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Anti-inflammatory and antioxidants properties of the ethanolic stem bark extract of *Cordia africana* Lam.

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Abstract

Cordia africana Lam. (Boraginaceae), a widely known shade tree, is used in Northern Nigeria and East Africa for the management of haemorrhoids, headache and fatigue, respectively. In this study, the ethanolic stem bark extract of the plant was screened for anti-inflammatory and antioxidant activities. The anti-inflammatory study was carried out *in vivo* in rats, using carrageenan-induced hind paw inflammation edema and formalin induced pain model while anti-oxidant activity was screened *in vitro* using DPPH radical scavenging assay. Following pilot study, intraperitoneal doses of 340, 424 and 509 mg/kg of the extract were used. Screening for anti-inflammatory effects revealed that the extract possess significant ($p<0.05$) anti-inflammatory activity at all doses when compared to placebo (normal saline). At high dose of 509 mg/kg, the extract revealed significant anti-inflammatory activity when compared to both placebo and positive control (piroxicam). Antioxidant study, EC_{50} values of 20.12 µg/ml, 32.07 µg/ml and 40.38 µg/ml were calculated for the ethanolic stem bark extract of *C. africana*, ascorbic acid and α-tocopherol acetate, respectively. These values revealed that the crude extract, in microgram concentration, is more potent than ascorbic acid and α-tocopherol acetate *in vitro*. Therefore, the ethanolic stem bark extract of *C.africana* possess anti-inflammatory and antioxidant effects, thus, rationalizingits ethnomedical use for the relief of pain and inflammation associated with piles in Northern Nigeria and East Africa

Key words : *Cordia africana* Lam., Haemorrhoids, carrageenan, DPPH, acute toxicity, antioxidant

1. Introduction

In the developing countries, many people rely on traditional healing practices and medicinal plants for their daily healthcare needs. In some Asian and African countries, 80% of the population depends on traditional medicine for primary healthcare. This is due to the fact that traditional medicine is a more affordable and accessible healthcare system when compared to modern medicine (Bhushan, 2005).

C. africana Lam. (Boraginaceae), is a small to medium-sized tree, widely distributed throughout Africa where it is commonly planted as a shade or roadside tree and is also used for wood or demarcation. In Northern Nigeria, the stem bark powder is used traditionally to treat pain and inflammation associated with piles (Mal. Garba personal communication, 4th January, 2013) while in East Africa, it is used for general healing of open wound, treatment of schistosomiasis, skin troubles and jaundice, and as stimulating tonic for fatigue, pain, headache and exhaustion (Obeng, 2010).

Due to the limitations of currently available nonsteroidal anti-inflammatory drug (NSAID) drugs in the management of inflammatory conditions, there is need for continuous search for new and better agents. In view of this and the fact that the ethnomedical claim of this plant in the traditional management of some inflammatory conditions has not been scientifically evaluated. This current study is aimed at investigating the ethanolic stem bark extract of *C. africana* for anti-inflammatory properties in rats and antioxidant activities *in vitro*.

2. Materials and Methods

2.1 Collection of plant material

The stem bark of *C. africana* Lam. was collected from Malali Village, Igabi L.G.A. Kaduna-Nigeria. The plant was identified and authenticated at the herbarium section in the Department of Biological Sciences, Ahmadu Bello University (A.B.U), Zaria-Nigeria, where it was compared with an existing voucher specimen number, 14666. The plant material was then dried under shade until constant weight was obtained before it was crushed into coarse powder, using wooden pestle and mortar.

2.2 Preparation of extract

1.5 kg of the powdered plant was extracted with 2 L of N-hexane. The marc (N-hexane residue) was extracted with 3 Litres of aqueous ethanol (70/30% v/v) for 72 h. at room temperature, using a percolator. The solvent was evaporated over a water bath at temperature 45°C. The extract was then stored in a desiccator and only prepared freshly for each study.

2.3 Animals

Adult Wistar rats of both sexes, weighing 150-200 g, respectively were obtained from the Animal House of Department of Pharmacology and Therapeutics, A.B.U., Zaria after obtaining animal ethical committee approval (ABU/AEC/Pharm-Sc/7610). The animals were maintained in a well-ventilated room, and fed on

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standard feed and clean tap water *ad libitum*. They were kept in clean cages under normal light/dark cycle and allowed to acclimatize to the laboratory environment for a period of 24 h. before the commencement of each experiment.

2.4 Drugs and chemicals

The following drugs and chemicals of analytical grade were used:ethanol, N-hexane, carrageenan (Sigma Aldrich),Piroxicam (Hovid), α-tocopherol, Ascorbic acid (Sigma Aldrich) and 2,2-Dipenyl-1-picrylhydrazyl (DPPH - Sigma Aldrich).

2.5 Equipment

UV spectrophotometer, electronic weighing balance, stopwatch, needles and syringes, spatula, animal cages, pestle and mortar, test tubes, beakers, digital calliper, gloves, cotton wool, labelling tape, red and blue markers.

2.6 Phytochemical screeing

Standard tests as described by Evans (2009) were employed in screening the ethanolic stem brark extract of *C. africana* for different phytochemical constitutents.

2.7 Carrageenan-induced paw oedema model in rats

Thirty rats were divided into five groups each, composing of six rats. Acute inflammation was induced by injecting 0.1ml of 1% saline suspension of carrageenan into the subplanter surface of each rat's left hind paw (Winter *et al.*, 1962). Normal saline (1 ml/kg), extract (340 mg, 424 mg and 509 mg/kg body weight), and piroxicam 10 mg/kg body weight were administered intraperitoneally to the animal 30 min. before carrageenan injection. The paw diameter was measured with a digital Vernier calliper at 0, 1,2,3,4 and 5 h. following carrageenan injection. The difference between reading at 0 h. and different time intervals were taken as the measurement of oedema.

2.8 Formalin test in rats

The method described by Dubuisson and Dennis (1997) was adopted. 30 rats were divided into five groups, each containing 6 rats, and were, respectively treated with normal saline (1 ml/kg), three different doses of the extract (340 mg/kg, 424 mg/kg and 509 mg/kg) and morphine (5 mg/kg) intraperitoneally. Thirty minutes after this treatment, 50 µl of a freshly prepared 2.5% solution of formalin was injected subcutaneously under the planter surface of the left hind paw of each rat. The rats were monitored for one hour and the severity of pain was recorded for each rat based on the following scale: 0: rat walked or stood firmly on the injected paw; 1: the injected paw was favoured or partially elevated; 2: the injected paw was clearly lifted off the floor; 3: the rat licked, chewed or shook the injected paw. The antinociceptive effect was determined in two phases: the early phase (phase I) was recorded during the first 5 min. after formalin administration, while the late phase (phase II) was recorded during the last 45 min. with 10 min. lag period in between both phases.

2.8 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The determination of DPPH radical scavenging activity of ethanolic stem bark extract of *C. africana* was carried out according to the method described by Mensor *et al.* (2001) with slight modification. Six different concentrations (5 µg/ml, 7.5 µg/ml, 10 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml) of the plant extract and standard references (α-tocopherol acetate and ascorbic acid) were prepared.

To 0.5 ml of the extract in 1.5 ml of ethanol, 0.5 ml of 1 mm DPPH was added. The standard reference drugs were similarly prepared and a blank solution containing only DPPH in the same amount of ethanol was also prepared. The test samples were then allowed to stand in a dark chamber for 30 min. before their absorbances were measured at 518 nm using U.V. spectrophotometer.

The antioxidant activity of the plant extract was calculated as % inhibition of DPPH radical absorbance activity using the formula below:

Percentage inhibition of absorbance

$$= \frac{\text{Ab control} - \text{Ab sample}}{\text{Ab control}} \times 100$$

$Ab_{control}$ = is the absorbance of DPPH radical in ethanol; Ab_{sample} = is the absorbance of DPPH radical + sample extract or standard in ethanol.

2.9 Statistical analysis of results

All results obtained were expressed as Mean ± SEM and analysed by analysis of variance, using SPSS software, version 19. Results obtained from carrageenan induced paw oedema model were analysed using one-way ANOVA and Tukey's post hoc test while Kruskal-Walis and Mann Whitney's test was used for Formalin test. Also, student t-test was used to analyse results of DPPH radical scavenging antioxidant assay tests. At $p<0.05$ differences in means were considered significant. Results were also presented and analysed in the form of graphs.

3. Results

3.1 Preliminary phytochemical screening

Preliminary phytochemical screening of the extract revealed a percentage yield of extract = 16% w/w and the presence of alkaloids, flavonoids, saponins and triterpenes (Table 1).

3.2 Median lethal dose (LD_{50})

The median lethal dose (LD_{50}) of the ethanolic stem bark extract of *C. africana* Lam. (EECA) in rats was found to be greater than 5000 mg/kg *via* oral route and 1265 mg/kg *via* intraperitoneal route (Table 2).

3.3 Carrageenan-induced oedema in rats

The ethanolic stem bark extract of *C. africana* (EECA) administered *i.p.*route, at 340 mg/kg dose, significantly ($p<0.05$) reduce the paw oedema size at the 2, 4 and 5 h. while at 424 mg/kg dose, EECA significantly ($p<0.05$) reduce the paw oedema size at the 2, 3, 4 and 5 h. when compared to negative control, normal saline. At 509 mg/kg *i.p.* doses, EECA showed significant paw oedema size reduction at the 2, 3, 4, and 5 h. when compared to both controls (Figure 1).

3.4 Formalin test

In the first phase (phase I), the ethanolic stem bark extract of *C. africana* (EECA) did not show any statistically significant reduction in pain at all oral treatment doses when compared to both controls (normal saline and morphine). In the second phase, 340 mg/kg, 424 mg/kg and 509 mg/kg doses showed significant ($p<0.05$) reduction in pain when compared to both controls. However, morphine (5 mg/kg) inhibited pain significantly ($p< 0.05$) in both phases (Figure 2).

3.5 DPPH radical scavenging assay

At 518 nm wavelength using UV spectrophotometer, the extract significantly ($p < 0.05$) decreases absorbance of DPPH free radical when compared with controls, ascorbic acid and α-tocopherol acetate. The EC_{50} value for the extract was calculated to be 20.12 µg/ml as against 32.07 µg/ml and 40.38 µg/ml for the standard references (ascorbic acid and α-tocopherol acetate), respectively as shown in Figures 3, 4, 5 and 6.

Table 1: Preliminary phytochemical screening result of ethanolic stem bark extract of *C. africana* Lam.

Test	Constituents	Results
Dragendoff's, Meyer's and Wagner's	Alkaloids	+
Shinoda's, Dragendoff's and NaOH	Flavonoids	+
Frothing/Haemolysis	Saponins	+
Liberman Buchard	Triterpenes	+
Molisch and Fehling's A&B	Carbohydrates	+
Borntrager's Test	Anthraquinone	-
Lead acetate/Ferric Chloride	Tannins	-

+ = present, - = absent.

Table 2: Median lethal dose (LD_{50}) values of ethanolic stem bark extract of *C. africana* Lam. in rats

Route of administration	Animal species	LD_{50} Values (mg/kg)
Oral	Rats	>5000
i.p.	Rats	1265

i.p. = intraperitoneal

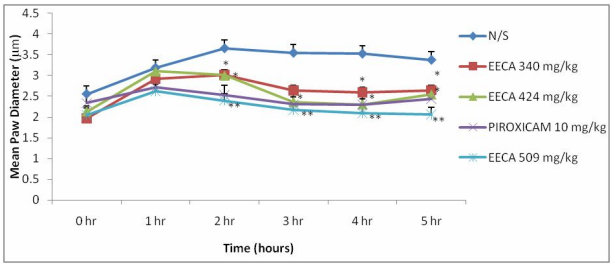

Figure 1: Effects of *i.p.* administration of ethanolic stem bark extract of *C. africana* Lam. (EECA) on carragee-nan induced paw oedema size in rats

Each value represent Mean ± SEM in µm for six rats (n=6), *p < 0.05 when compared with group treated with normal saline (1ml/kg), **p < 0.05 when compared to both controls using one-way ANOVA. EECA = ethanolic stem bark extract of *C. africana* Lam., N/S = normal saline.

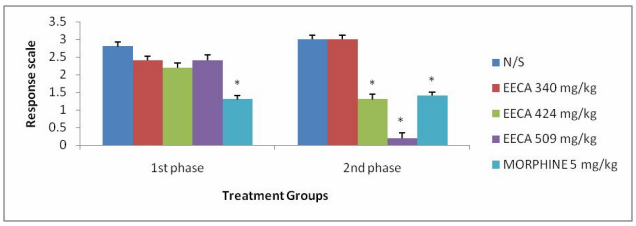

Figure 2: Effects of *i.p.* administration of ethanolic stem bark extract of *C. africana* Lam. and morphine on formalin-induced pain in rats

Each value represents Mean ± SEM for six rats (n=6). *p<0.05 significant compared to both controls using one-way ANOVA. EECA = ethanolic stem bark extract of *C. africana* Lam., N/S = normal saline.

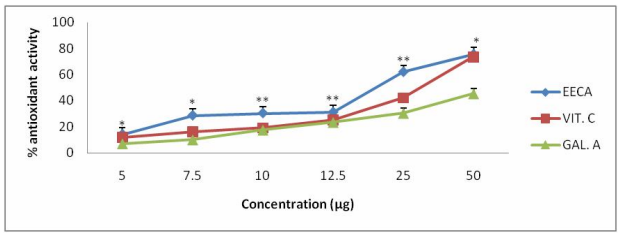

Figure 3: Percentage antioxidant activity of ethanolic stem bark extract of *C. africana* Lam., ascobic acid and and α - tocopherol on absorbance activity of DPPH free radical using UV spectrophotometer at 518 nm wavelengths

Each value represent Mean ± SEM (n = 3). *p< 0.05, **p < 0.001 significant compared to control (ascorbic acid and α - tocopherol) using student t-test. EECA = ethanolic stem bark extract of *C. africana* Lam., VIT. C = ascorbic acid, GAL. A. = α - tocopherol.

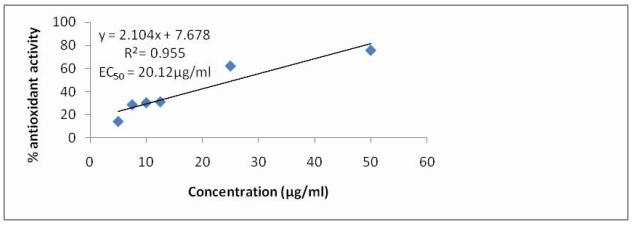

Figure 4: Regression coefficient graph and EC_{50} of the ethanolic extract of *C. africana* Lam. on absorbance activity of DPPH stable free radical using UV spectrophotometer at 518 wavelength

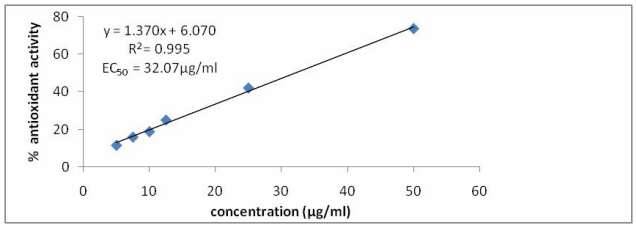

Figure 5: Regression coefficient graph and EC_{50} of ascobic acid on absorbance activity of DPPH free radical using UV spectrophotometer at 518 wavelength (n = 3)

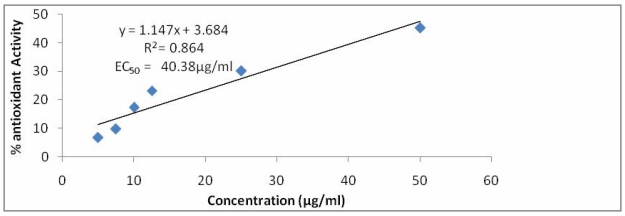

Figure 6: Regression coefficient graph and EC_{50} of α-tocopherol on absorbance activity of DPPH free radical using UV spectrophotometer at 518 wavelengths (n = 3)

4. Discussion

The ethanolic stem bark extract of *C. africana* Lam. contains phytochemicals such as alkaloids, saponins, glycosides and tannins which have been shown to be bioactive and possess desirable pharmacologic activities such as antinociceptive, antidiabetic, anti-inflammatory and antioxidant activities (Okwu, 2005; Chindo *et al*., 2010; Sankaradoss *et al*., 2012; Lanthers *et al*., 1992; Perez, 2001; Meshikles, 2004; Park *et al*., 2008).

Investigation into the acute toxicity profile of the crude extract in rats using Lorke's method (1983) revealed oral LD_{50} value of greater than 5000 mg/kg and *i.p*. LD_{50} value of 1265 mg/kg. According to Lorke's postulate (1983), lethal doses of substances are classified according to their LD_{50} values: for LD_{50} e" 1mg/kg, substance is highly toxic; for LD_{50} e" 5 mg/kg, substance is toxic; for LD_{50} e" 100 mg/kg, substance is moderately toxic, for LD_{50} e" 1000 mg/kg, substance is slightly toxic; and for LD_{50} e" 5000 mg/kg, substance is non-toxic. Therefore, extract may be said to be orally non-toxic and slightly toxic when administered through intraperitoneal route. This result could be a logical explanation as to the history of safety of the crude plant extract in traditional medicine (Obeng, 2010).

Preliminary pilot study showed the minimum effective dose of the extract as 340 mg/kg body weight *i.p*. This, coupled with the LD_{50} values (for safety reasons) of the plant, informed the choice of 340 mg/kg, 424 mg/kg and 509 mg/kg body weight *i.p*. doses used in the course of this study.

Studies on carrageenan-induced paw oedema model in rats have demonstrated that the inflammatory effect induced by carrageenan is biphasic in nature; first phase resulting from the rapid production of several inflammatory mediators such as histamine, serotonin and bradykinin and a second phase by the release of prostaglandin and nitric oxide with peak at 3 h. produced by inducible isoforms of cyclo-oxygenase (COX-2) and nitric oxide synthase (NOS), respectively (Seibert *et al*., 1994; Ogonowski *et al*., 1997). Results obtained in this model can be used to investigate anti-inflammatory effect of compounds (Shenawy *et al*., 2002). The ethanolic stem bark extract of *C. africana* Lam. at 340 mg/kg dose significantly ($p<0.05$) reduced the size of induced paw oedema at 2^{nd},4^{th} and 5^{th} h. while, at 424 mg/kg dose, it significantly ($p<0.05$) reduced the size of induced paw oedema at 2^{nd}, 3^{rd}, 4^{th} and 5^{th} h. after carrageenan administration only when compared to negative control, normal saline. However, in comparison to both piroxicam and normal saline, the extract at 509 mg/kg dose, yielded significant ($p<0.05$) reduction in the size of induced paw oedema at 2, 3, 4 and 5 h. after carrageenan administration. Thus, the extract possesses anti-inflammatory activity in a dose-dependent fashion, which may be as a result of inhibition of production of proinflammatory mediators (Tjolsen *et al*., 1992). The lack of activity at the 3rd h. after administration of carrageenan at doses 340 mg/kg can be explained by possible biological differences between test groups and interaction between constituents of extract sample (Vongtau *et al*, 2004).

Formalin induced pain model is employed to screen drugs of both central and peripheral antinociceptive activity. It consists of two phases of nociceptive response termed early (phase I) and late phase (phase II). In phase I (0-5 min. after drug administration), pain is as a result of stimulation of nociceptive receptors in the paw depicting neurogenic pain caused by the direct effect of formalin on the sensory C fibres. In phase II (15-60 min. after drug administration), pain is as a result, inflammatory response and the release of nociceptive mediators such as serotonin, histamine, bradykinin and prostaglandin. It is well known that drugs such as morphine that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs such as NSAIDs inhibit the late phase (Hunskaar and Hole, 1987). Using this model, results obtained showed that the extract only significantly ($p < 0.05$) reduced pain in the second phase (phase II) at the doses 424 mg/kg and 509 mg/kg body weight while morphine (10 mg/kg) significantly ($p < 0.05$) reduce pain in the two phases (phase I and II). The ability of the extract to significantly ($p < 0.05$) inhibit pain in the second phase, shows that it possess the ability to manage peripheral pain as a result inflammation caused by release of inflammatory mediators such as serotonin, histamine, bradykinin and prostaglandin while morphine's ability to inhibit both phases of formalin induced shows it has the ability to control both centrally and peripherally mediated pain (Dubuisson and Dennis, 1997).

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay is a quick, reliable, and reproducible *in vitro* test to screen extracts for antioxidant activity (Aliyu *et al*., 2009; Goncalves *et al*., 2005). It utilizes a dark coloured crystalline powdered substance called 2, 2-diphenyl-1-picrylhydrazyl (DPPH) which is composed of stable free radical molecules that can readily be scavenged by extract that possess antioxidant activity. This kind of reaction is evident by colour change (from violet to yellow) of the compound DPPH (free radical) due to its reduction either by the process of electron transfer or hydrogen ion donation and can be detected as change in absorbance using UV spectrophotometer at specific wavelength. Any extract or substance capable of performing this reaction can be regarded as possessing antioxidant activity (Dehpour *et al*., 2009). This model is based on the fact that production of oxyradicals as well as nitric oxide plays an important role in various models of inflammation (Cuzzocrea *et al*., 1998; Moncada *et al*., 1991). The extract showed significant ($p<0.001$) decrease in absorbance of DDPH free radical with calculated EC_{50} (the concentration or amount of substance that reduce absorbance by 50%) of 20.12 µg/ml, 32.07 µg/ml, 40.38 µg/ml for the extract, ascorbic acid and α-tocopherol, respectively. This result shows that the extract, (*in vitro* and in µg concentration) is one and half times (1.5) more potent than ascorbic acid and two (2) times more potent than α-tocopherol acetate.

5. Conclusion

Bassed on the results obtained from this study, it can be concluded that the ethanolic stem bark extract of *C. africana* Lam. possess anti-inflammatory and antioxidant properties. These findings support

the ethnomedical use of *C. africana* bark in the relief of inflammation and pain associated with piles and other conditions in Northern Nigeria and East Africa.

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Conflict of interest

We declare that we have no conflict of interest.

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