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Anti-amnesic effect of ethanol stem bark extract and fractions of *Milicia excelsa* (Moraceae) in mice

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Abstract

Background: Amnesia is associated with normal aging and neuropsychiatric disorders with no known medical cure. Medicinal plants used in traditional medicines to combat neuropsychiatric disorders may be a veritable vehicle towards providing the appropriate drug candidate(s).

Methods: Hence, this study assessed the anti-amnesic potential of *Milicia excelsa* stem bark upon its widespread use in traditional medicine for treating mental illnesses. The anti-amnesic potentials of ethanol stem bark extract and fractions were investigated using mouse models of scopolamine-, and diazepam-induced amnesia on Y-maze.

Results: The result obtained showed that the crude ethanol extract, ethyl acetate and *n*-butanol fractions at all the doses used (75, 150 and 300 mg/kg, p.o.) significantly ($p<0.05$) reversed the amnesia induced by scopolamine (1 mg/kg, i.p.) and diazepam (1 mg/kg, i.p.) in mice. The *n*-hexane at 300 mg/kg and aqueous fraction at 75 and 150 mg/kg significantly ($p<0.05$) ameliorated the amnesia induced by scopolamine in scopolamine-induced amnesia.

Conclusion: This study, therefore, concludes that the extract and its fractions may possess anti-amnesic effect. However, further studies may be carried out to isolate and characterize the anti-amnesic bioactive principle(s) in ethyl acetate and n-butanol fractions that showed consistent anti-amnesic potentials in the scopolamine-, and diazepam-induced amnesic models used. The in-vivo antioxidant and acetylcholinesterase assays of these active fractions should also be carried out to corroborate the observed anti-amnesic effect.

Keywords: Scopolamine-induced amnesia; diazepam-induced amnesia; Y-maze; ethylacetate fraction; *n*-butanol fraction.

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Background

Human societies have relied on their environment for food and medicines since their formation [1]. Thus, every culture of the world maintains its health care needs with the knowledge and understanding of the traditional medicinal substances at its disposal since ancient time [2] to recover from diseases [3], and multiple of evidences have indicated the valuable potentials of medicinal plants employed in different traditional settings [4]. Moreso that medicinal plants have been used as the vehicle for the development of new drugs and will continue to play significant roles in the process of drug discovery [5].

Amnesia is a cognitive deficit characterized by a profound memory loss which is a syndrome of some specific neurodegenerative disorders such as Alzheimer's disease (AD) and vascular dementia [6, 7]. Dementia is a general term describing disorders associated with progressive cognitive decline resulting mostly from neurodegeneration with no known conventional treatment for the behavioural and neurological consequences associated with this disorder [8]. The most well-known type of dementia is AD with no known medical cure except symptomatic treatment [8]. Dysfunction of central cholinergic system is associated with the pathogenesis of AD, hence scopolamine-induced amnesia has been utilized to screen plant extract with potential efficacy against amnesic symptom of dementia-like AD [6].

Medicinal plants have been used to forestall the etiological factors which cause incidence of amnesia, dementia, and Alzheimer's disease such as elevated oxidative stress, increasing age, reduced brain cholinergic firing, hypercholesterolaemia, neuroinflammatory reactions, amyloid protein deposits and restore memory [9-11] with phytochemicals having anti-inflammatory, antioxidant effects, anti-amyloidogenic, anti-cholinesterase, and hypolipidemic effects [11, 12-15]. This may provide alternative therapy to the currently available synthetic drugs such as acetylcholinesterase inhibitors (e.g donepezil), N-methyl-D-aspartate receptor antagonist (e.g memantine) and nootropics (e.g piraceram) with minimum effectiveness, symptomatic relief, slow disease progression and target late phase of the disease [16-19] and acetylcholinesterase inhibitors and nootropics are associated with negative health [20] which have limited their use [7]. Thus, numerous research groups have focused on the identification of anti-amnesic agents in herbal medicines from traditional medications [12]. As a result, we are currently witnessing increasing tendencies in people towards the use of traditional medicine [21].

Milicia excelsa (Welw.) CC Berg, otherwise known as *Chlorophora excelsa* is a member of the mulberry family otherwise known as Moraceae [22]. It is called 'Iroko tree' in Yoruba, 'Loko' in Hausa and 'Oje' in Igbo speaking tribes of Nigeria, West Africa. It is a large deciduous tree which grows naturally in the humid area of West Africa to a height of 30 m [22]. In traditional medicine, the stem bark is used as an antipsychotic [23], anticonvulsant [24], antiaging [25], aphrodisiac [22] and as sedative and for treating mental illness [26]. The anti-inflammatory [27], and sedative-hypnotic [28] effects of the stem bark have been scientifically reported.

Consequent upon the use of Milicia excelsa stem bark in treating mental illnesses among the Hausa ethnic group of Northern Nigeria [26]. This study aimed to evaluate the anti-amnesic effect of the hydro alcohol extract and fractions in mice.

Methods

Plant identification and authentication

Milicia excelsa (Moraceae) stem bark was collected by Mr Adebayo M. A from Temporary Site of the Igbinedion University, Okada in March 2019. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Sciences of the Obafemi Awolowo University, Ile-Ife Nigeria and herbarium number Ife-17482 was obtained.

Preparation of the plant materials

The extraction of the *Milicia excelsa* stem bark and fractionation were carried out as previously reported [28]. Briefly, pieces of the stem bark were air-dried and ground into fine powder. Exactly, 8.29 kg of the powder was macerated in 15 liters (w/v) of seventy percent (70%) ethanol for 2 days (48 h). The ethanol extract was concentrated using rotary evaporator set at 40°C and subsequently freeze-dried to yield 220.7 g (2.65%) crude extract (ESB). Two hundred gram (200 g) of ESB was successively fractionated into n-hexane, ethyl acetate, *n*-butanol and water to obtain *n*-hexane fraction (HF), ethyl-acetate fraction (EAF), *n*- butanol fraction (BF) and aqueous (AF) fractions respectively.

Drugs

Diazepam (Roche, Basel, Switzerland), Scopolamine hydrobromide, Piracetam, Tween 80 (Sigma Aldrich, St. Louis Missouri, USA), and physiological saline (Unique Pharmaceutical Limited, Lagos, Nigeria). The ESB and all its fractions were dissolved separately with 3% Tween 80 and made up to the required concentrations with normal saline. The ESB and its fractions were prepared fresh on each day of the behavioral investigation.

Laboratory Animals

Adult albino mice of both sexes (18–25 g) were obtained from the Central Animal House of the Igbinedion University, Okada, Edo State. The mice were fed with standard animal pellets and water ad libitum. The mice were allowed to acclimatize to the laboratory conditions for 2 weeks before the commencement of the behavioral evaluations. The experimental procedures adopted in this study followed the approved institutional animal ethical committee guidelines, which is in accordance with the internationally accepted principles for Laboratory Animal Use and Care [29].

Pharmacological experiments

General experimental design

Adult mice of either sex were randomized into 32 groups (n=5) as follows:
Group I (control group): mice received 3% Tween 80 in physiological saline (10 mL/kg) and 1 hour post oral treatment, mice were observed on Y-maze for 8 minutes and the sequence of arms visited was recorded. Group II: (scopolamine group): mice received 3% Tween 80 in physiological saline (10 mL/kg) + scopolamine (1 mg/kg, i.p.), Group III: mice received scopolamine (1 mg/kg, i.p.) + piracetam (200 mg/kg, p.o.), Groups IV-VI: mice received ESB (75, 150 and 300 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.), Groups VII-IX: mice received HF (75, 150 and 300

mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.), Groups X-XII: mice received EAF (75, 150 and 300 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.), Groups XIII-XV: mice received BF (75, 150 and 300 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.), Groups XVI-XVIII: mice received AF (75, 150 and 300 mg/kg, p.o.) + scopolamine (1 mg/kg, i.p.), Group XIX: mice received 3% Tween 80 in physiological saline (10 mL/kg) + diazepam (1 mg/kg, i.p.), Group XX: mice received piracetam (200 mg/kg, p.o.) + diazepam (1 mg/kg, i.p.), Groups XXI-XXIII: mice received HF (75, 150 and 300 mg/kg, p.o.) + diazepam (1 mg/kg, i.p.), Groups XXIV-XXVI: mice received EAF (75, 150 and 300 mg/kg, p.o.) + diazepam (1 mg/kg, i.p.), Groups XXVII-XXIX: mice received BF (75, 150 and 300 mg/kg, p.o.) + diazepam (1 mg/kg, i.p.), Groups XXX-XXXII: mice received AF (75, 150 and 300 mg/kg, p.o.) + diazepam (1 mg/kg, i.p.).

Scopolamine-induced amnesia

Scopolamine, an amnesic agent was used to induce memory deficit as earlier reported in literature [30, 31]. Thirty minutes following oral ingestion of the vehicle [3% Tween 80 in physiological saline (10 mL/kg)] in Group II or piracetam (200 mg/kg, p.o.) in Group III or the fractions in Groups IV-XVIII, mice were administered scopolamine (1 mg/kg, i.p.). Thirty minutes after scopolamine injection, each mouse was gently put on arm A of the Y-maze (40 x 3 x 12) with angles of 1200 between each of the three arms and the sequence of arms visited on consecutive choices in 8 minutes was recorded. The number of triads was recorded as 'percentage alternation' which indicates the assessment of short-term memory [32], according to this equation:

An alternation is defined as an entry into all three arms on consecutive choices

$$\% \text{ Alternation} = [(\text{Number of alternations})/ (\text{Total arm entries} - 2)] \times 100.$$

Diazepam-induced amnesia

Diazepam was used to induce memory impairment at a dose found to cause cognitive decline [33]. Thirty minutes following oral ingestion of the vehicle [3% Tween 80 in physiological saline (10 mL/kg)] in Group XIX or piracetam (200 mg/kg, p.o.) in Group XX or the fractions in Groups XXI-XXXII, mice were administered diazepam (1 mg/kg, i.p.). Thirty minutes after diazepam injection, each mouse was gently put on arm A of the Y-maze and the sequence of arms visited on consecutive choices in 8 minutes was recorded. The alternation score (%) for each mouse was scored and calculated as done for scopolamine-induced amnesia above.

Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc analysis. The level of significance for all tests was set at $p < 0.05$ compared to the control group.

Results

Effect of ESB and its fractions on scopolamine-induced amnesia on Y-Maze task.

There was significant ($p<0.05$) reduction on spontaneous percentage alternation in scopolamine treated control group when compared to the control treated group. This reduction in spontaneous percentage alternation was significantly ($p<0.05$) ameliorated by ESB at 150 and 300 mg/kg, HF at 300 mg/kg and EAF, BF and AF at all the doses of 75, 150 and 300 mg/kg compared to the scopolamine treated control group. Piracetam (200 mg/kg, p.o.), a reference anti-amnesic agent significantly ($p<0.05$) reversed the reduced percentage alternation observed in scopolamine treated mice. The result is presented in Figure 1.

Effect of ESB and its fractions on diazepam-induced amnesia on Y-Maze task.

There was significant ($p<0.05$) reduction on spontaneous percentage alternation in diazepam treated control group when compared to the control treated mice. This reduction in spontaneous percentage alternation was significantly ($p<0.05$) ameliorated by ESB, EAF and BF at all the doses of 75, 150 and 300 mg/kg compared to the diazepan treated control group. Piracetam (200 mg/kg, p.o.), a reference anti-amnesic agent significantly ($p<0.05$) reversed the reduction in percentage alternation observed in diazepam treated mice. The result is presented in Figure 2.

Discussion

The present study reported the anti-amnesic potential of ethanol stem bark extract of *Milicia excelsa* (*M. excelsa*) and fractions using scopolamine-, and diazepam-induced amnesic models in mice. The findings revealed that the crude ethanol extract and fractions might possess anti-amnesic effect. Previous scientific finding has reported the oral acute toxicity (LD50) of the ethanol stem bark extract of *M. excelsa*, *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of the crude ethanol extract to be greater than or equal to 5000 mg/kg in mice [28]. Hence doses of 75, 150 and 300 mg/kg were used in the behavioral assessments carried out in this study in mice.

Scopolamine and diazepam have been used to induce memory impairment in mice [34, 35], probably via different mechanism. Scopolamine induced memory deficit via the blockade of the central muscarinic cholinergic receptors while diazepam induce amnesia either via agonistic or antagonistic action on the GABAA/benzodiazepine receptor pathway and GABAA/benzodiazepine receptor complex controls acetylcholine release or via oxidative stress [36, 37]. Hence these amnesic agents were employed to evaluate the anti-amnesic potential of the extract and its fractions in this study.

The reduction in percentage alternation brought about by scopolamine in this study suggests the induction of amnesia in experimental mice. This observation conforms with earlier investigations of the induction of amnesia by scopolamine [6, 31]. However, the reversal of amnesia by the extract and fractions in scopolamine-induced amnesia is suggestive of anti-amnesic effect. This finding adds to the existing body of knowledge of the anti-amnesic potential of medicinal plants in scopolamine-induced amnesia [6, 31].

Although the mechanism of anti-amnesic effect of the extract and fractions was not investigated in this study, but previous studies have demonstrated that scopolamine, a central muscarinic cholinergic blocker induce amnesia by inhibiting cholinergic neurotransmission either via blocking the muscrainic actetycholine receptor or enhanced acetylcholinesterase activity or via the induction of oxidative stress [38, 39]. Since the crude ethanol extract and fractions reversed the amnesia induced by scopolamine, it is probably therefore, to suggest that the crude ethanol extract and fractions may be acting to antagonize the various mechanism via which scopolamine exerts its amnesic effect.

The observed reduction in percentage alternation following diazepam administration in this study suggests the induction of amnesia in experimental mice. This observation agrees with earlier investigation of the induction of amnesia by diazepam [40]. However, the reversal of amnesia by the extract and fractions in diazepam-induced amnesia is indicative of anti-amnesic effect. This finding may as well add to the existing body of scientifically validated medicinal agents with potential anti-amnesic effects in diazepam-induced amnesic model [41, 42].

The mechanism of amnesic effect induced by diazepam has been reported to be mediated via GABAergic system and supported by the fact that the hippocampus and related brain regions affected by aging and mediating memory processing are largely controlled by the GABAergic system [43]. The reversal of amnesia induced by diazepam in diazepam-induced amnesia by the crude ethanol extract, ethyl acetate and *n*-butanol fractions in this study suggest that the crude ethanol extract and these fractions may be exerting antagonistic effects on benzodiazepine-receptor to bring about the anti-amnesia effect since flumazenil (a benzodiazepine-receptor antagonist) has been demonstrated to reverse benzodiazepine-induced amnesia [44].

The ameliorating effect of the crude ethanol extract and its fractions on scopolamine-, and diazepam-induced amnesia in this study suggest that the extract and fractions may possess anti-amnesic effect possibly via the facilitation of cholinergic transmission or GABA-benzodiazepine blockade.

Previous quantitative phytochemical estimation from our laboratory has shown flavonoids to be the most abundant phytoconstituents assayed in the ethanol stem bark extract of *M. excelsa*, *n*-hexane, ethyl acetate, n-butanol and aqueous fractions of the extract [28]. This plant secondary metabolite may therefore be responsible for the observed anti-amnesic effect in this study. Since flavonoids such as luteolin and apigenin isolated from other plants of Moraceae family [45-46] have been reported to show beneficial effect in amnesia and Alzheimer's disease [47-50].

It is noteworthy that the ethanol stem bark extract of the *M. excelsa*, ethyl acetate and *n*-butanol fractions of the stem bark extract gave consistent anti-amnesic results in both scopolamine-, and diazepam-induced amnesia in this study. Therefore, effort is ongoing in our laboratory to isolate and characterize the anti-amnesic bioactive principles in these fractions as well as carry out *in-vivo* antioxidant and acetylcholinesterase assays and object recognition test for the isolated compound(s).

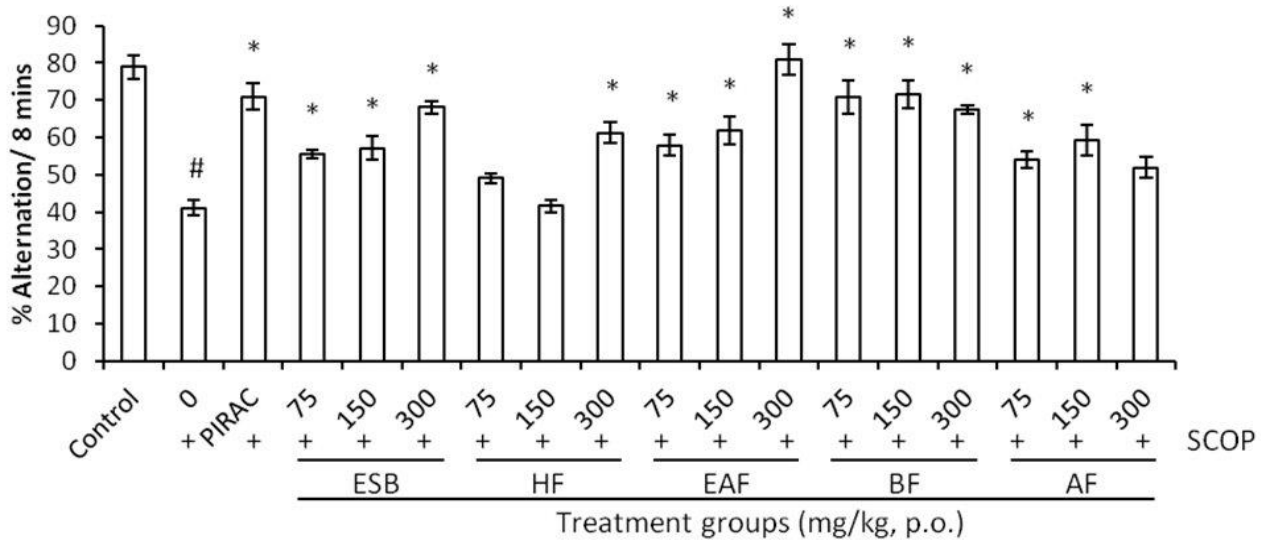

Figure 1. Anti-amnesic effect of ESB and its fractions on scopolamine-induced amnesia in mice.
Each bar represents Mean ± SEM, n=5. #p<0.05 and *p<0.05 compared to the control and scopolamine treated group, respectively. PIRAC; piracetam (200 mg/kg, p.o.), SCOP; scopolamine (1 mg/kg, i.p.), ESB; ethanol stem bark extract of *Milicia excelsa*, HF, EAF, BF and AF represent *n*-hexane, ethyl

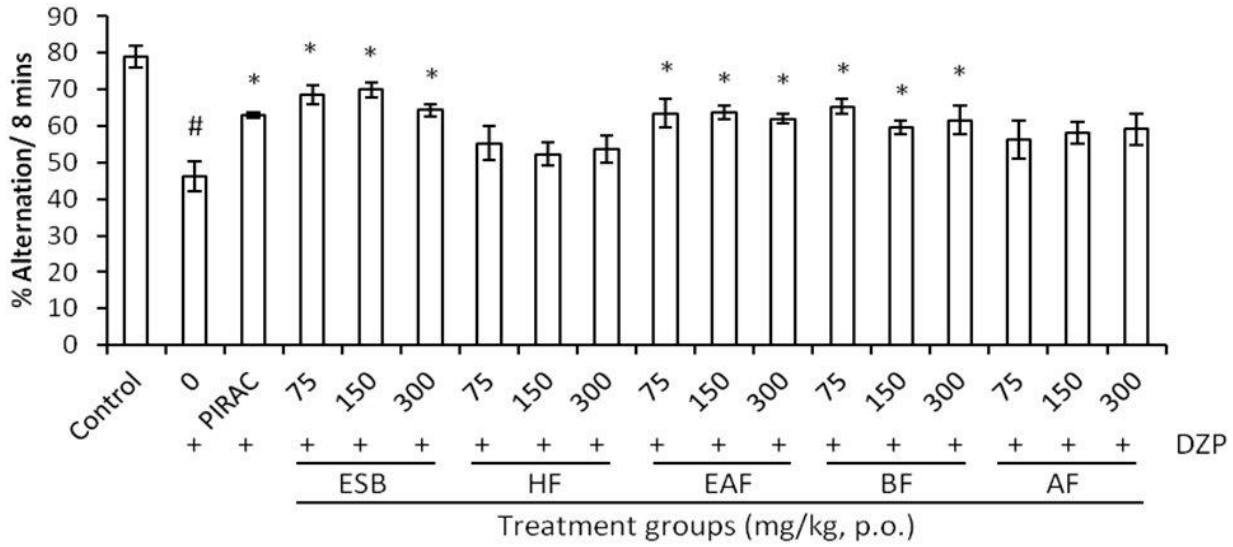

Figure 2. Effect of ESB and its fractions on diazepam-induced amnesia in mice.
Each bar represents Mean ± SEM, n=5. #p<0.05 and *p<0.05 compared to the control and diazepam treated group respectively. PIRAC; piracetam (200 mg/kg, p.o.), DZP; diazepam (1 mg/kg, i.p.), ESB; ethanol stem bark extract of *Milicia excelsa*, HF, EAF, BF and AF represent *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of *Milicia excelsa* stem bark extract respectively.

Conclusions

This study concludes that the ethanol extract and its fractions may possess anti-amnesic effect. The neural mechanism of action might probably involve cholinergic and/or GABAergic receptor pathways.

Abbreviations

AD, Alzheimer's disease; ESB; ethanol stem bark extract of *Milicia excelsa*, HF; *n*-hexane fraction of *Milicia excelsa*, EAF; ethyl acetate fraction of *Milicia excelsa*, BF; n-butanol fraction of *Milicia excelsa*, AF; aqueous fraction of *Milicia excelsa*, SEM, standard error of mean; ANOVA, analysis of variance; *M. excelsa*, *Milicia excelsa*; LD_{50}, median lethal dose; GABA, gamma amino butyric acid, DZP, diazepam; PIRAC, piracetam, SCOP, scopolamine.

Authors' Contribution

The research work reported in this study was carried by collaboration among all authors. Author LAA designed the study, managed the literature search, carried out the statistical analysis and wrote the first draft of the manuscript. Authors OMA, RTA and CJM carried out the laboratory work under the supervision of LAA. Author MAA collected the plant material, assisted in some laboratory works, provided some materials and contributed to the writing of the manuscript. Author IAO reviewed and edited the manuscript.

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Conflict of interest

Authors declare we have no competing interest

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