



ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF ETHYLACETATE FRACTION OF *CISSUS CORNIFOLIA* PLANCH

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ABSTRACT

Cissus cornifolia (Vitaceae) is used traditionally in the treatment of malaria, septic tonsils, gonorrhoea and other diseases. The analgesic and anti-inflammatory activities of crude methanol extract of *Cissus cornifolia* leaf had earlier been investigated. In this study, the antinociceptive, anti-inflammatory and antioxidant properties of ethylacetate fraction of crude methanol leaf extract was evaluated using the abdominal writhing, hot plate, carrageenan induced edema and DPPH radical scavenging tests. Doses used were 15, 30 and 60 mg/kg body weight in mice and rats. The ethylacetate fraction produced significant ($P < 0.05$ - $P < 0.001$) reduction of number of writhes in the acetic acid induced test in mice. Thermal nociception induced by hot plate was significantly ($P < 0.05$) inhibited at the dose of 30 mg/kg of the fraction, and the effect that was blocked by naloxone. The ethylacetate fraction also showed significant ($P < 0.001$) anti-inflammatory effect in the carrageenan induced edema test. The DPPH radical scavenging activity was greater than that of ascorbic acid. These findings suggest that the ethylacetate fraction of *C. cornifolia* leaf possesses antinociceptive and anti-inflammatory activities which might have been enhanced by its antioxidant property.

KEYWORDS: *Cissus cornifolia*, Carrageenan, Hot plate, Pain, Inflammation, writhing

INTRODUCTION

Pain is a global problem and most people experience pain at some time in their lives. Many diseases that need drug treatments involve common pathological symptoms of pain. Inflammation is the body's attempt at self-protection. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects on pain and inflammation [1, 2].

Cissus cornifolia (Bak) planch is an annual, sub-erect herb that is distributed in the rocky suburbs and bush savanna in Ghana and Northern Nigeria and across Africa to Sudan [3]. It belongs to the family Vitaceae and is locally known as *Rigar biri*, *Duwawun biri* (Hausa in Nigeria), *Dagaari sinkatora* (Ghana) and *Manding- Dyula* (Ivory coast). The plant is traditionally used by the Shona speaking people as a remedy for gonorrhoea when taken with native natron while the leaf-sap is used by the

Tanganyika of Tanzania as a sedative in cases of mental derangement [3]. The root decoction is also used for malaria, septic tonsil, cardiac problems, pharyngitis, and diabetes [3]. The plant has been investigated for various activities, including the analgesic and anti-inflammatory effects of the methanol leaf extract [4]. This study investigated the antinociceptive and anti-inflammatory activity of ethylacetate fraction of methanol leaf extract of *C. cornifolia* using experimental animal models

MATERIALS AND METHODS

Collection of plant material

Leaf of *C. cornifolia* (Bak) Planch was collected from Samaru Zaria, Kaduna State Nigeria. The leaf was identified and authenticated by Malam Musa M. of the herbarium unit Department of Biological Sciences, Ahmadu Bello University, Zaria, where a voucher specimen number 024 was deposited.

Animals

Wistar rats (110-170 g) and Swiss albino mice (18-25 g) of both sexes were used. The animals were obtained from the animal house facility, Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria and kept at the animal house Department of Pharmacology and Therapeutics, Bayero University Kano. The animals were kept in clean cages in a well-ventilated room under standard laboratory conditions. They were fed on standard animal feeds and water *ad libitum*. All the experiments were performed in accordance with Institute of Laboratory Animal Resources, Commission on Life Sciences (National Research Council 1996). The study was approved by the Animal Research Ethical Committee, Department of Pharmacology and Therapeutics Bayero University, Kano.

Drugs and Chemicals

Methanol (70%) Diclofenac Sodium (Novartis, Switzerland), Morphine (Warsaw Pharm. Polfa, Poland), Naloxone hydrochloride (Verve Human Care, U.K) Acetic acid (Sigma Aldrich), Carrageenan (Sigma Aldrich), Ascorbic acid (Nutribiotic, UK), Diphenyl-Picryl-Hydrazyl (DPPH) (Sigma Aldrich), Ethylacetate (KESHI Pharmaceuticals, China).

Preparation of plant extract

The leaves of *C. cornifolia* were air dried under shade to constant weight, then grounded into powder using mortar and pestle. 1600 g of powdered leaf was extracted using cold maceration with 9L of 70% methanol for four days with occasional stirring. The extract was filtered and placed on a water bath to dry at 50°C. After evaporation, the yield was found to be 9.25%w/w. The extract was then partitioned with ethylacetate. The resultant fraction was kept in air tight containers maintained at 21±1°C until use.

Equipment

Hot plate, digital vernier caliper, electronic weighing balance, digital thermostat, water bath.

Phytochemical screening

Phytochemical screening of the ethylacetate fraction of *C. cornifolia* leaf extract was carried out using method described by Evans [5].

Acute toxicity study

Acute toxicity study was conducted using the method of Lorke [6]. In the first phase, 9 mice were divided into three groups of three mice each and

then treated with the ethylacetate fraction of *C. cornifolia* leaf extract at doses of 10, 100 and 1000 mg/kg intraperitoneally. The mice were observed for the first four hours and 24 hours after treatment for signs of toxicity including death. In the second phase, four groups of one mouse each were treated with the fraction at selected doses of 140, 225, 370 and 600 mg/kg *i.p* based on the result of the first phase. The mice were observed for the first four hours and 24 hours for signs of toxicity including death. The median lethal dose (LD₅₀) was calculated as the geometric mean of the lowest dose that caused death and the highest dose at which all the animals survived.

Acetic acid-induced writhing test in mice

The test was conducted using the method described by Koster *et al.* [7]. Swiss albino mice were randomly distributed into five groups of six mice each. Group I received normal saline 10 ml/kg. Groups II, III and IV received ethylacetate fraction at doses of 15, 30 and 60 mg/kg body weight *i.p* respectively. Group V received diclofenac sodium 10 mg/kg, *i.p*. After thirty minutes; mice in all the groups received 0.6 % acetic acid (10 ml/kg body weight *i.p*). Five minutes after acetic acid injection, the mice were placed individually in different cages and the numbers of abdominal contractions were counted for each mouse for a period of 10 minutes, after five minutes latency. A writhe was indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. Percentage inhibition of writhes was calculated using the formula below:

$$\text{Percentage inhibition} = \frac{\text{Inhibition of control} - \text{Inhibition of extract}}{\text{Inhibition of control}} \times 100$$

Hot plate test in mice

The method described by Eddy and Leimbach [8] was adopted for this test. Mice were placed on the hot plate maintained at 55± 1°C and the time between placement and shaking or licking of limb or jumping was considered as pain reaction time. Thirty pre-screened (pain response within 5 min) mice were grouped into five groups of six mice each. Group I received normal saline 10 ml/kg, groups II, III and IV received graded doses of ethylacetate fraction (15, 30 and 60 mg/kg body weight *i.p*), while group V received morphine (5 mg/kg *i.p*). The reaction time was recorded at 30, 60, 90 and 120 minutes following treatments.

Carrageenan-induced paw edema test in rats

The method described by [9] was adopted. Rats were divided into five groups of six rats each. The groups were pre-treated intraperitoneally as follows; group I received normal saline 1ml/kg. Groups II, III and IV received graded doses of ethylacetate fraction of *C. cornifolia* leaf extract (15, 30 and 60 mg/kg body weight *i.p.*), whereas group V was treated with diclofenac 10 mg/kg. Thirty minutes after, acute inflammation was produced by the sub-plantar administration of 0.1ml (1% w/v carrageenan in distilled water) in the right hind paw of rats. The paw diameter was measured at 0, 1, 2, 3, and 4 hours using vernier caliper to determine the diameter of the edema. The difference between the reading at time 0 h and at different time intervals was taken as the thickness of edema.

DPPH radical scavenging assay

The 1, 4-diphenyl-2-picryl-hydrazyl (DPPH) assay was performed using the method described by [10]. About 1.0 ml of the 0.04% methanol solution of DPPH was added to 1ml of various concentrations (20–100 µg/ml) of ethylacetate fraction of *C. cornifolia* leaf extract respectively, and (20 – 100 µg/ml) of vitamin C. The mixture was vortexed thoroughly and left at room temperature for 30 minutes in the dark. The absorbance was measured at 517 nm. Radical scavenging activity was calculated as percentage inhibition using the equation below:

$$\text{Percentage Inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

Where, Abs control is the absorbance of DPPH + methanol and Abs sample is the absorbance of DPPH radical + sample (fraction or standard)

Control Abs=1.091

IC₅₀ value was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus % inhibition.

Statistical analysis

Results were expressed as mean ± SEM and analysed using One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparison among groups. Statistical significance was set at $P < 0.05$.

RESULTS

Phytochemical constituents

Phytochemical constituents detected in the ethylacetate fraction of *C. cornifolia* leaf extract

include glycosides, terpenoids, phenols, tannins, and flavonoids.

Acute toxicity (LD₅₀ determination)

The intraperitoneal median lethal dose (LD₅₀) of ethylacetate fraction of *C. cornifolia* leaf extract in mice was estimated to be 288 mg/kg body weight.

Acetic acid induced writhing in mice

The ethylacetate fraction significantly ($P < 0.05$ - $P < 0.001$) reduced the mean number of writhes in mice compared to normal saline treated group. The percentage inhibition (82.3%) of writhes exhibited by the highest dose (60 mg/kg) was higher than that of the standard drug diclofenac (74.0%) (Table 1).

Hot plate test

The ethylacetate fraction significantly ($P < 0.05$) increased the mean response time to thermally induced pain compared to the negative control (normal saline) at the dose of 30 mg/kg at ninety minutes (90 min). Similarly, morphine 5 mg/kg increased the mean reaction time significantly ($P < 0.05$, $P < 0.005$) but much earlier at 30 and 60 min respectively (Table 2).

Interactive study with naloxone in hot plate test

The ethylacetate fraction of *Cissus cornifolia* leaf extract at the dose of 30 mg/kg in the presence of naloxone did not affect the mean response time to thermally induced pain compared to the negative control group. Similar response was observed with morphine 10 mg/kg (Table 3).

Carrageenan-induced hind paw edema in rats

The injection of 1% carrageenan suspension into the sub plantar region of the hind paw of the rats produced a local edema reaching its maximum at the fourth hour. The ethylacetate fraction at the dose of 15 mg/kg significantly ($P < 0.05$) reduced the mean paw diameter at the first and second hours; while at the dose of 60 mg/kg significant ($P < 0.005$) reduction was observed at the second hour. At the fourth hour, all the tested doses of the fraction and diclofenac significantly ($P < 0.001$) reduced paw edema in rats (Table 4).

DPPH radical scavenging activity

The percentage scavenging of DPPH radical of EAF was found to be within the range of 89.3 - 90.3%, while that of ascorbic acid was within 40.9 - 43.1%. The IC₅₀ value of EAF was 0.557 µg/ml, which was lower than the standard ascorbic acid (1.221 µg/ml) (Table 5).

Table 1: Effect of ethylacetate fraction of *C. cornifolia* leaf extract on acetic acid induced writhing in mice

Treatment (mg/kg)	Mean No. of Writhes	Percentage Inhibition
NS 10 mg/ml	17.8±1.49	--
EAF(15)	5.0±2.80**	70.5
EAF(30)	6.8±2.61*	64.7
EAF(60)	3.1±1.57***	82.3
DCL (10)	4.7±1.0**	74.0

Values presented as Mean ± SEM, n=6, *P < 0.05, ** P < 0.005, *** P < 0.001 compared to control using one way ANOVA, followed by dunnett's test. NS- Normal saline, EAF = Ethylacetate fraction, DCL = Diclofenac

Table 2: Effect of ethylacetate fraction of *C. cornifolia* leaf extract on thermally induced pain in mice

Treatment (mg/kg)	Mean Response Time (Sec)				
	0 min	30 min	60 min	90 min	120 min
NS 10 ml/kg	2.1±0.20	2.0±0.41	2.5±0.27	1.8±0.11	2.6±0.38
EAF(15)	1.7±0.11	1.5±0.14	2.0±0.32	3.5±0.33	3.7±0.63
EAF(30)	1.6±0.17	3.5±0.60	3.5±0.45*	4.8±1.24**	3.9±0.94*
EAF(60)	2.0±0.28	2.0±0.16	3.2±0.50	4.1±0.85	3.4±0.64
MPH (5)	1.9±0.16	7.6±2.60***	3.0±0.35*	4.3±0.81*	2.7±0.77

Values presented as Mean ± SEM, n=6, significant compared to 0 min (*P < 0.05, ** P < 0.005, *** P < 0.001) using repeated measure ANOVA followed by dunnette's tests. NS =Normal saline, EAF =Ethylacetate fraction, MPH =Morphine

Table 3: Effect of naloxone on ethylacetate fraction of *C. cornifolia* leaf extract in hot plate test

Treatment (mg/kg)	Mean Response Time (Sec)				
	0 min	30 min	60 min	90 min	120 min
NS 10 ml/kg	1.2±0.56	1.1±0.94	1.1±0.32	0.9±0.82	1.1±0.86
NAL(1) + EAF(30)	1.1±0.72	1.1±0.68	1.1±0.81	0.9±0.71	1.0±0.56
NAL(1) + MPH (10)	1.5±0.25	1.8±0.55	1.2±0.12	1.2±0.15	1.2±0.18

Values presented as Mean ± SEM, n=6, NS =Normal saline, NAL =Naloxone, EAF =Ethylacetate fraction, MPH =Morphine

Table 4: Effect of ethylacetate fraction of *C. cornifolia* leaf extract on carrageenan induced hind paw edema in rats

Treatment (mg/kg)	Mean Paw Diameter (mm)			
	1h	2h	3h	4h
NS 1 ml/kg	1.19±0.59	1.18±0.11	1.70±0.36	2.18±0.10
EAF (15)	0.53±0.78*	0.62±0.84*	0.97±0.12	1.17±0.12***
EAF(30)	0.88±0.15	0.71±0.11	0.92±0.12	0.92±0.17***
EAF(60)	0.75±0.91	0.40±0.72**	1.06±0.28	0.77±0.12***
DCL (10)	0.87±0.10	0.98±0.24	0.66±0.15*	0.53±0.20***

Values presented as Mean ± SEM, n=5, (*P < 0.05, ** P < 0.005, *** P < 0.001) ANOVA followed by dunnette's tests. NS =Normal saline, EAF =Ethylacetate fraction, DCL =Diclofenac

Table 5: DPPH radical scavenging activity of ethylacetate fraction of *C. cornifolia* leaf extract

Concentration (µg/ml)	Percentage Inhibition	
	EAF (0.557)	AA (1.221)
20	89.3	40.9
40	90.8	42.8
60	90.7	41.2
80	90.7	41.9
100	90.3	43.1

Absorbance of control (DPPH + methanol) = 1.091 nm; Wavelength of UV spectrophotometer used = 520 nm
Values in parenthesis = IC₅₀ (µg/ml); EAF =Ethylacetate fraction, AA =Ascorbic acid

DISCUSSION

Cissus cornifolia has long history of use for several health conditions including pain and inflammation. This folkloric claim was studied on the crude methanol leaf extract of the plant [4]. The present work studied the analgesic and anti-inflammatory effects of ethylacetate fraction (EAF) of the crude methanol leaf extract of *C. cornifolia*.

Phytochemical constituents of plants have been implicated in their pharmacological activities. Terpenes are recognized for having anti-inflammatory and analgesic properties, and therefore have been considered as potential candidates for new drugs intended to control painful syndromes and inflammatory diseases [11, 12]. Flavonoids, saponins and glycosides have also been reported to possess analgesic and anti-inflammatory effects [13, 14]. The phytochemical constituents detected in this study include glycosides, terpenoids, phenols, tannins, and flavonoids which were largely corroborative of the work of [4, 15] who reported presence of glycosides, flavonoids, saponins, steroids, terpenoids, and tannins in the crude methanol leaf extract.

In the acetic acid induced writhing test EAF significantly reduced abdominal contractions of mice and the percentage inhibition of writhes was even higher than diclofenac at the highest dose. The acetic acid-induced writhing is a peripheral pain model which is generally used for screening plants and new agents for analgesic properties [16, 17]. It is a sensitive procedure used to evaluate peripherally acting analgesics and represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from the tissue phospholipid [18]. It can therefore be suggested that EAF may be acting by inhibiting the release of these mediators.

The hot plate test measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity [19]. It is an established fact that any agent that causes a prolongation of the hot plate latency using this test must be acting centrally [20]. As observed in our results EAF exhibited antinociceptive effect in the hot plate test at 30 mg/kg dose by significantly delaying the reaction time of thermally-induced pain, suggesting a central antinociceptive activity. However the effect of morphine had an earlier onset compared to EAF. To confirm the central activity of EAF, an interactive study with naloxone, a competitive antagonist of opioid receptors was carried out. From the results, naloxone blocked the analgesic activity of EAF and morphine in the hot plate test emphasizing the possible involvement of EAF with opioid receptors. Our findings in this study therefore showed that both peripheral and central mechanisms are involved in the analgesic effect of EAF.

Results obtained from the carrageenan induced hind paw edema test showed that EAF exhibited inhibition of inflammation which reached a peak at 4 h at the tested doses. A similar pattern of activity was observed with diclofenac. Diclofenac like other NSAIDs prevents inflammation by inhibition of cyclooxygenase enzyme involved in the conversion of arachidonic acid into prostaglandins [21]. It can be deduced that EAF could be acting by a similar mechanism to prevent carrageenan induced paw edema

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl, hydrogen peroxide radical, and peroxy nitrite participate in the process of inflammation in various tissues [22]. Excessively produced ROS can injure cellular biomolecules, such as nucleic acids, proteins, carbohydrates, and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation [23, 24]. The antioxidant effect of EAF was studied to

see the possible radical scavenging property of the fraction as a contributory factor to its anti-inflammatory activity. Data obtained from our results showed that EAF exhibited significant DPPH scavenging activity which was higher than standard synthetic antioxidant ascorbic acid.

Based on the results of this study, it can be concluded that EAF exhibited antinociceptive and anti-inflammatory effects. These findings seem to justify the use of the plant in traditional medicine in the management of pain and inflammation.

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