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Evaluation of antimicrobial and anti-inflammatory properties of leaf extracts of *Crateva religiosa*

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Abstract
Crateva religiosa leaf extract is used in traditional medicine for treatment of otitis media. In this work, anti-inflammatory and antimicrobial activity of the diethyl ether and methanolic extracts, extracted successively were evaluated. Phytochemical classes of constituents present in the extracts and leaf powder were determined. The lethal doses of the extracts in mice were determined intraperitoneally. Aspirin, diclofenac, chloramphenicol and gentamicin were used as control drugs. The LD_{50} of the extracts were greater than 5mg/kg, while the yield was 7.2% for diethyl ether and 16.7% for methanol. The major classes of compounds in the extracts are alkaloids, steroids, terpenoids, flavonoids, resins and proteins. The diethyl ether extract had anti-inflammatory activity that was concentration dependent and significant (P<0.001) AT 100 mg/kg with percentage inhibition of edema value of 65.6. The methanol extract had antimicrobial activity comparable to those of the standard drugs. Analytical thin layer chromatographic studies on the methanol extract using silica gel pre coated plates and different solvent system gave n-hexane chloroform (1:2) as the best solvent system which afforded 10 bands on preparative thin layer chromatography. Antimicrobial studies on the chromatographic fraction confirmed the antimicrobial active fractions to be steroidal terpenoids.

Keywords: Crateva religiosa, anti-inflammatory, antimicrobial, LD_{50}, chromatography, steroidal terpenoids

Introduction
Crateva religiosa leaves are used in Nigeria for treatment of otitis media of which inflammation and microbial infection are implicated in such disease condition. Inflammation is a localized reaction of tissue to irritation, injury or infection. Some drugs associated with many shortfalls are used in the treatment of inflammation (Vane and Bothling, 1993) [13]. Though there are numerous orthodox medicine for the management of microbial infection, but the ever increasing rate of development of resistance to the drugs by the pathogens call for the need for newer antimicrobial agents. Many plants such as *E. polycarpa* have a promising antimicrobial agent and the newer, more effective antimicrobial agent can easily be gotten from plants (Ajali, 2000) [1]. Present research aims to evaluate the antimicrobial and anti-inflammatory activities of different extract fractions of Crateva religiosa.

Materials and methods
Reagents
The reagents are sourced commercially, which included methanol, ethylacetate, diethylether, n-hexane, chloroform, dimethylsulphoxide (DMSO) (Products of Sigma-Aldrich Laborhe-Mikalien GmbH, Germany). Other reagents used were of laboratory standards. Gentamycin and chloramphenicol used were a gift sample from Emzor Pharmaceuticals, Lagos, Nigeria.

Plant
The fresh leaves of *Crateva religiosa* were harvested from Okigwe, Imo State, Nigeria, in February, 2008. They were authenticated by Mr. A. Ozioko of Bioresource, Conservative and Development Program Centre, Aku Road, Nsukka. The leaves were dried at room temperature, pulverized and stored in a dry container.

Animals
Adult swiss albino mice (15-35g) and rat (110-179g) of either sex were obtained from the animal house of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were housed in stainless metal cages and fed with standard animal feed and water ad-libitum. They were allowed to stay for 7 days in the laboratory to acclimatize before use.

Microorganisms
Microorganisms were clinical isolates maintained at the department of pharmaceutics, University of Nigeria, Nsukka. They include Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Escherichia coli, and Candida albicans.

Extraction
A 280g of the leaf powder was successively and exhaustively extracted with diethyl ether and methanol using a soxhlet extractor. The solvents were distilled off to obtain the residue with a rotary evaporator. Analytical thin layer chromatography (TLC) studies using silica gel precoated plates (poly gram^R SILG / UV_{254}) and different solvent systems were carried out on the methanol extracts.
Preparative TLC studies using 0.5cm silica gel coated glass plates and n-hexane: chloroform (1:2) gave ten prominent bands which were scraped off and eluted with methanol to obtain 10 fractions. The different bands were studied for antimicrobial and anti-inflammatory activity.

Acute Toxicity
The acute toxicity of the diethyl ether and methanol extracts were evaluated in mice following the method of Lorke (1983). Oral doses of 10,100 and 1000mg/kg of each extract in tween 80 were given to 3 groups of mice (n=3). The animals were observed for 24 hours and the number of deaths were recorded. In the second stage, doses of 2000, 3000, 4000 and 5000 mg/kg were given orally to four mice respectively. They were observed for 24 hours and the number of deaths recorded.

Antimicrobial Evaluation of the Extracts
The extracts and fractions were evaluated with the aid of DMSO. A 10mg/ml of each extract was screened for sensitivity (Bradshaw, 1997) [3]. The minimum inhibitory concentration (MIC) was determined using serial dilution method (Okeke *et al*, 2001) [8]. Serial dilutions of the solution were prepared to obtain solutions with lower concentrations ranging from 10-0.1mg/ml. A 0.1ml of each test organism (3.0×10^8 cfu/ml) was carefully seeded to a sterile Petri dish and molten nutrient agar was added. The dish was swirled gently to homogenize and allowed to set. Holes of about 8mm in diameter were bored with sterile cork borer. One millimeter of each solution was placed in each hole and was allowed 30 minutes to diffuse. The plates were incubated at 37 ^oC for 24 hours for bacteria and 25 ^oC for 48 hours for fungi. The inhibition zone diameters (IZD) were measured and plots of IZD^2 against log concentration was prepared. The MIC was determined as the antilogarithm of the intercept on the log concentration axis.

Phytochemical Evaluation
The chemical classes of phytocompounds present in the extracts, leaf powder and fractions were determined following standard methods (Trease and Evans, 1983; Harbone, 1988) [11, 5]. The classes tested for included glycosides, saponins, carbohydrates, tannins, flavonoids, oils, resins and proteins.

Anti-Inflammatory Test
The rat hind paw oedema was used (Muko *et al*, 2000) [7]. Increase oedema sizes induced by the subplanter injection of fresh albumin was used as a measure of acute inflammation (Bani *et al*, 2000) [2]. The rats were divided into groups of five rats each. Doses of 50 and 100 mg/kg of each extract in 10% (v/v) tween 80 were given orally to four groups respectively. Two other groups received 100mg/kg of aspirin and diclofenac respectively. Another group was given 0.4ml of 10% (v/v) tween 80. One hour later, inflammation was induced by injection of 0.1ml of undiluted egg albumin into the subplanter of the right hind paw of the rats. The sizes of the inflammation were measured by water displacement using plethysmometer at 1 hour intervals. The percentage inhibition of edema was calculated and used as an index for inflammatory effect.

Statistical Analysis
The data were analyzed statistically and reported as mean ± standards deviation. Comparison was done using student t-test and recorded as significant at $p<0.001$.

Results
The diethyl ether and methanol successive extraction gave percentage yield of 7.2% and 16.4% respectively. The chemical classes of constituents present in the extracts and the leaf powder are shown as table 1. Methanol and diethylether extracts had an LD_{50} greater than 5g/kg. Chromatographic fractionation of the methanol extract using silica gel and different solvent system gave n-hexane: chloroform (1:2) as the best resolution solvent medium among the solvent system tried. The solvent medium gave 10 bands. The classes of constituents color and Rf values of the bands are shown as Table 2. From the antimicrobial screening, the methanol extract had broad spectrum antimicrobial activity. It has no antifungal activity. The diethyl ether extract had no antimicrobial activity. The MIC is shown in Table 3. Antimicrobial tests on the bands indicated that bands 4, 5, 6, 8, 9 and 10 had strong antibacterial activity while phytochemical tests confirmed the bands to be steroidal terpenoids. The MIC of the bands are shown as Table 4.
The percentage inhibition of acute inflammation by the extracts and standard drugs are shown as Table 5.

Table 1: Classes of phytocompounds present in Methanol Diethyl ether extracts and leaf powder

Classes of phytocompounds	Diethylether extract	Methanol extract	Leaf powder
Carbohydrate	-	+	+
Reducing sugar	-	-	-
Alkaloids	+	+	+
Glycosides	-	-	-
Saponins	-	-	-
Tannins	-	+	+
Flaonoids	+	+	+
Resins	+	+	+
Protein	+	+	+
Oil	+	-	-
Steroids	+	+	+
Terpenoids	+	+	+

+ Means present
- Means absent

Table 2: Characteristics of the n-hexane: chloroform (1:2) TLC bands of the methanol extract

	BANDS									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Color	**Light green**	**Dark green**	**Yellow**	**Yellow**	**Green**	**Yellow**	**Green**	**Yellow**	**Yellow**	**Yellow**
RF value	0.02	0.07	0.10	0.13	0.15	0.22	0.29	0.33	0.45	0.92
Phytocompounds										
Flavonoids	+	+	-	-	-	-	-	-	-	-
Alkaloids	+	+		-	-	-	-	-	-	-
Resins	+	+	-	-	-	-	-	-	-	-
Steroids	+	+	-	+	+	+	+	+	+	+
Terpenoids	+	+	-	+	+	+	+	+	+	+
Fats and Oils	+	+	-	+	+	+	+	+	+	+
Reducing sugars	-	-	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-	-	-	-	-
Carbohydrate	-	-	-	-	-	-	-	-	-	-

+ Means present
- Means absent

Table 3: The Minimum Inhibitory Concentration (MIC) of the extracts and standard drugs

Microorganisms	**MIC (mg/ml) of agents**			
	Methanol extract	**Diethylether extract**	**Gentamycin**	**Chloramphenicol**
Bacillus substilis	1.06	-	0.67	1.21
Staphylococcus aureus	0.70	-	0.94	0.98
Klebsiella pneumonia	1.72	-	0.70	0.70
Escherichia coli	1.68	-	-	-
Pseudomonas aeruginosa	1.47	-	-	-
Salmonella typhii	1.38	-	-	0.98
Candida albicans	-	-	-	-

-Means not determined because it is insensitive

Table 4: Minimum Inhibitory Concentration (MIC) of the bands and the Standard drugs

Microorganisms	**MIC (mg/ml) of the bands/standard drugs**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**CP**	**GM**
Bacillus substilis	-	-	-	0.74	0.91	0.79	0.73	0.82	0.76	0.53	1.21	0.67
Staphylococcus aureus	-	-	-	0.40	-	-	-	0.64	-	0.35	0.98	0.94
Klebsiella pneumonia	-	-	-	-	-	-	-	-	-	-	0.70	0.70
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	0.38	0.31	0.84	0.74	-	0.74	-	-	-
Salmonella typhii	-	-	-	-	-	-	-	-	-	-	0.98	-
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	-

Means not determined because it is insensitive
CP Chloranphenical GM Gentamycin

Table 5: Percentage inhibition of acute inflammation by the extracts and standard drugs

Agent	**Doses (mg/kg)**	**Percentage inhibition of edema [1 hour interval]**			
10% tween 80	0.40ml	0.00	0.00	0.00	0.00
Methanol Extract	50	14.3	4.11	3.91	2.83
	100	20.1	7.12	5.34	4.3
Diethylether extract	50	-10.0	0.00	13.8	40.7
	100	0.00	17.8	52.4	65.6
Aspirin	100	-100	0.00	12.7	31.6
Diclofenac	100	10	27.4	49.6	53.7

Discussion

The percentage extraction yield of 7.2% and 16.4% supported the high secondary metabolite composition of the leaves as indicated in Table 1. Most of the classes of phytocompounds found in the extracts are known antimicrobial compounds like tannins and flavonoids. The acute toxicity test result suggests that the extracts are relatively safe for consumption. The fact that antimicrobial activity resides in the methanol fractions suggested that the possible constituents are antimicrobial constituent(s) are slightly polar. The antimicrobial activity of the methanol extracts when compared to those of gentamycin and chloramphenicol aeruginosa, Bacillus subtilis and Salmonella typhii. The lowest MIC value of 0.70mg/ml for Staphylococcus aureus shows that it was more susceptible to the antimicrobial effect of the methanol extract. The diethylether extract had no antimicrobial activity while the methanol extract had a broad spectrum of antimicrobial activity. The extract had no antifungal effect. The chromatographic fractionation confirmed the antimicrobial agent to be steroidal terpenoids.

These extracts had anti-inflammatory activity that was concentration dependent. The diethylether extract had better anti-inflammatory activity than the methanol extract and even better than aspirin and diclophenac. The extracts had flavonoids, which its mechanism of action was suggested to be a consequence of its inhibitory action on arachidonic acid metabolism, cyclooxygenase and 5-lipogenase pathway (Sharma *et al*, 1996; Hajare *et al*, 2001) [10, 4]. The suppression of inflammation 1 hour post injection of phlogistic agent suggests that the agent is likely to possess an antihistamine effect, whereas subsequent suppression after then suggests an inhibition of arachidonic acid pathway (Willoghby and Flower, 1993) [13]. The diethylether extract inhibited the inflammation after 1 hour post injection of phlogistic agents, indicating that the major anti-inflammatory agent may likely be flavonoids. Inflammation and microbial infection are implicated in otitis media and the extracts were found to have positive anti-inflammatory and antimicrobial effects. It could be suggested that the leaf extract of Crateva religiosa can be used locally in Nigeria, and since the extracts were found to be safe, holistic formulation is recommended for treatment of otitis media but further studies are necessary for proper formulations.

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