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Butanol soluble fractions of *Cissus cornifolia* methanolic leaf extract and behavioural effects in mice

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ABSTRACT

Cissus cornifolia Baker - Planch (Family: Vitaceae) is used for various central nervous system disorders. The present study reported the sedative and central nervous depressant effects of fractions (butanol soluble portion and its flavonoid rich fraction) obtained from methanolic leaf extract of *Cissus cornifolia* using diazepam induced sleep, head-dip and motor coordination tests in mice at doses between 5 to 600 mg/kg body weight. The flavonoid rich column fraction 3 (CF3) significantly ($p < 0.05$) prolonged the duration of sleep in mice at the dose of 10 and 20 mg/kg. Similarly, the butanol soluble portion significantly ($p < 0.001$) prolonged the duration of diazepam induced sleep at 150, 300 and 600 mg/kg in a dose dependent manner. It also significantly ($p < 0.05$) decreased the onset of sleep at the dose of 150 and 600 mg/kg. A dose dependent and significant ($p < 0.001$) decrease in the mean number of head-dips was produced by the butanol soluble portion at all the doses tested. The butanol soluble portion at all the doses tested significantly ($p < 0.005$) prolonged the time to complete the beam walk, however the extract did not produce a significant increase in number of foot slips. The results demonstrated that the butanol soluble fractions obtained from methanolic leaf extract of *Cissus cornifolia* posses sedative and central nervous system depressant activity, thus supporting its ethno medicinal use as a sedative in the management of central nervous system disorders.

Keywords: Behaviour, *Cissus cornifolia*, Diazepam, Flavonoid.

INTRODUCTION

Herbal medicines are an inherent part of African traditional medical practices and are once again becoming popular throughout the developing and developed world [1]. The practice of herbal medicine continues to exist in developing nations and on this basis, it becomes relevant to search for potent, effective and relatively safe plant medicines as well as to scientifically validate success claims about plants already in use by traditional medicine practitioners and their potential for drug discovery.

Cissus cornifolia Bak.- Planch (Family: Vitaceae) is one of the plants used in managing variety of illness such as cases of mental derangement. The herb is distributed mainly in the rocky suburbs and Savannah regions of Ghana and Northern Nigeria. It is locally called "Rigarbiri" or "Duwawun biri" literally meaning "robe of the monkey" among the Hausa speaking people of Nigeria. The plant parts are also used in ethno medicine for variety of illness such as gonorrhoea when taken with native natron, as a sedative, in pharyngitis and in treatment of malaria [2].

Our earlier studies on *Cissus cornifolia* revealed that the crude methanolic extract possess central nervous system depressant and sedative properties in mice [3] [4]. This study therefore aimed at investigating the active constituents responsible for such activities.

MATERIAL AND METHODS

Collection and Preparation of Plant Material

The leaves of *Cissus cornifolia* was freshly collected from Basawa, Sabon-Gari Local Government area of Kaduna State, Nigeria in the month of October, 2008. It was identified and authenticated at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, where a voucher specimen (No. 024) was deposited for future reference. The leaves were air dried under shade until constant weight was obtained and then pulverized using pestle and mortar.

Animals

Albino mice (18-25 g) of either sex maintained in the Animal House of the Department of Pharmacology

and Therapeutics, Ahmadu Bello University, Zaria were used for the study. The animals were housed in well ventilated rooms (at room temperature), fed on pelletized Vital Feed® (Bukuru, Jos) and water *ad libitum.* All experimental protocols were approved by the Ahmadu Bello University Animal Ethics Committee.

Drugs and chemicals

Diazepam (Roche, France), Chloroform, Ethylacetate, Methanol and Butanol solvents (Fluka-Aldrich).

Extraction and preparation of butanol soluble portion

The powdered leaves (1200 g) was macerated in 2 liters of 70% $^{v}/_{v}$ aqueous methanol solution, with occasional shaking for 14 days and then filtered. The filtrate was evaporated to dryness *in vacuo* at 40^{o}C to yield 115.51 g of residue referred to as *Cissus cornifolia* methanolic leaf extract. The methanolic extract (100 g) was then treated successively with chloroform, ethyl acetate and butanol to obtain their respective portions [5].

Column chromatography

The butanol soluble portion of *Cissus cornifolia* (5 g) was chromatographed over silica gel packed column (60-120 mesh size). The sample was gradiently eluted with chloroform, chloroform/methanol mixture and then methanol. Forty five fractions of 50 ml each were collected and based on the thin layer chromatographic profile produced six major column fractions. The column was washed with methanol to afford the seventh fraction [5].

Gel filtration of active column fraction

Gel filtration was performed using sephadex LH-20. The gel filter was suspended in methanol, allowed to swell for 24 hours and then poured to a glass column. About 200 mg of column fraction 3 (CF_3) was dissolved in methanol and then loaded on top of the column. The sample was gradiently eluted with 100% methanol.

Phytochemical Screening

Phytochemical analysis of the butanol soluble portion and its column fraction 3 (CF3) was carried out to determine their chemical composition using standard methods [6] [7].

Acute toxicity studies

The intraperitoneal median lethal dose (LD_{50}) of the butanol soluble portion of *Cissus cornifolia* leaf extract was determined in mice [8]. The study was carried out in two phases; in the initial phase, three groups of mice each received the extract at doses of 10, 100 and 1000 mg/kg and then observed for signs of toxicity and death within 24 hrs. In the second phase, three mice were treated with more specific doses (which depended on the result of the first phase) of the extract and observed for signs of toxicity and death within 24 hours. The LD_{50} value was calculated as the geometric mean of the lowest lethal dose and the highest non-lethal dose.

Behavioural Studies

Diazepam induced sleeping test

Sleep potentiating effect of the butanol soluble portion at doses of 150, 300 and 600 mg/kg was studied in four groups of mice that received diazepam at a dose of 20 mg/kg intraperitoneally, 30 minutes after intraperitoneal administration of the butanol portion [9]. There were 6 mice in each group consisting of 3 treatment groups and positive control (normal saline). The criteria for sleep was considered to be loss of righting reflex [10] and sleeping time was measured as the time between disappearance and recovery of righting reflex [11]. Similar study was carried out on three major column fractions (CF_3, CF_5 and CF_7) each at doses of 5, 10 and 20 mg/kg to determine the most active fraction.

Hole-board test

The influence of the butanol soluble portion on the exploratory activity of mice was determined using the hole-board test [12]. The apparatus used consists of a white wooden board (40cm x 40cm) with 16 evenly spaced holes (1cm diameter x 2cm depth). Six groups of 6 mice each were used and the test was carried out 30 minutes after intraperitoneal treatment with the extract. Normal saline (10 ml/kg) and diazepam (1 and 0.25mg/kg) were used as negative and positive controls respectively. Each mouse was placed at one corner of the board and the animal moved about and dipped its head into the holes indicating exploratory behaviour. The number of head dips in 5 minutes was recorded.

Mouse beam-walking assay

Mice were trained to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by a wooden support to a goal box. Three trials were performed for each mouse, and were designed such that the mice tested would be aware that there was a goal box that could be reached. A ruler was used because the mouse found this easy to cross, and at the same time, it induced minimum anxiety.

The mice that successfully walked along the ruler were randomly grouped into five groups each containing six mice. The first group received normal saline at the dose of 10 ml/kg *i.p*. The second, third and fourth groups received the butanol soluble portion at doses of 150, 300 and 600 mg/kg respectively, *i.p*., while the sixth group received diazepam (0.25 mg/kg, *i.p*.). Thirty minutes post-treatment, each mouse was placed on the beam (60 cm long, 8 mm in diameter and 30 cm elevated above the bench) at one end and allowed to walk to the goal box. Mice that fell were returned to the position they fell from, with a maximum time of 60 seconds allowed on beam. The measurements taken were time to complete the task, the number of foot slips (one or both hind limbs slipped from the beam) and the number of falls [13].

RESULTS

Successive partitioning of methanolic extract (100 g) of *Cissus cornifolia* leaf yielded butanol, ethyl acetate, chloroform and residual aqueous portions (Table 1).

Table 1: Yield of partition portions of *Cissus cornifolia* methanolic leaf extract

Portions	Yield (g)	Percentage yield ($^{w}/_{w}$)
Butanol	8.40	8.40
Ethyl acetate	2.36	2.36
Chloroform	2.08	2.08
Residual aqueous	10.04	10.04

Phytochemical contents

The phytochemical constituents of butanol soluble portion of *Cissus cornifolia* methanolic leaf extract and its respective column fractions are presented in table 2

Table 2: Phytochemical constituents of butanol soluble portion of *Cissus cornifolia* methanolic leaf extract

Constituents	Butanol soluble portion	Column fraction (CF3)
Saponins	+	-
Flavonoids	+	+
Anthraquinones	-	-
Alkaloids	+	-
Steroids	-	-

Key: (+) = present; (-) = absent

Column chromatography of butanol portion of *Cissus cornifolia* methanolic leaf extract

Fractionation of butanol soluble portion using silica gel column chromatography yielded seven fractions when eluted with chloroform 100%, chloroform methanol mixture and methanol 100% (Table 3). The subfractions obtained after gel filtration of the major eluate are presented in (Table 4).

Table 3: Eluates obtained from column chromatography of butanol soluble portion of *Cissus cornifolia*

Eluates	Eluting solvent	Weight (g)	No. of spots
1	$CHCl_3$: MeOH (95:5)	0.20	5
2.	$CHCl_3$: MeOH (90:10)	0.10	5
3.	$CHCl_3$: MeOH (80.20)	0.96	3 major
4.	$CHCl_3$: MeOH (70:30)	0.23	5
5.	$CHCl_3$: MeOH (60:40)	0.30	5
6.	$CHCl_3$: MeOH (50:50	0.19	4
7.	MeOH (100%)	0.80	6

MeOH = Methanol

Table 4: Eluates obtained from gel filtration of fraction 3

Sub fraction	No. of Spots
1	3
2	3
3	2 major; 2 minor
4	1 major; 2 minor
5	3 major
6	4
7	2 major

Acute toxicity studies

The intraperitoneal median lethal dose of the butanol soluble portion of *Cissus cornifolia* methanolic leaf extract was estimated to be 2154.1 mg/kg body weight in mice.

Behavioural studies

The butanol soluble portion showed significant difference ($p < 0.05$) in onset of sleep between normal saline (3.8±0.4) and 600 and 150 mg/kg (2.8±0.4 and 2.6±0.3 respectively). The decrease in onset was not dose dependent. However, a dose dependent increase in duration of sleep was observed from 52.3±11.7 (Normal saline) to 269.0±31.6 (600 mg/kg), 225.6±26.0 (300 mg/kg) and 197.2±13.4 (150 mg/kg). Significant difference ($p < 0.05$) was also observed between the extract doses of 600 and 150 mg/kg body weight (Fig. 1).

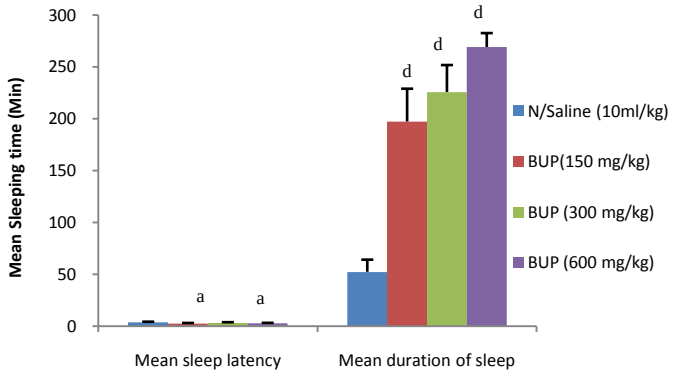

Figure 1: Effect of butanol soluble portion (BUP) of *Cissus cornifolia* methanolic leaf extract on diazepam-induced sleep in mice. a = $p < 0.05$, d = $p < 0.001$ - Student t-test, n = 6

The three column fractions (CF_3, CF_5 and CF_7) at all the doses tested (5, 10, and 20mg/kg) did not show any significant difference in sleep latency when compared with normal saline. Column fraction 3 which produced four major spots on TLC significantly ($p < 0.05$) prolonged the duration of sleep at doses of 20 and 10 mg/kg when compared to normal saline treated group. Fraction 5 which also showed five major spots on TLC significantly ($p < 0.05$) prolonged the duration of sleep at 20 mg/kg when compared with normal saline. Fraction 7 which produced two major and four minor spots on TLC plate produced no effect on mean duration of sleep at all doses tested when compared with normal saline (Fig. 2)

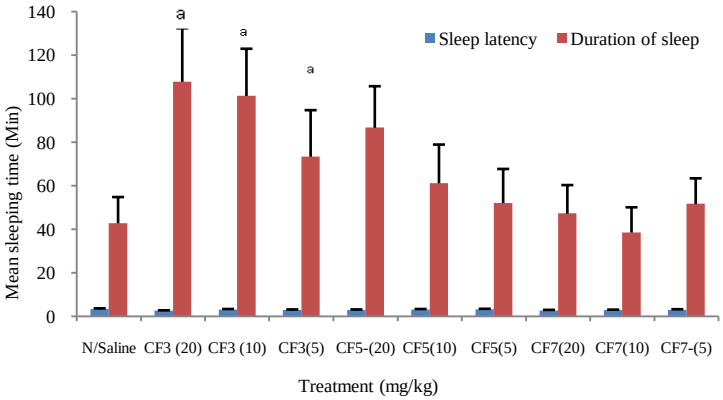

Figure 2: Effect of three major column fractions (CF3, CF5 and CF7) obtained from butanol soluble portion of *Cissus cornifolia* methanolic leaf extract on diazepam induced sleep in mice. a = $p < 0.05$ – Student t-test, n = 6

In the head dip test, the butanol soluble portion significantly ($p < 0.001$) and dose dependently decreased the number of head dips from 14.2±1.8 in normal saline treated group to 3.8±0.4, 5.0±0.4 and 5.5±0.5 at doses of 600, 300 and 150 mg/kg respectively. Diazepam at higher dose of 0.5 mg/kg significantly ($p < 0.001$) reduced the number of head dips from 14.2±1.8 to 4.8±0.3, while at lower dose of 0.25 mg/kg, it significantly ($p < 0.001$) increased exploration in mice to 27.3±2.0 when compared to normal saline (14.2±1.8) (Fig. 3).

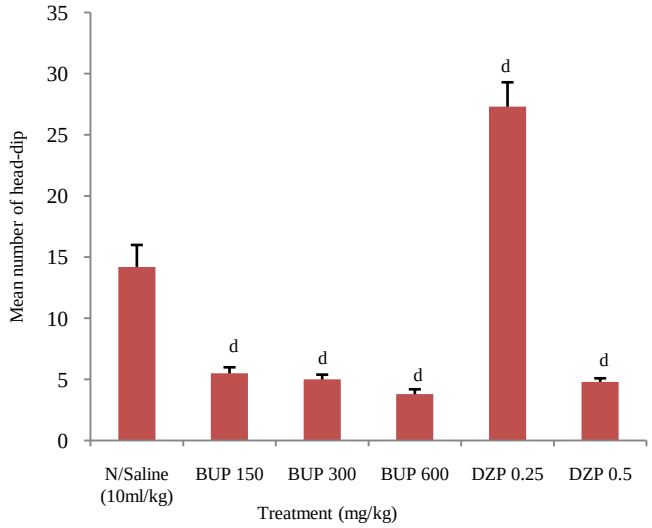

Figure 3: Effect of butanol soluble portion (BUP) of *Cissus cornifolia* methanolic leaf extract on exploratory behaviour in mice. d = $p < 0.001$ – student t-test, n = 6

The butanol soluble portion significantly ($p < 0.005$) delayed the time to reach the goal box when compared with normal saline treated group. It also produced foot slips at doses of 600, 300 and 150 mg/kg. Diazepam, showed a significant ($p < 0.001$) increase in number foot slips as well as the time to reach goal box when compared with normal saline and the extracts ($p < 0.05$) at the doses tested (Fig. 4a and 4b).

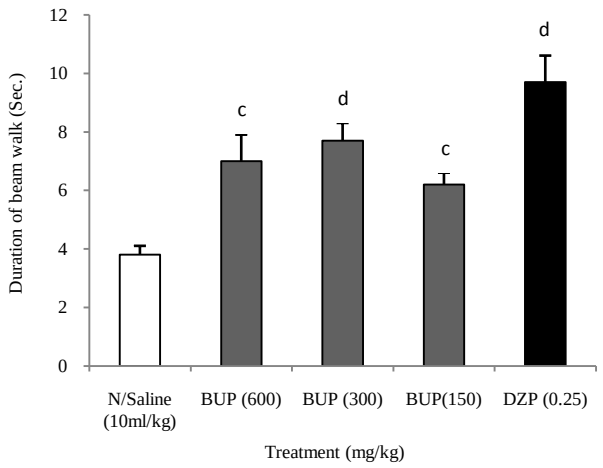

Figure 4A: Effect of butanol soluble portion (BUP) of *Cissus cornifolia* methanolic leaf extract on motor coordination in mice. c = p< 0.005, d= p< 0.001 – Student t-test, n = 6

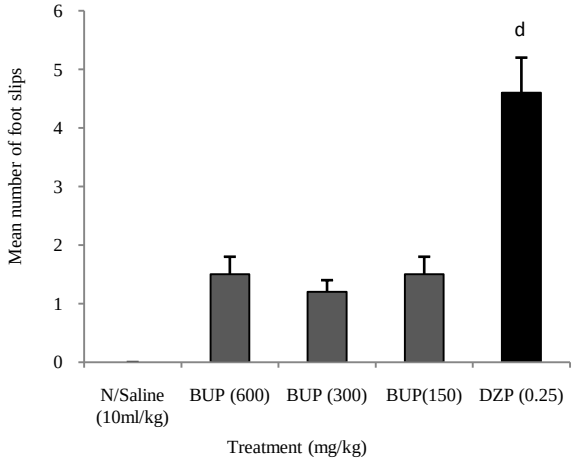

Figure 4b: Effect of butanol portion of *Cissus cornifolia* methanolic leaf extract on motor coordination in mice. d= p< 0.001 – Student t-test, n = 6

DISCUSSION

The butanol soluble portion of *Cissus cornifolia* leaf was found to retain saponins, flavonoids and alkaloids already identified in the crude methanolic extract of the leaf [2]. The positive chemical reaction shown by CF_3 when subjected to shinoda and ferric chloride tests for flavonoids indicates that CF_3 has flavonoid nucleus with some free phenolic hydroxyl groups [14], while negative frothing test for saponins produced by the CF_3 showed that it was devoid of saponins. The absence of dark red fluorescence by CF_3 after been exposed to ammonia vapour denotes the absence of chalcone nucleus [15]. Therapeutic benefits of traditional remedies depends upon a combination of constituents [16], such as those identified in this plant.

Assessment of toxicity profile of any compound is important in drug discovery. In this study, the median lethal dose of butanol soluble portion of *Cissus cornifolia* was determined and according to classification of LD_{50} values, the extract is moderately toxic in mice following intraperitoneal administration [17].

By potentiating the diazepam-induced sleep, the butanol soluble portion and the column fraction (CF_3) all seems to possess sleep inducing properties [9] [18]. Sedative-hypnotic agents act to increase GABA-mediated synaptic inhibition either by directly activating $GABA_A$ receptors or, more usually by enhancing the action of GABA on $GABA_A$ receptors. Benzodiazepines and barbiturates are examples of widely used therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors [19]. Benzodiazepines act by enhancing postsynaptic inhibition through a specific benzodiazepine receptor which is an integral part of the $GABA_A$ receptor chloride channel complex. The receptor is gated primarily by GABA and modulated by secondary ligands which include benzodiazepines. The modulatory benzodiazepine receptor increases the frequency of chloride channel opening induced by submaximal concentration of GABA [20]. The ability of the extracts to potenitate the sedative property of diazepam suggests it may possibly act by interacting with GABA-mediated synaptic transmission. The column fraction 3 was found to be the most active amongst the column fractions (CF_3, CF_5 and CF_7). Phytochemical screening of the butanol soluble portion revealed the presence of saponins, flavonoids, and alkaloids, while that of CF_3 revealed the presence of flavonoids. Alkaloids and saponins have been reported to show potent sedative activity [21], [22]. Saponins and flavonoids obtained from ziziphus seeds have also been reported to possess sedative effects [23]. Terpenoids cross the blood-brain barrier and are known to cause sedative and anxiolytic effects [24].

The butanol soluble portion at all doses tested significantly reduced the number of head dips in the hole-board experiment (Fig 3). This further supports the claims that the plant contains substances that are sedative in nature. The hole-board test is a measure of exploratory behaviour in animals [12]. It has been accepted as an experimental model for the evaluation of sedative and anxiety condition in animals [25] [26]. A decrease in number of head dips reveals a sedative behaviour [27], while anxiolytic drugs have been shown to increase the number of head dips in the hole-board test [28]. Thus, diazepam at a high dose of 0.5 mg/kg decreased the hole-board exploration (similar to the butanol soluble portion of *Cissus cornifolia* methanolic leaf extract) and was sedating, while the significant increase in the hole exploration caused by diazepam at lower dose showed that it was anxiolytic.

The butanol soluble portion significantly ($p < 0.05$) prolonged the time required to reach goal box and produced a dose-dependent increase in number of foot slips made by mice (Fig. 4a and 4b). The number of foot slips made has been found to be a sensitive measure at determining benzodiazepine-induced motor coordination deficits and is a good predictor of doses producing clinical sedation [13]. By increasing the number of foot slips made by the mice, the butanol soluble portion seemed to posses sedative property.

CONCLUSION

The butanol soluble fractions of *Cissus cornifolia* methanolic leaf extract possess good sedative activity compared to standard drugs such as diazepam used in most tests conducted. Potentiation of diazepam-induced sleep as well as reduction in mean number of head dips by the extract strongly suggests CNS depressant effect of *Cissus cornifoia* in mice.

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