Investigational
Medicinal Chemistry & Pharmacology

Research Article Open Access

Antidepressant potential of butanol fraction of *Milicia excelsa* (Moraceae) leaf in mice

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Abstract

Background: *Milicia excelsa* is used to treat mental illnesses among the Hausa ethnic group of Northern Nigeria, but there is lack of scientific investigation to validate this ethnomedicinal claim. Hence, this study investigated the antidepressant-like effect of butanol fraction of *Milicia excelsa* leaves in mice (BFME).
Methods: The antidepressant-like effect of BFME was investigated using forced swim (FST) and tail suspension (TST) tests in mice. The probable neural mechanism of its antidepressant-like effect was investigated using receptor antagonists, nitric oxide precursor and inhibitors in the forced swim test. The phytoconstituents in BFME were also quantified.
Results: The BFME significantly ($p<0.05$) decreased the immobility time of mice in FST and TST indicating an antidepressant-like effect. This effect was significantly ($p<0.05$) reversed by prazosin (62.5 µg/kg, i.p.), yohimbine (1 mg/kg, i.p.), cyproheptadine (3 mg/kg, i.p.), methylene blue (10 mg/kg, i.p.), L-NG-Nitroarginine (10 mg/kg, i.p.) while L-arginine (750 mg/kg, i.p.) potentiated it suggesting the involvement of adrenergic, serotonergic and nitric oxide signaling pathways. Alkaloids and phenols were the most abundant phytoconstituents in BFME.
Conclusion: The study therefore, concluded that BFME may possess antidepressant-like effects, which may be due to the synergistic or additive effects of the phytoconstituents present while the mechanism(s) may involve adrenergic, serotonergic and nitric oxide signaling pathways.

Keywords: *Milicia excelsa;* antidepressant; phytoconstituents; adrenergic pathway; serotonergic neurotransmission; nitric oxide signaling pathways.

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Citation on this article: Lateef A. Akinpelu, Julius K. Olaonipekun, Samuel S. Agboola, Theophilus. A. Adegbuyi, Idowu J. Olawuni, Idris A. Oyemitan, Gbola Olayiwola. Antidepressant potential of butanol fraction of Milicia excelsa (Moraceae) leaf in mice. Investigational Medicinal Chemistry and Pharmacology (2020) 3(2):41; Doi: https://dx.doi.org/10.31183/imcp.2020.00041

Background

Depression is the most prevalent mental disorder [1]. It is characterized by mood disturbances accompanied by alterations in behaviors such as appetite, sleep, energy and weight gain [2]. It has been projected by the World Health Organization that depression will rank as the second leading diseases in the developed countries by 2020 [3].

Several hypotheses have been propounded based on the neurotransmitters involved in depression. One of such is the monoamine hypothesis, which suggests changes in norepinephrine and serotonin concentrations in the brain of patients with major depression [4]. Dopamine has also been demonstrated to be involved in the pathophysiology and treatment of depression [5], and the abnormality of glutamate function in the neural substrate of depressed patients has been reported [6]. Nitric Oxide, a second messenger in the brain has also been implicated in depression [7].

Different classes of antidepressant drugs such as monoamine oxidase inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors, 5-HT_2 receptor antagonists, specific serotonin-norepinephrine reuptake inhibitors, and other heterocyclics are clinically employed as drug therapy for depression [8]. However, due to an array of negative health such as sexual dysfunction, apathy, fatigue and cognitive impairment are among the side effects associated with these medications [9], hence, depression continues to be a major medical problem [10], thereby necessitating the search for new effective antidepressants with less side effects and better tolerability.

Milicia excelsa (Welw.) C.C. Berg is a member of the family Moraceae. It is called 'Iroko tree' among the Yoruba tribe of South Western Nigeria. It is commonly called African teak. *Milicia excelsa* (*M. excelsa*) is a large deciduous tree which grows up to 50 m high and occurs in the natural humid forests of West Africa region [11]. The different plant parts of *M. excelsa* including its ashes are used in African traditional medicine to prepare traditional medication [12] for the management of mental illnesses [12, 13], rheumatism [14] among other diverse folkloric uses.
The anticonvulsant [15], anti-stress [16], anti-inflammatory [17] and anti-amnesia [18] effects of the leaf extract have been reported.

Previous scientific report of ultraviolet visible spectroscopic analysis of butanol fraction of *Milicia excelsa* leaf extract (BFME) revealed the presence of flavonoids and terpenoids [17]. The Fourier Transform Infra-Red spectroscopy of BFME showed the presence of polyhydroxyl compounds, alcohol, phenols, aromatic compounds, amide, alkanes, methoxy methyl ether and phosphate compounds which were suggested to confer therapeutic benefits to the leaf [17].

This study aimed to assess the potential antidepressant effect of BFME, its probable neural mechanism(s) of action, as well as to determine quantitatively the phytoconstituents therein.

Methods

Plant identification and authentication

Milicia excelsa (Moraceae) leaves were collected on the campus of the Obafemi Awolowo University in January 2017. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Science, OAU, Ile-Ife and herbarium voucher Ife 17482 was obtained.

Preparation of plant materials

Milicia excelsa leaves were extracted with seventy percent (70%) ethanol for 72 h and partitioned as previously reported [19]. The *n*-butanol fraction (BFME) of the crude ethanol extract showed central nervous system (CNS) excitatory effect following its assessment on novelty-induced behaviors on open field apparatus, making it suitable for this study.

Drugs

The following drugs were used as either positive control drugs, receptor antagonists, nitric oxide inhibitors or precursor: Diazepam (Roche, Basel, Switzerland), prazosin hydrochloride, yohimbine hydrochloride, cyproheptadine hydrochloride, (±) sulpiride, atropine sulphate, ascorbic acid, methylene blue, LNG- Nitroarginine, L-arginine, imipramine hydrochloride and fluoxetine hydrochloride (Sigma Aldrich, St. Louis, MO, USA), and physiological saline.

Laboratory animals

Adult male albino mice were purchased from the Animal House of the Faculty of Pharmacy, OAU, Ile-Ife, Osun State, Nigeria. The mice were housed in cages with 5 mice per cage. They were maintained on standard animal pellets and water *ad libitum*. The experimental protocols adopted in these behavioral investigations adhered to the approved institutional guidelines which align with the internationally accepted principles for Laboratory Animal Use and Care [20].

General experimental design

Adult male albino mice (18 to 25 g) were randomly grouped into 5 groups (n=5). Group 1 (vehicle-treated control group) mice were orally ingested with 2% Tween 20 dissolved in physiological saline (10 mL/kg). Group 2-4 (treatment groups) received orally BFME at 100, 200 and 400 mg/kg respectively, while Group 5 (positive control group) mice received orally imipramine at 20 mg/kg for forced swim (FST) test, fluoxetine (20 mg/kg, p.o.) for tail suspension (TST) test and diazepam (1 mg/kg, i.p) for open field test.

Forced-swim test

The FST was carried out using the modified Porsolt test [21]. One hour after oral treatments with the vehicle, BFME, and imipramine, each mouse was individually forced to swim in an inescapable cylindrical glass jar (25 x 12 cm) filled with fresh water up to 15 cm height and maintained at 25^0C. After 2 minutes of initial vigorous struggling to escape from the jar, each mouse assumed a characteristic immobile posture, without struggling but only making minimum movements of its limbs essential to keep it afloat. In a pre-test session, each animal was placed into the cylinder for 15 minutes, 24 hours prior to the 6 minutes swimming test, in which the duration of immobility was recorded for the last 4 minutes of the 6 minutes [22].

Tail-suspension test

One hour after oral treatments with the vehicle, BFME or fluoxetine. Each mouse was suspended with adhesive tape 50 cm above the floor on the edge of a table. The adhesive tape was placed approximately 1 cm from the tip of the tail of each mouse. The first 2 minutes of vigorous activity in water was disregarded for

every mouse, while the duration of immobility was recorded for the last 4 minutes of the 6 minutes of test session for each mouse. Each mouse was considered immobile when it ceased from making any movement and hang passively [23].

Open field test (OFT)

One hour after post oral treatments with the vehicle, BFME and 30 minutes after injection of diazepam (1 mg/kg, i.p). Each mouse was gently placed in an observation cage (100 cm x 100 cm x 30 cm) subdivided into 16 equal squares. The number of square crossed for 5 minutes duration was recorded [24].

Mechanism of the antidepressant effect of BFME

The highest dose of BFME (300 mg/kg, p.o.) was used for the delineation of the probable neural mechanism of action of BFME. Mice were pretreated with adrenergic receptor blockers [Prazosin (62.5 µg/kg, i.p., α_1-receptor blocker); yohimbine (1 mg/kg, i.p., α_2-receptor blocker)], 5-HT_2 serotonergic receptor blocker [cyproheptadine (3 mg/kg, i.p.)], dopaminergic receptor blocker [sulpiride (50 mg/kg, i.p., D_2 receptor blocker)], muscarinic cholinergic receptor blocker [atropine (1 mg/kg, i.p.)], ascorbic acid [100 mg/kg, i.p., a putative neuromodulator that antagonizes *N*-methyl-D-aspartate (NMDA) receptor], nitric oxide synthase (NOS) precursor [L- arginine (750 mg/kg, i.p.), [nitric oxide synthase (NOS) inhibitor [L-NNA (10 mg kg-1, i.p.)] and methylene blue (10 mg/kg, i.p., a direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase), 15 minutes before BFME (300 mg/kg, p.o.). Sixty minutes after oral ingestion of BFME, mice were subjected to FST. The doses of the receptor antagonists, nitric oxide synthase precursors, and nitric oxide inhibitors were selected from literature [6, 25-27].

Preliminary phytochemical quantification of BFME

Determination of total alkaloid in BFME

To 1 (mg/ml) of BFME was added 1ml of 2 N HCl and thereafter filtered. The resulting filtrate was transferred to a separating funnel. Exactly 5 ml of bromocresol green solution and 5 ml of phosphate buffer were both added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform successively by vigorous shaking and collected in a 10 ml volumetric flask which was diluted to the required volume with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 µg/ml) were prepared in the same way as described above. The absorbance for the test and standard solutions were determined against the reagent blank at 470 nm with UV/Visible spectrophotometer. The total alkaloid content was estimated in mg of atropine equivalent (AE)/g of BFME [28, 29].

Determination of total phenols in BFME

The total phenolic content of BFME was estimated using Folin-Ciocalteau reagent. About 20 µg of BFME was taken separately and made up to 1 ml with distilled water. Then 500 µL of diluted Folins-phenol reagent (1:1 ratio with water) and 2.5 ml of sodium carbonate (20%) were added. The mixture was shaken well and incubated in the dark for 40 minutes for the development of color. After incubation, the absorbance was measured at 725 nm. A calibration curve of gallic acid was constructed and linearity was obtained in the range of 10-50 µg/ml. The total phenolic content in BFME was expressed as mg of gallic acid equivalent (mg GAE/g extract) by using the standard curve [30].

Determination of total flavonoids content in BFME

Total flavonoid content was measured by the aluminum chloride colorimetric assay. The reaction mixture consists of 1 ml of BFME and 4 ml of distilled water taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was added and after 5 minutes, 0.3 ml of 10 % aluminum chloride was mixed. After 5 minutes, 2 ml of 1 M sodium hydroxide was added and diluted to 10 ml with distilled water. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of quercetin equivalent (QE)/g of extract [31].

Determination of tannin content in BFME

The Folin - Ciocalteu method was used to quantify tannins in BFME. To a 10 ml volumetric flask containing 7.5 ml of distilled water and 0.5 ml of Folin Ciocalteu phenol reagent was added about 0.1 ml of BFME, 1 ml of 35 % Na_2CO_3 solution was diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions with gallic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for BFME and standard solutions were measured against the blank at 725 nm with the aid of a UV/Visible spectrophotometer. The tannin content of BFME was therefore expressed in mg of GAE /g of extract [32].

Atomic absorption spectrometer (AAS) measurement

Exactly, 0.5 g of BFME was weighed into a digesting flask, 5ml of the mixture of nitric and perchloric acid in the ratio (4:1) was added, it was heated at 100°C until the solution changed color. On cooling, it was made up to 50 ml with distilled water. The dilute filtrate was used for the analysis of elements of interest (Mg, Zn, Cd, Cr, and Pb) by AAS (Perkin Elmer Analyst 400 Atomic Absorption Spectrometer) using suitable hollow cathode lamps. The concentrations of various elements were determined by a relative method using A.R. grade solutions of elements of interest [29].

Statistical analysis

Results are expressed as mean ± S.E.M. The significance of difference between groups were analyzed using one-way Analysis of Variance (ANOVA), followed by Dunnett's post hoc test. The level of significance for all tests was set at $^*p<0.05$ and $^{\#}p<0.05$.

Results

Effect of BFME on forced swimming (FST) and tail suspension (TST) tests

The BFME at all the doses used significantly ($p<0.05$) reduced the immobility time in FST and TST when compared to the vehicle treated control group. The activities of BFME at 200 and 300 mg/kg were comparable to that of the standard antidepressant drug imipramine for FST and fluoxetine for TST (Table 1).

The result of mechanism of the antidepressant effect of BFME

The pretreatment of mice with prazosin, yohimbine, cyproheptadine, L-NNA and methylene blue before the oral ingestion of BFME (300 mg/kg) significantly ($p<0.05$) reversed the antidepressant effect of BFME in FST (Figure 1). However, pre-treatment of mice with sulpiride, atropine, and ascorbic acid, before oral administration of BFME, did not reverse the anti-immobility effect of BFME (Figure 2). The L-arginine significantly ($p<0.05$) potentiated the antidepressant-like effect of BFME in FST (Figure 2).

Results of BFME on locomotor activity of mice in Open field test.

The BFME at all the doses used and diazepam (1 mg/kg) did not show any significant ($p>0.05$) effect on locomotor behaviour of the experimental mice when compared to the vehicle treated control group (Table 2).

Results of quantitative phytochemical estimations of BFME

The results of phytochemical estimation of BFME revealed that BFME total alkaloid is 79.80 ± 3.70 mg atropine equivalent/g of BFME with reference to standard calibration curve ($y = 0.0051x - 0.078$, $R^2 = 0.9765$), total phenols is 37.60 ± 0.20 mg gallic acid equivalent/g BFME with reference to standard calibration curve ($y = 0.0042x + 0.0221$, $R^2 = 0.9947$), tannin (24.36 ± 0.86 mg gallic acid equivalent/g BFME with reference to standard calibration curve ($y = 0.0049x + 0.0596$, $R^2 = 0.9594$) and total flavonoids 2.83 ± 0.02 mg gallic acid equivalent/g BFME with reference to standard calibration curve ($y = 0.01177x + 0.0635$, $R^2 = 0.9731$) (Table 3).

Results of atomic absorption spectroscopy of BFME

The results of AAS analysis of BFME revealed the presence of the following elements in the order of magnitude: Mg > Zn > Pb > Cd. Chromium was not detected. The BFME showed negligible concentration of Pb and Cd (Table 4).

Discussion

This study provides evidence for the antidepressant-like effects of the butanol fraction of *Milicia excelsa* leaf extract (BFME), proposes its neurobehavioral mechanism of action, and assayed for the phytoconstituents, as well as its elemental compositions.

From the previous oral LD_{50} determination of *n*-butanol fraction of *Milicia excelsa*, which was found to be greater than or equal to 5000 mg/kg per oral [19], lower doses of 100, 200 and 300 mg/kg per oral, which did not affect locomotor behavior were used in this study.

The forced swim and tail suspension mouse models are the two most widely used models of depression [33], which may be therapeutically effective in human depression [7, 33]. The reduction of immobility time in FST and TST is consistent with antidepressant-like effect. This finding is in agreement with earlier report of antidepressant-like effect of *Citrus maxima* leaves [22].

An earlier report has shown that agent that brings about alteration in general motor activity of mice may result in false positive/negative outcome in both FST and TST [23]. Therefore, the doses of BFME used in this study were selected such that they did not affect the spontaneous locomotor activities of mice suggesting that the antidepressant-like effects of BFME may not be due to psychostimulant effect [34].

In this study, the reversal of the antidepressant-like effect of BFME by prazosin and yohimbine suggest that BFME may be acting via the interaction with the adrenoceptors [35].

Subsequently, the reversal of the antidepressant-like effect of BFME by cyproheptadine indicates the interaction of BFME with serotonergic pathway since inhibition of serotonin *via* $5\text{-}HT_2$ receptor pathway is crucial to the antidepressant effect of some antidepressant agents in FST [36].

The non-reversal of the antidepressant effect of BFME by sulpiride, atropine and ascorbic acid, suggest the non-involvement of dopaminergic, muscarinic cholinergic, and NMDA receptor pathways in the antidepressant-like effect of BFME.

Nitric Oxide (NO) plays a significant neuromodulatory role in the CNS [6]. The L-arginine-nitric oxide-cyclic guanosine monophosphate is a signaling pathway implicated in the study of mechanism of action of an antidepressant agent in FST [37]. In this study, L-NNA and methylene blue (nitric oxide inhibitors) reversed the antidepressant-like effect of BFME while L-arginine (nitric oxide precursor) potentiated this effect in FST suggesting the involvement of nitric oxide signaling pathway in the antidepressant-like effect of BFME. The potentiation of the antidepressant effect of BFME by L-arginine found in this study may at least in part be supported by the antidepressant effect of L-arginine [38].

The phytochemical estimation in this study revealed that alkaloids and phenols are the most abundant phytoconstituents, among other secondary metabolites in BFME, which could be responsible for the observed antidepressant-like effect of BFME. A review of the literature for the last decade reported plant-based alkaloids [39] and phenolic compounds [40] as emerging therapies for depression [39, 40]. Particularly *via* the inhibition of monoamine oxidase enzymes secondary to increased serotonergic, dopaminergic and noradrenergic neurotransmission in some selected brain regions [41].

Previous report has shown the isolation of ursolic acid from *Milicia excelsa* leaf [42]. A scientific report has also shown that ursolic acid isolated from *Rosmarinus officinalis* plant showed antidepressant-like activities [43]. It could be suggested therefore, that the observed antidepressant effect of BFME may partly be due to the presence of ursolic acid which may act in additive or synergy with other compounds in BFME.

The need for the quality control measures of herbal drugs has been suggested, this is because of the likelihood of being contaminated by heavy metals as a result of environmental pollution [44]. For example, St. John's wort (*Hypericum perforatum* L.) used as an anti-depressive agent for centuries [45], have now been shown to have high cadmium contents [46]. Hence, the determination of toxic metals in the medicinal plant is pertinent and imperative. The negligible concentrations of Pb and Cd in BFME below the hazardous levels set by the World Health Organization [47] together with the non detection of Cr suggest that BFME may be safe from Cr, Pb and Cd toxicities. Since toxic metal like lead has strongly been linked to depressive and anxiety disorders via disruption of monoaminergic systems [48]. The presence of magnesium and zinc in BFME may add additional therapeutic benefit to BFME since the antidepressant effects of magnesium and zinc have been reported [49].

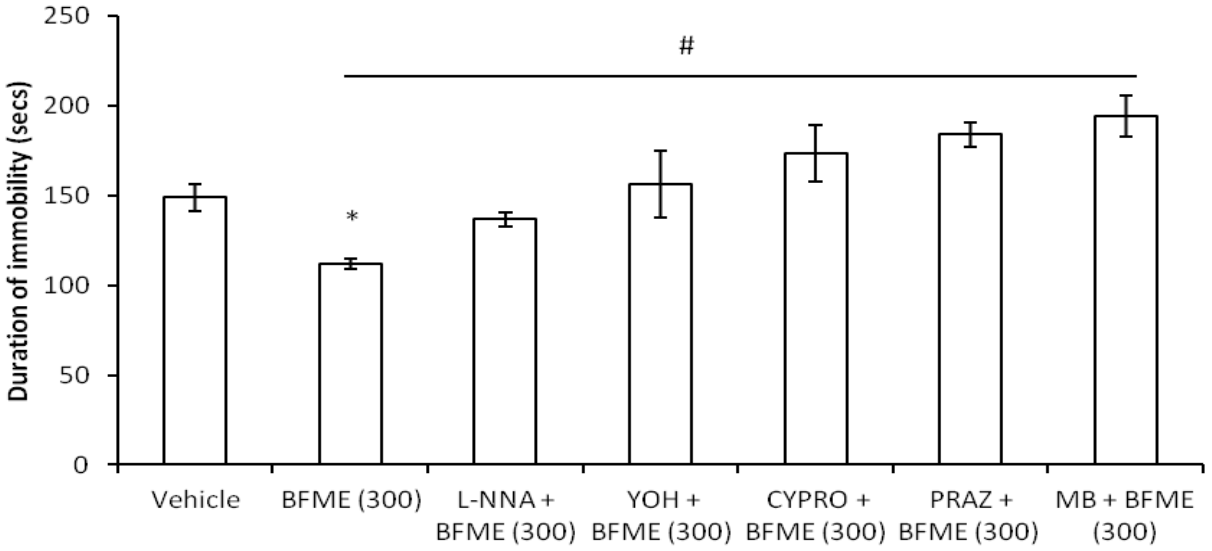

Figure 1. The effect of L-nitroarginine (L-NNA), yohimbine (YOH), cyproheptadine (CYPRO), prazosine (PRAZ) and methylene blue (MB) on the anti-immobility of n-butanol fraction of *Milicia excelsa* leaf extract (BFME).

Each column represents Mean ± SEM (n=6). *p<0.5 compared to the vehicle treated control group. #p<0.05 compared to BFME treated group.

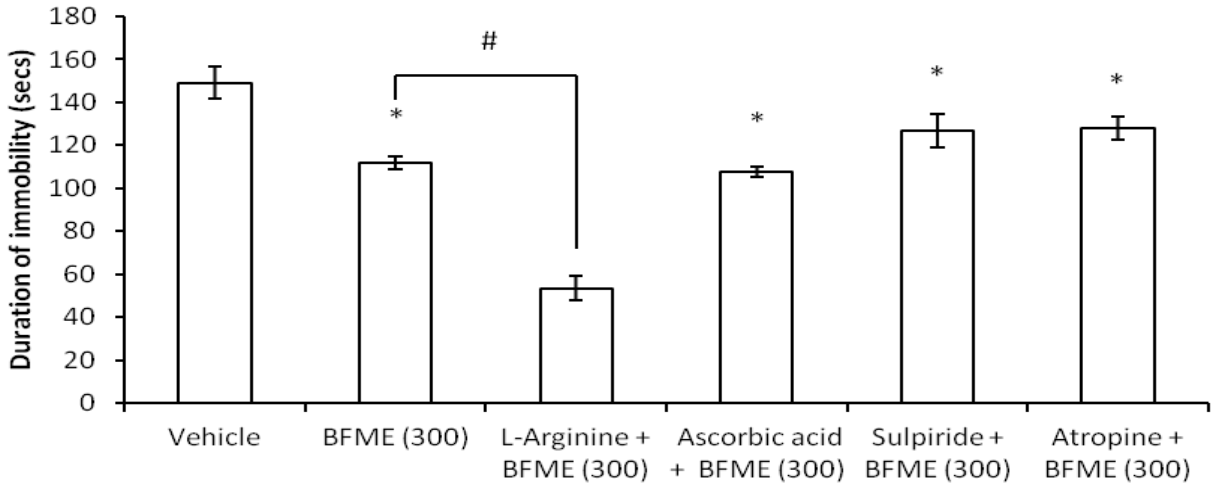

Figure 2. The effect of L-arginine, ascorbic acid, sulpiride and atropine on the anti-immobility of n-butanol fraction of *Milicia excelsa* leaf extract (BFME).

Each column represents Mean ± SEM, (n=6). *p<0.5 compared to the vehicle treated control group. #p<0.05 compared to BFME treated group.

Table 1. Effects of BFME on FST and TST behavioral models in mice

Group	Doses (mg/kg)	FST	TST
		Duration of Immobility (secs) (Mean ± S.E.M)	Duration of Immobility (secs) (Mean ± S.E.M)
Vehicle	(10 mL/kg)	149.0 ± 7.5	144.5 ± 8.0
BFME	100	126.5 ± 3.3*	121.3 ± 8.5*
	200	111.0 ± 4.2*	114.5 ± 3.9*
	300	111.8 ± 3.0*	95.3 ± 7.0*
Imipramine	20	125.3 ± 2.9*	-
Fluoxetine	20	-	125.5 ± 3.7*

Each group represents Mean ± SD (n=6). *p<0.05 compared to the vehicle treated control group. BFME; n-butanol fraction of *Milicia excelsa* leaf extract.

Table 2. Effects of diazepam and BFME on behavior of mice in Open field paradigm

Group	Doses (mg/kg)	Number of square crossed (Mean ± S.E.M)	Frequency of rearing (Mean ± S.E.M)	Total (Mean ± S.E.M)
Vehicle	(10 mL/kg)	89.2 ± 5.4	28.3 ± 2.1	117.5 ± 6.0
BFME	100	104.3 ± 6.9	28.5 ± 1.8	132.8 ± 7.0
	200	94.2 ± 4.9	35.3 ± 1.1	129.5 ± 3.9
	300	98.2 ± 5.4	33.5 ± 1.8	111.8 ± 3.0
Diazepam	1	94.0 ± 3.6	18.0 ± 3.7*	112.0 ± 4.4

Each group represents Mean ± SD (n=6). *p<0.05 compared to the vehicle treated control group. BFME; n-butanol fraction of *Milicia excelsa* leaf extract.

Table 3. Quantitative phytochemical estimation of BFME

Phytochemicals	Amounts
Total Alkaloid content	79.80 ± 3.7 mg of AE/g BFME
Total phenol	37.60 ± 2.0 mg GAE/g BFME
Tannin content	24.36 ±0.86 mg of GAE /g BFME
Total flavonoid content	2.38 ± 0.02 mg QE/g BFME

Values are means of triplicate determination ± Standard deviation; where AE, GAE and QE are atropine, gallic acid and quercetin equivalents respectively.

Table 4. Result of the AAS Analysis of the BFME

Metals	BFME (mg/100 g)
Magnesium (Mg)	695.200 ± 0.040
Zinc (Zn)	13.340 ± 0.010
Lead (Pb)	0.024 ± 0.010
Cadmium	0.015 ± 0.005
Chromium (Cr)	ND

The result of each of the parameter is in triplicate and presented in mean ± standard deviation.
ND means not detected

Conclusion

In conclusion, the present study provides a scientific rationale for the use of the leaves of *Milicia excelsa* in treating depression. The antidepressant activity of BFME may be due to the abundance of alkaloid and phenols, which may either in synergy, additive or counter interaction with other phytoconstituents in BFME. This work further suggests the involvement of multiple pathways as probable neuronal mechanisms of the antidepressant-like effect of BFME.

Abbreviations

BFME, butanol fraction of *Milicia excelsa* leaves; FST, Forced-swim test; TST, Tail-suspension test; OFT, open field test; NOS, nitric oxide synthase; L-NNA, L-nitroarginine; AE, atropine equivalent; GAE, gallic acid equivalent; QE, quercetin equivalent; AAS, atomic absorption spectroscopy; AR, analar grade; SEM, standard error of mean; ANOVA, analysis of variance; LD_{50}, median lethal dose; NMDA, *N*-methyl-D-aspartate; CNS, central nervous system; NO, nitric oxide; YOH, yohimbine; CYPRO, cyproheptadine; PRAZ, prazosine; MB, methylene blue.

Authors' Contribution

The research work reported in this study was carried by collaboration among all authors. Author LAA designed the study, carried out some laboratory experiments, managed the literature search, carried out the statistical analysis and wrote the first draft of the manuscript. Author JKO carried out the extraction and partitioning of the plant material and contributed to the writing of the first draft. Author SSA participated in the experimental design and carried out some laboratory experiments. Author TAA carried out some laboratory experimental work and contributed to the writing of the manuscript. Author IJO carried out the phytochemical estimations. Author IAO supervised the work, provided some materials and contributed to the editing of the manuscript, Author GO reviewed and edited the manuscript.

Acknowledgments

We are thankful to Mrs J.O Omotayo and Mr E.A Adeyemi of the Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile Ife, for providing technical assistance.

Conflict of interest

The authors declare no conflict of interest

Article history:

Received: 20 May 2020
Received in revised form: 9 June 2020
Accepted: 10 June 2020
Available online: 10 June 2020

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