *Piper interruptum* Opiz., seeds of *Piper nigrum* L., showed significant inhibitory activity on acetyl cholinesterase in Ellman's colorimetric method [17]. As Long pepper belongs to the *Piper* genus, it may contain such similar phenolic compounds in the aqueous methanol fraction.

There is a huge demand for new drugs for neurodegenerative diseases but the search is not economical and efficient evaluation methods are lacking. In recent times, a change in the strategy from a single-target to a multi-target drug therapy is seen in the treatment of chronic and complex diseases. Many phytochemicals have shown promising anti-cholinesterase activity [4] and as a result, the search for AChE inhibitors from plants has received greater momentum in recent years. Ayurvedic traditional remedies and their constituents have been increasingly employed as nerviness 'nervines' to enhance the nervous system functioning to prevent and restore loss of memory [18]. The presence of active constituents like flavonoids, saponins and alkaloids seems to be responsible for the inhibitory activity observed in the present study. The selected indigenous plants could be important in the management of AD and identification of active constituents and further evaluation of their clinical efficacy and potential toxicity in larger trials is very much required.

IV. Figures And Tables

Table 1: Phytochemical investigation of methanolic extracts

PHYTOCNSTITUENTS	Plant extracts				
	Mandookaparni	Liquorice	Gaduchi	Long pepper	Ukshi
Carbohydrates	+	+	+	+	+
Proteins	-	-	-	+	-
Amino acids	+	+	+	+	+
Fats and Fixed oil	-	-	-	-	-
Steroids	-	-	-	-	-
Triterpenoids	+	+	-	-	-
Glycosides	-	-	-	-	-
Saponins	+	+	-	-	-
Flavonoids	+	+	+	+	+
Alkaloids	-	-	+	+	-
Tannins/phenols	+	+	+	+	+

'+': phytochemical present; '-': phytochemical absent

Fig. 1: AChE inhibition bioautogram of Mandookaparni and Liquorice

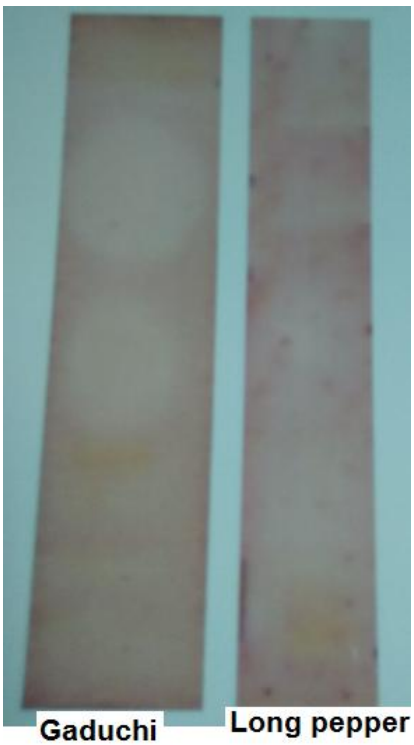

Fig. 2: AChE inhibition bioautogram of Gaduchi and Long pepper

V. Conclusion

Herbal medicine offers several options to modify the progress and symptoms of AD. There has been a new trend in the preparation and marketing of drugs based on medicinal plants, and their scientific and commercial significance appears to be gathering momentum in health-relevant areas. The strong knowledge base of Ayurveda can be coupled with combinatorial sciences and high-throughput screening techniques in drug discovery campaigns and development process thereby providing new functional leads for AD and other age-associated neurodegenerative diseases. In the present study, indigenous plants like Mandookaparni, Liquorice,

Gaduchi and Long pepper have shown significant acetylcholinesterase inhibition in TLC bioautographic method. The plants need to be studied further for identification of active constituents and clinical efficacy.

Acknowledgements

The authors are thankful to Vision Group on Science and Technology (VGST), Dept. of Science & Technology, Government of Karnataka for the financial support and also to the Principal and Management of BLDEA's SSM College of Pharmacy and Research Centre, Vijayapur, Karnataka for providing facilities.

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E.N. Gaviraj " Screening of indigenous plants for acetylcholinesterase inhibition by TLC bioautographic method.." .IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.5 (2018): 21-25.