

Acute Toxicity and Sedative-hypnotic Effects of Ethanol Stem Bark Extract and Fractions of *Milicia excelsa* (Moraceae) in Mice

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Abstract **Aim:** *Milicia excelsa* stem bark is used as sedative and for treating mental illnesses among the Hausa tribe of Northern Nigeria, but there is no scientific rationale for its use. Hence, this study investigated the oral acute toxicity and sedative potential of ethanol stem bark extract, n-hexane, ethylacetate, n-butanol and aqueous fractions of the stem bark extract in mice. The phytoconstituents in the extract and fractions were quantified. **Methodology:** The acute toxicity of the extract and fractions were investigated using OECD guidelines 425 of 2008. The sedative effects of the extract and fractions were investigated using pentobarbital-, and ketamine-induced sleep tests. **Results and discussion:** The results obtained showed that the acute toxicity of the extract and fractions were ≥ 5000 mg/kg, suggesting that the extract and fractions may be safe. The extract, n-hexane, ethylacetate and aqueous fractions significantly ($p < 0.05$) reduced sleep latencies, indicative of sedative effects, effective for sleep induction, while the extract and all the fractions significantly ($p < 0.05$) prolonged the sleep durations, suggesting sedative effects, effective for sleep maintenance. **Conclusion:** This study therefore, concluded that the extract and fractions may be safe. The study further concluded that the sedative effect of the extract and fractions may be due to the abundance of flavonoids in the extract and fractions. Thus, providing scientific rationale for its ethnomedicinal use. However, further study may be warranted to isolate and characterize the sleep promoting bioactive principles as well as carry out GABA binding assay of the isolated compound(s) in ESB and its various fractions.

Keywords *Milicia excelsa*, Sedative, Ketamine,

Pentobarbitone, GABA

1. Introduction

Insomnia is one of the ailments which affects people of all ages worldwide [1]. It is often defined as sleeping problems which is characterized by difficulty in falling asleep (sleep induction), continuing asleep (sleep maintenance) or as having non refreshing sleep to a certain extent [2].

Insomnia can be treated pharmacologically, non-pharmacologically, or by the combination of the two [3]. The non pharmacological treatments is also known as behavioural therapies, which include relaxation, sleep restriction, stimulus control and sleep hygiene, while the pharmacological treatments involve prescription drugs such as benzodiazepines [4] or newer non benzodiazepines [5, 6].

Despite reported clinical success, these medications have in attendance side effects such as physical dependence [7], daytime fatigue [8], cognitive impairment [9] and non-effective response to the existing therapeutic drugs by some patients with sleep problems [10]. In order to circumvent these undesirable effects, scientists have resorted to alternative therapy that could improve sleep quality as well as devoid of undesirable effects [11]. This is evident from the previous studies of herbal drugs with promising sedative-hypnotic effect [12].

Milicia excelsa (welw.) C.C. Berg, otherwise known as

Chlorophora excelsa, is a large deciduous tree growing up to the height of 50 m. It is naturally found in the humid forest of the West Africa sub-region [13]. It belongs to the mulberry family (Moraceae) and popularly called Iroko tree or African teak [13]. The different plant parts of *Milicia excelsa* are used in ethnomedicines to prepare herbal remedies in African traditional settings [14]. For example the stem bark is used as sedative and for the treatment of mental illnesses [15] such as convulsion [16] and psychosis [17]. Pharmacologically, the antipsychotic [18], anticonvulsant [19], anti-stress [20], anti-amnesic [21] and wound healing effects [22] of the leaf have been scientifically evaluated. The anti-inflammatory effect of the stem bark [23] and the antidiarrheal effect of the root bark [24] have also been scientifically validated.

Consequent on its use as sedative among the Hausa tribe of Northern Nigeria [15], this study was designed to investigate the sedative effect of the ethanol stem bark extract and fractions in mice.

2. Materials and Methods

2.1. Plant Identification and Authentication

Milicia excelsa stem bark was collected within the campus of Obafemi Awolowo University (OAU), Ile Ife. 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.

2.1.1. Preparation of Plant Materials

The stem bark was cut into pieces and air dried at room temperature for two weeks. The dried stem barks were ground into powder by mechanical grinder and 8.29 kg of the powder was extracted by cold maceration with 15 litres of seventy percent (70%) ethanol for 48 hr. The extract was concentrated *in vacuo* at a temperature of 40°C and freeze dried to yield 220.7 g (2.65%) crude extract and coded ESB. Two hundred gram (200 g) of the crude extract was successively partitioned into n-hexane, ethylacetate, n-butanol and aqueous fractions. The fractions were again concentrated *in vacuo* to give n-hexane fraction (HF 9.2 g, 4.6%), ethyl-acetate fraction (EAF 20.8 g, 10.4%), n-butanol fraction (BF 12 g, 6%) and aqueous fraction (AF 19 g, 9.5%).

2.2. Drugs

Diazepam (Roche, Basel, Switzerland), Pentobarbital sodium, Tween 80 (Sigma Aldrich, USA), Ketamine hydrochloride (Rotex Medica Tritau, Germany), and normal saline (Unique Pharmaceutical Limited, Lagos, Nigeria). The ESB and its various fractions were dissolved with 3% Tween 80 and made up to the required volume

with normal saline. The extract and its fractions were freshly prepared on each day of the experiment prior to ingestion to mice.

2.3. 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 maintained on standard animal pellets and water *ad libitum*. 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 [25].

2.4. General Experimental Design

Mice were randomized into 5 groups containing 5 mice per group (n = 5) as follows:

Group I (negative control group): mice in this group received 10 mL/kg of the vehicle (3% Tween 80 in normal saline).

Groups II-IV: mice in these groups received ESB at the 125, 250 and 500 mg/kg per oral respectively.

Groups V: mice in this group received the reference drug, diazepam (2 mg/kg, i.p.)

The experimental procedures above were repeated for HF, EAF, BF, and AF at the same doses as ESB.

Preliminary phytochemical quantifications

The total flavonoids, total phenols, tannin content and total alkaloids were quantitatively determined in ESB, HF, EAF, BF and AF as previously described [26-29]. n=3.

2.5. Acute Toxicity Test

The acute oral toxicity (LD₅₀) of ESB was investigated using the Organization for Economic Cooperation and Development (OECD) guidelines 425 of 2008 [26]. The acute toxicity test was conducted in two phases. In the first phase, a single female mouse was orally ingested with the extract at a dose of 2000 mg/kg per body weight of ESB after fasting for 3 hours. The mouse was observed for any signs of acute behavioural toxicity within the first 24 h. From the result of the first phase; another 4 female mice were orally ingested with ESB at 2000 mg/kg per oral and subsequently observed for death in 24 hours and for any sign of delayed toxicity or death for the next 14 days. Based on the result of the second step, the two steps of the experiments were repeated for 5000 mg/kg per oral. The two phases as above were repeated for HF, EAF, BF, and AF. Thereafter the LD₅₀ for each of the extract and fractions were determined.

2.6. Sedative-hypnotic Experiments

2.6.1. Pentobarbital-induced Sleeping Test

The effect ESB on pentobarbital-induced sleep test was carried out as previously done [31]. Mice were pretreated with ESB for 60 minutes or diazepam (2 mg/kg, i.p.) for 30 minutes prior to the intraperitoneal injection of sodium pentobarbitone (30 mg/kg). The time in minutes between pentobarbitone injection and loss of righting reflex by each mouse was noted and taken as sleep latency (onset of sleep). The time duration between the loss and regain of righting reflex by each mouse was noted and taken as sleeping time. The procedures were repeated for HF, EAF, BF and AF respectively.

2.6.2. Ketamine-induced Sleeping Time

The effect ESB on ketamine-induced sleeping time was carried out as previously described [32]. Mice were pretreated with ESB for 60 minutes or diazepam (2 mg/kg, i.p.) for 30 minutes prior to the intraperitoneal injection of ketamine (100 mg/kg). The time in minutes between ketamine administration and loss of righting reflex by each mouse was noted and taken as sleep latency (onset of sleep). The time duration between the loss and regain of righting reflex by each mouse was noted and taken as sleeping time. The procedures were repeated for HF, EAF, BF and AF respectively.

2.7. Statistical Analysis

Results are expressed as mean \pm S.E.M. The significance of different between groups were analysed using one way analysis of variance (ANOVA), followed by Dunnett post hoc analysis using GraphPadInStat® Biostatistics software (GraphPad Software, Inc., La Jolla, USA). The level of significance for all tests was set at * $p < 0.05$.

3. Results

3.1. Result of Preliminary Phytochemical Estimations of ESB and Its Fractions

The result showed that total flavonoids in ESB > AF > EAF > HF > BF. The tannin content in EAF > AF > ESB > BF > HF. The total phenols in EAF > AF > ESB > BF > HF. The total alkaloids in EAF > AF > ESB > HF > BF. The result also showed that total flavonoids is the most abundant in ESB while tannins, total phenols and total alkaloids are the most abundant in EAF. Of all the fractions, AF contained the most abundant total flavonoids, while EAF contained the most abundant of tannins, total phenols and total alkaloids. The result is presented in Table 1.

Table 1. Preliminary phytochemical estimations of ESB and its fractions

Samples	Total flavonoids(mg QUE/g sample)	Tannin content(mg GAE/g sample)	Total phenols(mg GAE/g sample)	Total alkaloids(mg AE/g sample)
ESB	757.90 \pm 2.48	83.19 \pm 0.80	2.23 \pm 0.17	10.36 \pm 0.07
HF	363.16 \pm 20.31	19.24 \pm 2.57	0.27 \pm 0.01	4.33 \pm 0.06
EAF	610.53 \pm 25.18	113.75 \pm 1.60	87.60 \pm 0.75	14.03 \pm 0.12
BF	357.90 \pm 6.56	55.46 \pm 2.01	1.33 \pm 0.06	3.92 \pm 0.07
AF	740.35 \pm 7.98	101.87 \pm 1.60	20.32 \pm 0.29	13.96 \pm 0.38

Values are means of triplicate determination \pm Standard deviation; where QE, GAE and AE are quercetin, gallic acid, and atropine equivalents respectively. ESB; ethanol stem bark extract of *Milicia excelsa*, HF; n-hexane, EAF; ethylacetate, BF; n-butanol and AF; aqueous fractions of *Milicia excelsa* stem bark extract

3.2. Results of Acute Toxicity (LD₅₀) of ESB, HF, EAF, BF and AF

The LD₅₀ of ESB, HF, EAF, BF and AF were found to be > 5000 mg/kg, per oral in mice.

3.3. Results of Sedative-hypnotic Experiments

3.3.1. Effects of ESB, HF, EAF, BF and AF on Sleep

Latency in Pentobarbital-induced Sleeping Time in Mice

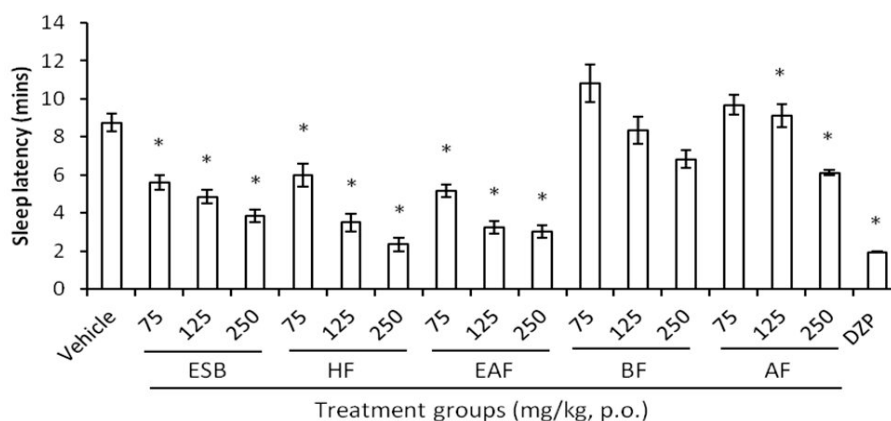
The ESB, HF, EAF significantly ($p < 0.05$) at all the doses of 75, 125 and 250 mg/kg, p.o shortened the sleep latency, AF significantly ($p < 0.05$) shortened the sleep latency at 125 and 250 mg/kg, p.o. when compared to the vehicle treated control group. However, BF at all the doses used did not show any significant ($p > 0.05$) reduction on sleep latency when compared to the vehicle treated control group. Diazepam, a positive control group at 2 mg/kg, i.p. significantly ($p < 0.05$) reduced the sleep latency when

compared to the vehicle treated control group in pentobarbital-induced sleeping time in mice. The result is presented in Figure 1.

3.3.2. Effects of ESB, HF, EAF, BF and AF on Sleep

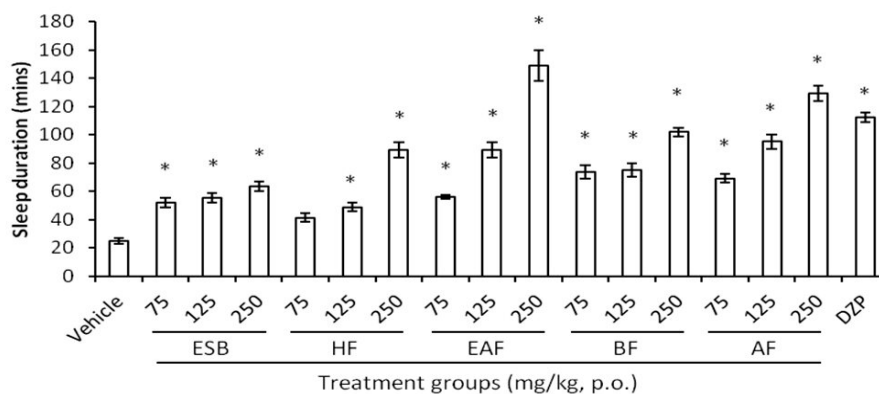
Duration in Pentobarbital-induced Sleeping Time in Mice

The ESB, EAF, BF and AF at all the doses used significantly ($p < 0.05$) and dose dependently prolonged the sleep duration when compared to the vehicle treated control group. The HF showed dose dependent increase in sleep duration but only significant ($p < 0.05$) at 125 and 250 mg/kg, when compared to the vehicle-treated control group. The positive control drug diazepam (2 mg/kg, i.p.) also significantly ($p < 0.05$) elongated the sleep duration when compared to the vehicle treated control group in pentobarbital-induced sleeping time in mice. The result is presented in Figure 2.



Each bar represents mean \pm SEM. (ANOVA, followed by Dunnett's post hoc), $n=5$, * $p < 0.05$ when compared to the vehicle-treated control group. ESB; ethanol stem bark extract of *Milicia excelsa*, HF; n-hexane, EAF; ethylacetate, BF; n-butanol and AF; aqueous fractions of *Milicia excelsa* stem bark extract

Figure 1. Sedative effect of ESB, HF, EAF and AF on sleep latency in pentobarbital-induced sleeping time in mice

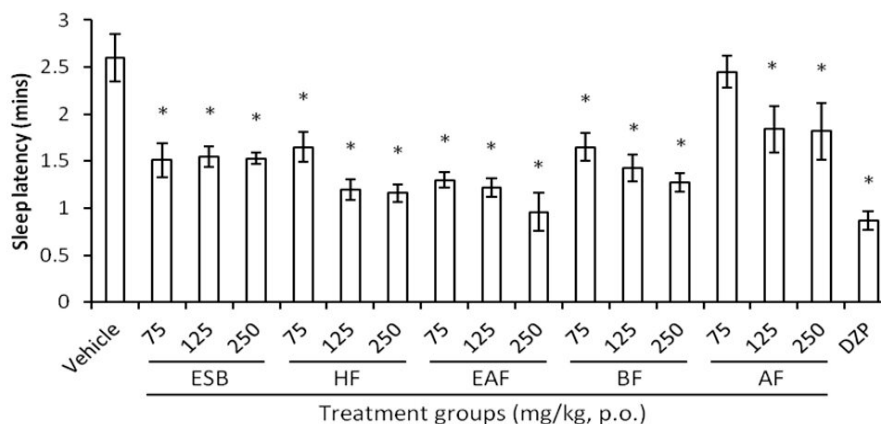


Each bar represents mean \pm SEM. (ANOVA, followed by Dunnett's post hoc), $n=5$, * $p < 0.05$ when compared to the vehicle-treated control group. ESB; ethanol stem bark extract of *Milicia excelsa*, HF; n-hexane, EAF; ethylacetate, BF; n-butanol and AF; aqueous fractions of *Milicia excelsa* stem bark extract

Figure 2. Sedative effect of ESB, HF, EAF, BF and AF on sleep duration in pentobarbital-induced sleeping time in mice

3.3.3. Effects of ESB, HF, EAF, BF and AF on Sleep Latency in Ketamine-induced Sleeping Time in Mice

The ESB, HF, EAF and BF significantly ($p < 0.05$) shortened the sleep latency at all the doses of 75, 125 and 250 mg/kg, p.o. AF significantly ($p < 0.05$) shortened the sleep latency at 125 and 250 mg/kg, p.o. while diazepam (2 mg/kg, i.p.) significantly ($p < 0.05$) reduced the sleep latency when compared to the vehicle-treated control group in ketamine-induced sleeping time in mice. The result is presented in Figure 3.

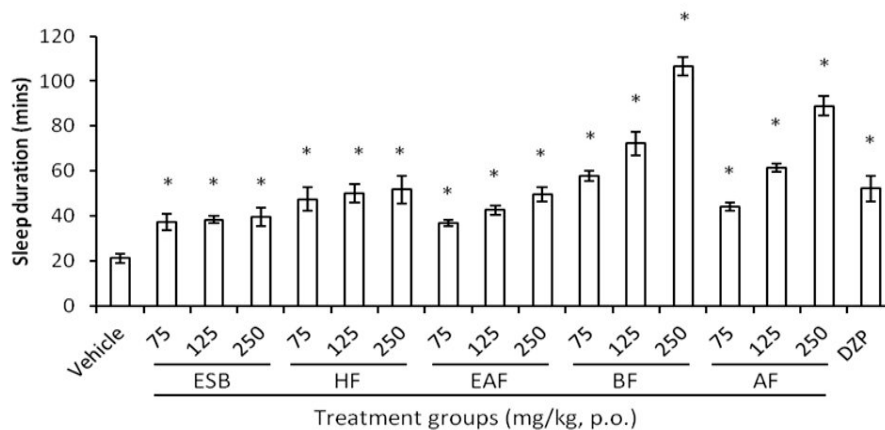


Each bar represents mean \pm SEM. (ANOVA, followed by Dunnett's post hoc), $n=5$, * $p < 0.05$ when compared to the vehicle-treated control group. ESB; ethanol stem bark extract of *Milicia excelsa*, HF; n-hexane, EAF; ethylacetate, BF; n-butanol and AF; aqueous fractions of *Milicia excelsa* stem bark extract

Figure 3. Sedative effect of ESB, HF, EAF, BF and AF on sleep latency in ketamine-induced sleeping time in mice

3.3.4. Effects of ESB, HF, EAF, BF and AF on Sleep Duration in Ketamine-induced Sleeping Time in Mice

The ESB, HF, EAF, BF and AF at all the doses used significantly ($p < 0.05$) and dose dependently prolonged the sleep duration when compared to the vehicle treated control group. The positive control drug diazepam (2 mg/kg, i.p.) also significantly ($p < 0.05$) elongated the sleep duration when compared to the vehicle treated control group in pentobarbital-induced sleeping time in mice. The result is presented in Figure 4.



Each bar represents mean \pm SEM. (ANOVA, followed by Dunnett's post hoc), $n=5$, * $p < 0.05$ when compared to the vehicle-treated control group. ESB; ethanol stem bark extract of *Milicia excelsa*, HF; n-hexane, EAF; ethylacetate, BF; n-butanol and AF; aqueous fractions of *Milicia excelsa* stem bark extract

Figure 4. Sedative effect of ESB, HF, EAF, BF and AF on sleep duration in ketamine-induced sleeping time in mice

4. Discussion

This study evaluated the acute toxicity (LD₅₀) of ESB, HF, EAF, BF and AF using oral route in mice. The study further investigated the sedative-hypnotic effects of the extract and fractions in mice. The findings showed that the extract and fractions were safe and possessed sedative effects in experimental models of sedative-hypnotic tests.

The LD₅₀ of HF, EAF, BF and AF were found to be greater than 5000 mg/kg indicating the safety of the extract and fractions in mice. Since according to Hayes [33], since no acute toxicity may be considered above 5 g/kg body weight.

The pentobarbital-induced sleep test is the most widely used experimental method used for screening sedative-hypnotic agents [34-36], especially those acting via GABAergic system [37, 38]. The ESB and all its fractions in this study reduced sleep latency and prolonged sleeping time in pentobarbital-induced sleep test suggesting that the extract and its various fractions may possess bioactive principles with sedative-hypnotic effect. Although the mechanism of action of ESB and its various fractions were not carried out in this study, but it is well known that barbiturates such as pentobarbital act on the GABA receptor's ionophore complex and allow the opening of chloride channels thus hyperpolarizing the membrane, leading to CNS depression which results in sedation and hypnosis [35]. It is also possible that the sleep modulating effect of ESB and its fractions may also be acting via mixed serotonergic and GABAergic mechanisms to activate the 5-HT_{2C} receptors expressed by GABAergic cells in sleep-related brain areas to bring about the reduction in the observed sleep latency and the prolongation of the sleeping time [11, 39].

The ketamine-induced sleep test has been employed to screen medicinal agents with sedative-hypnotic effect [32]. The reduction in sleep latency and potentiation of sleeping time by ESB and all the fractions suggest that these agents may have sedative effects. Ketamine, an antagonist of glutaminergic neurotransmission via the N-Methyl-D-Aspartate (NMDA) excitatory receptor system has been reported to cause sedation with additional γ -Amino Butyric Acid-A (GABA_A) receptor potentiation [40, 41]. Ketamine has also been reported to prevent the binding of glutamate; the major excitatory neurotransmitter in the CNS to the NMDA receptor, resulting in depressed activities [42, 43]. The observed sedative effects therefore, may be due to the involvement of GABA or NMDA receptor pathway. The finding of our study is in conformity with earlier published data of medicinal agents with sedative-hypnotic effect in ketamine-induced sleeping time [41, 43].

Earlier scientific findings have suggested that flavonoids from medicinal plants may possess sedative effects [44]. Flavonoids have further been reported to bind to the GABA_A benzodiazepine receptor site thereby resulting in

sedative effects [44, 45]. Therefore, the sedative effect of ESB and its various fractions may be attributed to the abundance of total flavonoids, which may act either in additive or synergy with other phytochemicals in the extract and fractions.

5. Conclusions

This study concludes that ESB and its fractions may possess sedative-hypnotic potentials. However, further studies may be warranted to elucidate the probable neural mechanism of the observed sedative effect using receptor antagonist and to carry out GABA binding assay to confirm the involvement of GABA in the sedative effect of ESB and its fractions reported in this study, as well as isolate and characterize the sleep promoting bioactive component(s) in ESB and its various fractions.

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