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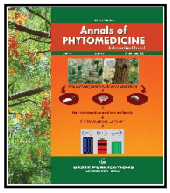

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Nootropic evaluation of methanolic bark extract of *Murraya koenigii* (L.) Spreng. against scopolamine and diazepam induced memory dysfunction in mice

Mahreen Siddique*,, Humaira Majeed Khan*, Kanwal Asif*,**, Farah Syed*, Humayun Riaz**, Muhammad Sameer Ashaq◆, Abdul Raheem Malik**, Fahad Asim**, Muzammil Raza** and Sanaullah Umair*****

Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan-250012, China

**Institute of Pharmacy, Lahore College for Women University, Jail Road, Lahore, Pakistan*

***Department of Pharmacology, Rashid Latif College of Pharmacy, 35-KM, Ferozepur Road Lahore, Pakistan*

****Indus Hospital, Mission Colony, Kot Radha Krishna Road, Raiwind City, Lahore, Punjab, Pakistan*

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Abstract

This study is aimed to investigate the nootropic potential of different doses (400, 500 and 600 mg/kg) of methanolic bark extract of *Murraya koenigii* (L.) Spreng. in healthy or amnesic mice induced by scopolamine and diazepam. The passive avoidance paradigm (PAP) and elevated plus maze (EPM) were used as behavioral models for memory assessment. Mice were subjected to scopolamine and diazepam induced amnesic models. Different concentrations of methanolic bark extracts of *M. koenigii* were administered orally for 15 days. Post behavioral analysis acetylcholine content was determined in brain extract. Preliminary qualitative phytochemical study of methanolic bark extract of *M. koenigii* expressed the existence of alkaloids, flavonoids, saponins, steroids, phenols, glycosides and tannins. There were significantly decrease in transfer latencies on EPM and increase in step down latencies on PAP. Additionally, 600 mg/kg dose significantly ($p<0.001$) attenuated the amnesic effect induced by scopolamine and diazepam. The 500 and 600 mg/kg concentrations of methanolic bark extract significantly ($p<0.05$) enhanced the role of acetylcholine in memory improvement. The carbazole alkaloids present in *M. koenigii* crude bark extract have significant nootropic effect as it increases acetylcholine content in brain at a dose of 500 and 600 mg/kg concentrations. Thus, the methanolic extract of *M. koenigii* bark may prove to be an effective nootropic agent in management of Alzheimer's disease having its underlying mechanisms that involves increasing cholinergic transmission.

1. Introduction

Alzheimer's disease (AD) is a multifactorial progressive age-related neurodegenerative disease with dementia as chief cause in aged people (Kumar and Singh, 2015). It is characterized by progressive loss of behavioral, mental, ability to learn and function. More than 80% of worldwide dementia cases in elders are due to AD (Anand *et al*., 2014). Pathogenesis of AD comprises of senile plaques consist of extracellular deposition of neurofibrillary tangles and beta-amyloid consist of dense fibers of altered microtubular-associated protein (tau protein) in neuron body (Akram and Nawaz, 2017). The currently available pharmacological agents against Alzheimer's disease are acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor antagonists (Silvestrelli *et al*., 2006). There are five drugs approved by FDA (Food and Drug Administration) for symptomatic relief of Alzheimer's disease along with cognitive improvement such as rivastigmie, galantamine, tacrine, donepezil, and memantine (Auld *et al*., 2002).

Corresponding author: Dr. Muhammad Sameer Ashaq

Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan-250012, China

E-mail: muhammadsameer615@gmail.com

Tel.: +86-17865137857

Ethnomedicinal studies are valuable source to identify potentially active drugs (Physostigmine from *P. venenosum*) which also provided the lead compound to develop synthetic drugs (Rivastigmine based on chemical structure of physostigmine) to cure the symptoms of AD. Although, the drug development with optimal efficacy and safety by modification in phytochemical of botanical origin is one of the aim, but the ethnomedicine is still popular (Howes and Houghton, 2012). *M. koenigii* plant is also known as curry leaf tree with habitat in Asia and originating all over Indian subcontinent. It is not only used to enhance food flavor but its leaves, fruits and bark are also used as folk medicine to cure a number of disorders such as diabetics, cancer, bacterial infections, hyperlipidemia, amnesia and pain (Chauhan *et al*., 2017). The phytochemical constituents of *M. koenigii* plant include a wide variety of carotenoids (lutein, carotene, α tocopherol), carbazole alkaloids and essential oils (Tee and Lim, 1991).

The current investigation was carried out to study nootropic evaluation of methanolic bark extract of *M. koenigii*, its possible protective potential in memory deficit mice models and to identify its mechanism of action by assessing its effect on brain acetylcholine content in mice.

2. Materials and Methods

2.1 Preparation of methanolic extract

Freshbarks *of M. koenigii* were taken from local garden of Lahore, Pakistan. These were authenticated and preserved (LCWU-15-84)

in the herbarium of Botany Department of Lahore College for Women University (LCWU), Pakistan.

The collected barks of *M. koenigii* were dried under shadow for 25 days following crushing into coarse powder. The coarse powder (300 g) was extracted with 95% methanol (1000 ml) utilizing the Soxhlet apparatus and dried by rotary evaporator (Anjaneyulu *et al*., 2017). Percentage yield of methanolic extract was calculated by below principle:

$$\text{Percentage (\%) yield} = \frac{\text{Weight of dried methanolic extract}}{\text{Weight of dry powdered bark sample}} \times 100$$

The extract was stored in closed container at 4°C till used for pharmacological studies by preparing suspension in water using tween 80 as suspending agent.

2.2 Experimental animals

Seventy locally bred male albino mice (25-35 g) were bought from U.V.A.S (University of Veterinary and Animal Sciences), Lahore and acclimatized at animal house of Institute of Pharmacy, LCWU, Lahore. The mice were fed at standard rodent diet with water *ad libitum* throughout experiment. All investigational measures were performed in accordance to approved guidelines from ECUA (Ethical Committee for use of Animal at Lahore College for Women University, Lahore, Reference: Dir./LCWU/ECUA-16-1510/01 May 24, 2021).

2.3 Phytochemical analysis

The methanolic bark extract of *M. koenigii* was qualitatively analyzed for the occurrence of phytochemicals, *e.g*., alkaloids, glycosides, tannins, saponins, steroid, flavonoids and phenolic content. Alkaloids were identified by Wagner's test by adding 1.0 ml of 1% HCl in 3 ml of extract then heat was provided for 20 min following cooling and filtration. Two drops of Wagner's reagent were added to 1.0 ml of filtrate, change in color was observed. Ferric chloride test was used for phenolic content adding two drops of 5 % ferric chloride in 1.0 ml of extract, change in the color was observed. For Salkowski test for cardiac glycosides, 1 ml extract was added in 2 ml chloroform or H_2SO_4 and change in the color was noted. Saponins were identified by Frothing test by shaking vigorously 2.0 ml of extract with water for 2 min and heated, appearance of froth upon warming was observed. Five drops of H_2SO_4 were instilled to 1 ml extract; color change was identified to ensure steroids. The presence of tannins was tested by addition of 1 ml extractin 1.0 ml of freshly formulated potassium hydrooxide solution, formation of precipitates was observed. Shinoda's test was used for flavonoids identification adding few drops of concentrated HCl in 1.0 ml of extract; change in the color was observed (Gul *et al*., 2017).

2.4 Acute toxicity evaluation

OECD (Organization for economic co-operation and development) guideline 423 was followed to study acute toxicity of methanolic bark extract of *M. koenigii*. Six male albino mice were administered 5 mg/kg dose of extract and mortality was observed for 24 h and further alertness, change in skin and fur were observed for 2 weeks (14 days). 5 mg/kg dose was mentioned as safe as no mortality was observed and further next dose levels 50 mg/kg, 300 mg/kg and 2000 mg/kg of methanolic extract were evaluated for acute toxicity studies in similar manner (Mani *et al*., 2012)

2.5 Experimental design

To evaluate the nootropic potential of methanolic bark extract of *M. koenigii*, mice were grouped by random selection (n = 5/group), weighed and marked. A pilot study was conducted at three dose levels, *i.e*., treatment group I (400 mg/kg), group II (500 mg/kg), group III (600 mg/kg) of methanolic bark extract of *M. koenigii*. Control group (water) and standard group (piracetam 400 mg/kg; i.p. for seven days), doses were administered orally for 15 consecutive days before behavioral analysis for memory scoring. After 15 days oral administration of extract, EPM (elevated plus maze) and PAP (passive avoidance paradigm) models were selected to determine the dose-dependent effect of methanolic extract of *M. koenigii* bark. After that, 500 and 600 mg/kg doses, *i.e*., treatment group A (500 mg/kg + scopolamine), treatment group B (600 mg/kg + scopolamine), treatment group C (500 mg/kg + diazepam) and treatment group D (600 mg/kg + diazepam) were given orally for 15 days and tested against memory deficit effect of scopolamine (induced group 1) and diazepam (induced group 2). At 15th day, 90 min after the last dose, 0.4 mg/kg scopolamine and 1mg/kg diazepam were injected intraperitoneally to respective groups. Behavioral analysis was done after 45 min of induced drug. After behavioral analysis, mice were decapitated, *via* cervical dislocation and brains were extracted for neurochemical analysis.

2.6 Memory assessment

2.6.1 EPM (elevated plus maze) model

EPM model was subjected for spatial long term-memory assessment. In two sessions, memory assessment was recorded. In training session, the mouse was placed at any of two opened arms facing opposite to central square of maze. Then, mouse was allowed to go in to one of closed arms with its all four paws. Time (sec) for entrance of mouse to closed arms was recorded as transfer latency. After 24 h in memory retention session, same procedure was done. After 24 h, decline in transfer latency (sec) was designated as memory improvement index (Batool *et al*., 2016).

2.6.2 PAP (passive avoidance paradigm) model

PAP apparatus was employed to estimate the long duration memory by negative reinforcement method. In the course of the training session, mouse was delicately positioned on the wooden platform, as it stepped down on the grid from platform with all of its paws; electric current was delivered to the grid for 15 s. Only those mice were considered to enter the second session showing step down latency ranging between 2-15 s. After 24 h, same procedure was applied without electric current and step down latency (sec) was noted with stopwatch. An increase in SDL (sec) after 24 h was taken as memory improvement index (Mani *et al*., 2012).

2.7 Neurochemical analysis

2.7.1 Brain acetylcholine content estimation

Hestrin (1949) proposed a method to estimate brain acetylcholine content which was used in this study. After the behavioral analysis, mice were decapitated and brains were isolated. The enzyme and acetylcholine release in brain was inactivated by boiling the brain for 10 min. 2 ml distilled water was used to homogenize the brain

tissues. Then alkaline hydroxylamine hydrochloric acid (1 ml) and hydrochloric acid solution (1:1) with water were added in homogenate following mixing and centrifugation. The supernatant was separated and 0.37 M ferric chloride solution of 0.5 ml was added to it. Brown colored solution was formed and further absorbance was measured at wavelength 540 nm on spectrophoto meter against reagent blank

2.8 Statistical analysis

The values of results were measured as mean ± SEM (Standard error of mean). SPSS (Version 17) software was used to evaluate data by using one-way ANOVA and Tukey Kramer Multiple comparison.

3. Results

Methanolic extract of *M. koenigii* was prepared and dried. Percentage yield of *M. koenigii* bark extracted in methanol was 11.14%.

3.1 Phytochemical analysis

The phytochemical analysis showed the occurrence of alkaloids, glycosides, saponins, steroids, phenols, flavonoids, and tannins. Dark reddish brown colored precipitates by adding wagner's reagent, indicated the presence of alkaloids. In salkowski test for glycosides, reddish brown colored interface indicated the presence of glycosides. Persisted froth on standing was obtained in form test which directed the saponins presence. Reddish coloration by addition of few drops of H_2SO_4 indicated the presence of steroids. Addition of ferric chloride solution with extract gave greenish black appearance to solution which indicated the presence of phenols. Red coloration in Shgalantainoda's test indicated the presence of flavonoids. In alkaline reagent test, no creamy white coloration indicated the absence of tannins.

3.2 Acute toxicity studies

As shown in Table 1, no mortality was observed at oral dose of 2000 mg/kg *M. koenigii* bark extract. Hence, remaining doses (400 mg/kg, 500 mg/kg, and 600 mg/kg) were subjected to assess more pharmacological activity.

3.3 Memory assessment

The methanolic bark extract of *M. koenigii* has not shown mortality up to 2000 mg/kg oral dose. Therefore, 400, 500 and 600 mg/kg doses were subjected for more pharmacological assessments. Dose related impacts of above doses of methanolic bark extract on memory function were calculated by EPM and PAP models. Groups II and III treated with 500 and 600 mg/kg significantly decreased the transfer latency (TL), whereas, the transfer latency of treatment group III treated with 600 mg/kg extract was comparable with group administered with standard (piracetam). On PAP model, all the treated groups exhibited significant increase in step-down latencies (SDL). Whereas, the step-down latency and memory enhancing effects of group treated with 600 mg/kg were comparable with group administered with standard (piracetam). These results demonstrated that 500 and 600 mg/kg dose levels of methanolic bark extract of *M. koenigii* had optimal memory enhancing effect in healthy mice and hence, such doses were designated for additional pharmacological investigations in amnesic mice induced by scopolamine and diazepam.

Table 1: Acute toxicity evaluation of *M. koenigii* bark extract

Extract doses	Vigilance	Skin or fur variations	Mortality
5 mg/kg	+	-	Survived
50 mg/kg	+	-	Survived
300 mg/kg	+	-	Survived
2000 mg/kg	+	-	Survived

Present +
Absent -

Table 2: Effect of methanolic bark extract of *M. koenigii* on memory scores in healthy and memory deficit mice model (n=5)

Groups	Transfer latency time on EPM (sec)	Step-down latency time on PAP (sec)
Control group (water)	26.7 ± 0.8	185.6 ± 1.7
Standard group (piracetam)	11.2 ± 0.6[a]	244.6 ± 2.0[a]
Treatment group I (400 mg/kg)	23.4 ± 0.9	200.0 ± 3.2[a]
Treatment group II (500 mg/kg)	18.0 ± 0.7[a]	206.6 ± 1.6[a]
Treatment group III (600 mg/kg)	13.6 ± 0.9[a]	231.4 ± 2.5[a]
Induced group 1 (scopolamine)	46.8 ± 0.8[a]	93.0 ± 1.6[a]
Induced group 2 (diazepam)	50.8 ± 1.2[a]	109.0 ± 2.3[a]
Treatment group A (500 mg/kg + scopolamine)	31.6 ± 1.5[ab]	156.6 ± 1.6[ab]
Treatment group B (600 mg/kg + scopolamine)	25.0 ± 1.1[b]	184.8 ± 1.4[b]
Treatment group C (500 mg/kg + diazepam)	36.8 ± 2.1[ac]	126.8 ± 4.0[ac]
Treatment group D (600 mg/kg + diazepam)	29.8 ± 1.4[ac]	170.4 ± 3.5[ac]

Values are expressed as mean ± SEM (p<0.05), [a] shows significant difference of control group with standard, induced and treatment groups, [b] shows significant difference of induced group with treatment group A and B and [c] shows significant difference of standard group with treatment group II, A and B.

Memory was assessed after administration of 500 and 600 mg/kg of extract in mice having memory impairment induced by scopolamine and diazepam. Transfer latency on EPM 24 h after training session (memory retention session) showed that induced group I (0.4 mg/kg scopolamine; I.P) remarkably elevated transfer latency in comparison to control group. Post hoc test showed that the transfer latency of treatment group A (500 mg/kg + scopolamine) and B (600 mg/kg + scopolamine) significantly decreased as compared to induced group 1 (scopolamine). In memory retention session (24 h after training session), step-down latency on PAP showed that induced group I (scopolamine) significantly decreased the step-down latency as compared to control group. Treatment group A (500 mg/kg + scopolamine) and B (600 mg/kg + scopolamine) significantly increased step-down latency as compared to induced group 1 (scopolamine). Results are expressed in Table 2.

In memory retention session (24 h after training session), transfer latency on EPM showed that the induced group II (diazepam1mg/kg; i.p) significantly increased the transfer latency as compared to control group. Transfer latency of treatment group D (600 mg/kg + diazepam) was significantly decreased from induced group 2 (diazepam). Whereas, transfer latency of treatment group C (500 mg/kg + diazepam) was not significantly different from induced group 2 (diazepam). In memory retention session (24 h after training session), step-down latency on PAP showed that induced group 2 (diazepam) significantly decreased step-down latency. The step-down latency of treatment group C (500 mg/kg + diazepam) and D (600 mg/kg + diazepam) were significantly increased from induced group 2 (diazepam) as expressed in Table 2.

Data are expressed as mean ± SEM (p<0.05), [a] shows significant difference of control group with standard, induced and treatment groups, [b] shows significant difference of induced group I with treatment group A and B and C shows significant difference of induced group II with treatment group C and D.

3.4 Estimation of brain acetylcholine contents

Table 3 is showing acetylcholine concentration (µM/mg) in brain after 15 days of oral administration of methanolic bark extract of *M. koenigii* at 500 mg/kg and 600 mg/kg. Both treatment groups II (500 mg/kg) and III (600 mg/kg) indicated note worthy rise in acetylcholine concentration, whereas treatment group III (600 mg/kg) increased acetylcholine concentration comparable with standard. Scopolamine injected group (induced group 1) showed significantly decrease in acetylcholine concentration. Whereas, scopolamine injected mice that were pre-administered with methanolic bark extract of *M. koenigii* at dose 500 and 600 mg/kg (treatment group A and B) showed significant level of acetylcholine as compared with induced group 1 (scopolamine).

Table 3: Effect of methanolic bark extract of *M. koenigii* on acetylcholine content in memory deficit mice models (n=5)

Groups	Acetylcholine (µM/mg)
Control (water)	3.06 ± 0.1
Standard (piracetam)	4.7 ± 0.1[a]
Treatment group II (500 mg/kg)	3.64 ± 0.1[ac]
Treatment group III (600 mg/kg)	4.98 ± 0.1[a]
Induced group 1 (scopolamine)	1.40 ± 0.0[a]
Treatment group A (500 mg/kg + scopolamine)	2.94 ± 0.1[abc]
Treatment group B (600 mg/kg + scopolamine)	4.00 ± 0.1[abc]

4. Discussion

The current experiment was conducted to study nootropic evaluation of methanolic bark extract of *M. koenigii*, its possible protective potential in memory deficit mice models and to identify its mechanism of action by assessing its effect on brain acetylcholine content in mice.

M. koenigii methanolic bark extract was prepared and by qualitative phytochemical analysis alkaloids, glycosides, saponins, phenolic contents, steroids and flavonoids were identified. The carbazole alkaloids, *e.g.*., murrayazolidine, murrayacine, mahanimbine, murrayazoline, koenioline, girinimbine, koenigine-quinone A, koenigine-quinone B and xynthyletin, were separated from *M.koenigii* bark stem (Chakrabarty *et al*., 1997; Saha and Chowdhury, 1998). In this study, methanolic bark extract of *M. koenigii* at 500 and 600 mg/kg doses exhibited significant decline in transfer latency (p<0.001), (p< 0.001) for EPM and for PAP increase in step-down latency (p< 0.001), (p<0.001), respectively at retention session 16th day after training session on 15th in comparison to scopolamine group on EPM and PAP models, respectively.

Acute oral toxicity studies were carried out up to dose of 2000 mg/kg; it was found safe. At three dose levels, 400, 500 and 600 mg/kg dose-related effects were studied by utilizing two exteroceptive models EPM and PAP. Such models were utilized to evaluate the memory score in animal models and widely accepted.

A scientifically approved probe, scopolamine (non-selective muscarinic receptor inhibitor) is used to cause deficiency of memory deficit in humans or animal models. The numerous plants and their ingredients (*i.e.*, alkaloids) have been investigated to provide significant anticipation of memory deficit induced by scopolamine in animal models such as *Uncariato mentosa*, *Panax ginseng*, and *Vitex negunda* which play important role to manage AD in human (de Bruin and Pouzet, 2006; Kanwal *et al.*, 2010). Diazepam (benzodiazepine) modulates amnesic effect by potentiating the inhibitory effect of γ-GABA *via* action on $GABA_A$ receptor complex. It was predicted that diazepam elevates the efficacy duration and breakdown of $GABA_A$ receptor-mediated inhibitory postsynaptic current in hippocampus neuron of rat brain slice (Rudolf *et al*., 1999; Xu and Sastry, 2005). In this study, a dose level 600 mg/kg of *M. koenigii* exhibited remarkable decrease in transfer latency and step-down latency on 16th day (retrieval session) after training session on 15th day when compared with diazepam group on EPM and PVP models, respectively.

Major cause of amnesia related to the neurodegenerative disorder and aging is damage to cholinergic neurons in brain. In accordance to cholinergic hypothesis of AD, declined amount of acetylcholine in cerebral cortex is the major reason of memory deficiency in AD (Overk *et al*., 2010).This study was undertaken to check acetylcholine levels in brain of healthy mice and scopolamine induced amnesic mice pre-administered with methanolic bark extract of *M. koenigii* at 500 and 600 mg/kg dose levels. Methanolic bark extract of *M. koenigii* at both dose levels significantly (p<0.01), (p< 0.001), respectively increased the brain acetylcholine level in healthy mice and also significantly (p<0.001) prevent the scopolamine induced decrease level of acetylcholine as compared to scopolamine injected induced group but these acetylcholine levels were significantly decreased as compared to the respective dose levels of methanolic bark extracts of *M. koenigii* (500 and 600 mg/kg) in normal healthy mice.

5. Conclusion

Conclusively, the current investigation infers that the methanolic bark extract of *M. koenigii* improved memory function, it prevented memory deficit caused through scopolamine or diazepam and increased the brain acetylcholine amount, too. The carbazole alkaloids in *M. koenigii* are responsible to enhance memory and to treat amnesia at a dose rate of 500 mg/kg or 600 mg/kg of methanol extract. Study was focused to investigate an alternative therapy to deal with memory issues. It is well documented that all the synthetic pharmacological agents to treat memory deficiency have severe life threatening adverse reactions. To counter this problem, there is dire need to investigate such medicinal plants which have more beneficial effects and less adverse effects. This research will help to find the medicinal ingredients present in natural plants which ensure their easy availability and cost effective as well. The issues of long term memory loss still require attention of researchers to reach plausible outcome for welfare of patients. *M. koenigii* should be further studied with different extracts to ensure its'antiamnesic and nootropic effect against various causative agents. This research will help pharmaceutical sector to develop a suitable dosage form with nootropic ingredient of selected plant.

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Conflicts of interest

The authors declare no conflicts of interest relevant to this article.

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