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Studies on the antiinflammatory, antinociceptive and antimicrobial activities of *Combretum calobotrys* (Combretaceae) leaf

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The methanol:dichloromethane extract of *Combretum calobotrys* Engl & Diels (Combretaceae) leaves (CCE) was subjected to solvent-guided fractionation to yield the hexane (HF), dichloromethane (DF) and methanol (MF) fractions. The anti-inflammatory effects of the extract and fractions were evaluated in rodents using xylene-induced topical ear edema, carrageenan-induced pedal edema, formaldehyde-induced arthritis and cotton pellet granuloma tests, respectively. The extract and fractions were screened for analgesic activity using acetic acid induced writhing and formalin test in rodents. They were subjected to antimicrobial assay and also analysed for phytochemical constituents, while the CCE was subjected to acute toxicity (Lethal dosage, LD_{50}) test using standard procedures. The extract and fractions (200 and 400 mg/kg) significantly ($P < 0.05$) inhibited topical, systemic and chronic inflammation. The extracts and fractions (200 and 400 mg/kg) elicited significant ($P < 0.05$) and dose-related inhibition of acetic acid induced abdominal writhing, and also reduced the number and duration of paw licking in both early and late phases of formalin test, compared to control. They exhibited varying degrees of antimicrobial activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Aspergillus niger* and *Candida albicans*. The CCE and MF tested positive to alkaloids, resins, tannins, saponins, steroids, terpenoids and flavonoids. The HF contained alkaloids and resins, while the DF tested positive to resins and flavonoids. The oral LD_{50} of CCE was estimated to be > 5,000 mg/kg. The results indicate that *C. calobotrys* leaf possess anti-inflammatory, analgesic and antimicrobial activities.

Key words: *Combretum calobotrys*, anti-inflammatory, analgesic, abdominal writhing, formalin test, agar dilution.

INTRODUCTION

Combretum calobotrys Engl. and Diels (Combretaceae) is a species of flowering plant widely distributed in tropical and South Africa, and in tropics of America and Asia, Madagascar and China (Lu and Philips, 2009). The morphology has been described (Hutchinson and Dalziel, 1954; Lu and Philips, 2009). The leaf and bark of *Combretum* species are used in many parts of Africa to manage abdominal discomfort, body pains, respiratory

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disorders, colds and fevers, schistosomiasis, hookworm, dysmenorrhoea and infertility in women, leprosy, syphilis, microbial infections, general body weakness, ear, eye and other ailments (Watt and Breyer-Brandwijk, 1962; Kokwaro, 1976; Hutchings et al., 1996).

In KwaZulu-Natal Province of South Africa and other parts of Africa, it is also claimed that extracts of some species of *Combretum* are effective remedies for the management and/or control of painful, arthritic and other inflammatory conditions (Ojewole, 2008). Earlier research in our laboratory demonstrated the antibacterial activity of leaf and stem extracts of *C. calobotrys* (Ezike et al., 2011). Although research on various *Combretum* species has been documented, reports on the pharmacological actions of *C. calobotrys* are sparse in the literature. The present study was therefore undertaken to investigate the anti-inflammatory, analgesic and antimicrobial properties of *C. calobotrys* leaf in line with its ethnomedicinal uses.

MATERIALS AND METHODS

Animals

Adult Swiss albino rats (150 to 200 g) and mice (19 to 22 g) of either sex bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka (UNN) were used for the study. The animals were maintained freely on standard pellets and water. All animal experiments were in compliance with National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85 – 23, revised 1985).

Microorganisms

Clinically isolated strains of *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Klebsiella pneumonia, Aspergillus niger and Candida albicans* obtained from the Pharmaceutical Microbiology Unit of the Department of Pharmaceutics, UNN were used for the study. The cultures were maintained in agar slants and used as such.

Preparation of extract

Fresh leaves of *C. calobotrys* were collected between November and December, and authenticated at the International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Nigeria. The leaves were cleaned, cut into small pieces, dried under shade for three days and pulverized to coarse powder using a mechanical grinder. The powdered leaf (3 kg) was extracted by cold maceration in a 1:1 mixture of methanol and dichloromethane for 48 h and concentrated in a rotavapor to afford the methanol-dichloromethane extract (CCE) (250 g; 8.33% w/w).

Solvent-guided fractionation of extract

The CCE (220 g) was subjected to solvent-guided fractionation in a silica gel column successively eluted with n-hexane, dichloromethane and methanol (100%) to obtain the hexane (HF: 50 g; 22.73% w/w), dichloromethane (DF: 40 g; 18.18% w/w) and methanol (MF: 50 g; 22.73% w/w) fractions. The extract and fractions were subjected to phytochemical analysis for identification of constituents using established procedures (Iwu, 1978; Trease and Evans, 1983; Harborne, 1998).

Acute toxicity tests

The acute toxicity and lethality (LD_{50}) of CCE was determined in mice using the method described by Lorke (1983). Briefly, nine mice randomly divided into three groups (n = 3) were orally administered 10, 100, and 1000 mg/kg of CCE, respectively and observed for 24 h for death. Since no death was recorded, 1,600, 2,900 and 5,000 mg/kg of CCE were administered, respectively to a fresh batch of animals (n = 1) and the number of deaths in 24 h recorded.

Antiinflammatory activity tests

Xylene-induced acute topical ear edema

The effect of the extract and fractions on topical inflammation was evaluated by a modification of the methods of Tubaro et al. (1985) and Atta and Alkohafi (1998). Adult Swiss albino mice of either sex were randomly divided into 6 groups (n = 5) to receive CCE, hexane fraction (HF), dichloromethane fraction (DF), or methanol fraction (MF) (5 mg/ear) applied on the anterior surface of the right ear. Topical inflammation was instantly induced on the posterior surface of the same ear by application of xylene (0.05 ml). Control animals received either equivalent volume of the vehicle [1:1 mixture of propylene glycol and Tween 20 (15% v/v)] or indomethacin (5 mg/ear). Two hours after induction of inflammation, mice were sacrificed by overdose of chloroform anaesthesia and both ears removed. Circular sections (6 mm diameter) of both the right (treated) and left (untreated) ears were punched out using a cork borer, and weighed. Ear edema in each animal was quantified as the weight difference between the two ear plugs. The anti-inflammatory activity was evaluated as percent edema inhibition in the treated animals relative to control animals using the relation:

$$\text{Edema inhibition (\%)} = 100\,[1 - (EE_T / EE_C)]$$

Where EE_T = mean ear edema of treated rats; EE_C = mean ear edema of control rats.

Systemic acute edema of the rat paw

Briefly, adult Swiss albino rats were randomly divided into 10 groups (n = 5) to receive oral administration of 400 or 800 mg/kg of CCE, HF, DF or MF, respectively. Control groups received piroxicam (50 mg/kg), or vehicle [1:1 mixture of propylene glycol and Tween-20 (15% v/v)] (5 ml/kg), respectively. One hour later, acute inflammation (paw edema) was induced by injecting 0.1 ml of 1% w/v carrageenan in normal saline into the sub-plantar region of the left hind paw of each rat (Winter et al., 1962). Edema was assessed in terms of the difference between the zero time paw volume of the treated paw and the volume at the different time intervals after carrageenan injection. The volume of the paw was measured by water displacement before and at 0.5, 1, 2, 3, 4, 5, and 6 h after carrageenan injection. The level of inhibition (%) of edema was calculated using the relation:

$$\text{Inhibition of edema (\%)} = 100\,[1 - (a - x / b - y)]$$

Where a = mean paw volume of treated rats after carrageenan

injection; x = mean paw volume of treated rats before carrageenan injection; b = mean paw volume of control rats after carrageenan injection; y = mean paw volume of control rats before carrageenan injection.

Formaldehyde-induced arthritis

The method of Seyle (1949) was used. Adult rats were randomly divided into 10 groups (n = 5) to receive oral administration of 200 or 400 mg/kg of CCE, HF, DF or MF, respectively. Control rats received piroxicam (20 mg/kg), or vehicle (5 ml/kg), respectively. On day 1 of the experiment, arthritis was induced by injecting 0.1 ml of 2% v/v formaldehyde solution into the sub-plantar region of the left hind paw of the rats. Paw volume was measured at 0 and 4 h after carrageenan injection. Formaldehyde injection was repeated on day 3 while animals were treated from day 1 to 10. Day to day changes in edema was evaluated by measuring the volume of water displaced by the inflamed paw once daily for the 10 days. The global edematous response to formaldehyde arthritis was quantified as the area under the curve (AUC) of the time-course of the arthritis event. The AUC was calculated using the trapezoid rule. The level of inhibition of arthritis was calculated using the relation 100 [1 - (AUC_T / AUC_C)]; where AUC_C = AUC of the control group; AUC_T = AUC of the treated group.

Granuloma tissue formation induced by cotton pellet

The effect of the extract and fractions on granulomatous inflammation was evaluated using the cotton pellet granuloma test (D'Arcy et al., 1960; Gepdiremen et al., 2004) with slight modifications. Adult rats were randomly divided into 10 groups (n = 5) to receive oral administration of 200 or 400 mg/kg of CCE, HF, DF or MF, respectively. Control rats received piroxicam (20 mg/kg), or vehicle (5 ml/kg), respectively. Sterile autoclaved cotton pellets (30 mg) were implanted one on each side of the subcutaneous dead space in the depilated axial region of rats under ketamine anesthesia.

The wounds were sutured with silk and mopped with an alcoholic swab and the animals placed in their individual cages. Animals were treated once daily via the oral route from day 1 to 7 post-implantation. On day 8, the animals were decapitated and the pellets carefully removed freely from extraneous tissues and dried in an oven at 60°C to a constant weight. The weight of the granuloma tissue formed was calculated as the difference in weight of the implanted and excised cotton pellet. The level of decrease (%) in the weight of granuloma tissue formed was calculated relative to the control.

Analgesic activity tests

Abdominal writhing induced by acetic acid

The method described by Koster et al. (1959) was used with slight modifications. Adult mice were randomly divided into 10 groups (n = 5) to receive oral administration of 200 or 400 mg/kg of CCE, HF, DF or MF, respectively. Control rats received acetylsalicylic acid (200 mg/kg), or vehicle (5 ml/kg), respectively. One hour later, animals received acetic acid [3% (v/v)] i.p. (10 ml/kg), and were transferred to a transparent plastic observation chamber. Five minutes after acetic acid administration, the number of full abdominal writhes was counted for a period of 30 min for each mouse.

Formalin-induced paw licking

In this test, adult mice were randomly divided into 10 groups (n = 5) to receive oral administration of 200 or 400 mg/kg of CCE, HF, DF or MF. Control rats received acetylsalicylic acid (200 mg/kg), or vehicle (5 ml/kg), respectively. One hour later, formaldehyde in normal saline (2.5% v/v) (0.05 ml) was injected into the foot pad of one of the hind paws of each mouse, which was immediately placed in a transparent plastic observation chamber. The number and duration of licking of the injected paw from 0 to 5 min and 20 to 30 min post injection of formalin were recorded (Dubuisson and Dennis, 1977).

Antimicrobial activity assay

Microbial sensitivity test

The sensitivities of clinical strains of *E. coli, B. subtilis, P. aeruginosa, S. typhi, S. aureus, K. pneumonia, A. niger and C. albicans* to the extract and fractions were evaluated using the agar dilution technique. The test organism suspension prepared to 0.5 McFarland standard was sub-cultured and maintained on nutrient agar media for bacteria (37°C) and Sabouraud agar media for fungi (25°C). A known volume of extract (5 mg/ml) dissolved in dimethyl sulphuoxide (DMSO) was mixed thoroughly by swirling with an appropriate volume (to make up to 20 ml of medium) of sterile molten nutrient agar of double strength. The mixture was poured into sterile petri dish and allowed to solidify. The nutrient agar surface was dried and allowed to cool. Using an inoculating loop, the plates were seeded with the microorganisms. The plates were allowed 30 min for diffusion and incubated in an inverted position for 24 h at 37°C for bacteria and 48 h at 25°C for fungi, respectively. Microbial growth was seen as colonies distributed on the matrix or surface of the solidified agar. The tests were performed in triplicates.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using the agar dilution method as described above. The organisms found sensitive in the sensitivity test were subjected to MIC determination, using 5, 2.5, 1.25, 0.625 and 0.3125 mg/ml of extract. Microbial growth was seen as colonies distributed on the matrix or surface of the solidified agar. The tests were performed in triplicates. The least concentration which inhibited microbial growth was taken as the MIC.

Determination of minimum biocidal concentration (MBC) and minimum fungicidal concentration (MFC)

The plates used for MIC determination were further incubated for 48 and 72 h for the determination of minimum biocidal concentration (MBC) and minimum fungicidal concentration (MFC), respectively. The tests were performed in triplicates.

RESULTS

Phytochemical constituents of extract and fractions

The CCE and MF gave positive reactions for alkaloids, saponins, flavonoids, tannins, terpenoids, steroids, resins, carbohydrates, reducing sugars and proteins. The

Table 1. Phytochemical constituents of *C. calobotrys* leaf.

Phytoconstituent	CCE	HF	DF	MF
Alkaloids	+++	+	-	++++
Saponins	+	-	-	++
Flavonoids	+++	-	+++	+
Tannins	+	-	-	++
Terpenoids	+	-	-	++
Steroids	+	-	-	++
Resins	++	+++	++++	++
Glycosides	+	-	-	+++
Carbohydrates	+++	-	-	++++
Reducing sugars	+	-	-	+
Proteins	++	-	+++	+
Fats and oils	+	++	+	+

CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction. - =absent, + = mildly present, ++ = moderately present, +++ = highly present, ++++ = abundantly present.

Table 2. Effect of extract and fractions on topical acute edema in mice.

Treatment	Dose (mg)	Edema (mg)	Inhibition of edema (%)
CCE	5	1.98±0.37	18.18
HF	5	1.38±0.33	42.98
DF	5	0.4±0.19*	83.47
MF	5	1.48±0.49	38.84
Indomethacin	5	1.82±0.63	24.79
Control	-	2.42±0.64	-

n = 5; *$P < 0.05$ (ANOVA; LSD post hoc test); CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction.

HF tested positive to alkaloids and resins, while DF gave positive reactions for flavonoids, resins and proteins. The extract and fractions also gave positive reactions for fats and oils (Table 1).

Acute toxicity (LD_{50})

Oral administration of up to 5,000 mg/kg of CCE to mice caused no death in the treated animals. Therefore, the oral LD_{50} of CCE in mice was estimated to be greater than 5,000 mg/kg.

Effect of extract and fractions on topical acute edema

Topical application of the extract and fractions inhibited the development of xylene-induced ear edema to varying extents. The magnitude of inhibition was of the order: DF > HF > MF > CCE. The inhibitory effect of the fractions was greater than that of indomethacin (Table 2).

Effect of extract and fractions on carrageenan-induced pedal edema

Sub-plantar injection of carrageenan caused a progressive formation of edema that peaked at 4 h over the course of the experiment. However, oral administration of the extract and fractions suppressed the edema formation to varying extents. The inhibitory effects were dose-related and appreciable at 800 mg/kg. The magnitude of suppression of paw edema was of the order DF > HF > CCE > MF (Table 3).

Effect of extract and fractions on arthritis induced by formaldehyde

Chronic administration of the extract and fractions significantly ($P < 0.05$) inhibited the global edematous response to formaldehyde arthritis to varying extents. Except for HF, the extract and the rest of the fractions caused a dose-related inhibition. The magnitude of inhibition was of the order MF > DF > HF > CCE (Table 4).

Effect of extract and fractions on granuloma tissue formation

Chronic administration of the extract and fractions elicited a significant ($P < 0.05$) and dose-related inhibition of granuloma tissue formation on implanted cotton pellets in rats. The order of magnitude of inhibition was CCE > HF > MF > DF (Table 5).

Effect of extract and fractions on abdominal writhing

The CCE and fractions elicited significant ($P < 0.05$) dose-related reduction in abdominal writhing induced by acetic acid in mice. The inhibitory effect produced by CCE, DF and MF (400 mg/kg), HF (200 mg/kg), respectively was greater than that of acetylsalicylic acid (Table 6).

Effect of extract and fractions on paw licking

The CCE and fractions reduced the duration and number of paw licking induced by formalin in both phases. The MF and DF caused a significant ($P < 0.05$) dose-related reduction in duration of paw licking. The effect of the extract and fractions was greater in the early than later

Table 3: Effect of extract and fractions on acute pedal edema in rats.

Treatment	Dose (mg/kg)	Edema (ml)						
		0.5 h	1 h	2 h	3 h	4 h	5 h	6 h
CCE	400	0.18±0.04 (18.18)	0.22±0.06 (15.38)	0.46±0.06 (NI)	0.56±0.07 (NI)	0.52±0.12 (18.75)	0.56±0.08 (9.68)	0.54±0.08 (12.90)
	800	0.12±0.04 (45.45)	0.22±0.1 (15.38)	0.36±0.09 (5.26)	0.56±0.07 (NI)	0.58±0.07 (9.38)	0.5±0.09 (19.35)	0.5±0.08 (19.35)
HF	400	0.2±0.03 (9.09)	0.32±0.08 (NI)	0.54±0.1 (NI)	0.68±0.12 (NI)	0.74±0.11 (NI)	0.74±0.1 (NI)	0.78±0.09 (NI)
	800	0.22±0.06 (0)	0.18±0.06 (30.77)	0.24±0.07 (36.84)	0.28±0.07 (44)	0.32±0.10* (50)	0.38±0.08 (38.71)	0.4±0.1 (35.48)
DF	400	0.16±0.05 (27.27)	0.3±0.08 (NI)	0.5±0.13 (NI)	0.6±0.16 (NI)	0.64±0.15 (0)	0.68±0.15 (NI)	0.74±0.13 (NI)
	800	0.08±0.05* (63.64)	0.18±0.05 (30.77)	0.3±0.03 (21.05)	0.42±0.09 (16)	0.44±0.05 (31.25)	0.5±0.06 (19.35)	0.48±0.07 (22.58)
MF	400	0.2±0.03 (9.09)	0.26±0.04 (0)	0.42±0.07 (NI)	0.56±0.09 (NI)	0.6±0.07 (6.25)	0.74±0.07 (NI)	0.68±0.10 (NI)
	800	0.12±0.02 (45.45)	0.18±0.02 (30.77)	0.32±0.02 (15.79)	0.48±0.06 (4)	0.52±0.07 (18.75)	0.6±0.05 (3.23)	0.6±0.05 (3.23)
Piroxicam	50	0.12±0.05 (45.45)	0.2±0.05 (23.08)	0.14±0.02* (63.16)	0.24±0.05 (52)	0.28±0.04* (56.25)	0.32±0.07 (48.39)	0.36±0.07 (41.94)
Control	-	0.22±0.02	0.26±0.02	0.38±0.06	0.50±0.07	0.64±0.05	0.62±0.06	0.62±0.07

n = 5; *P < 0.05 (ANOVA; LSD post hoc test); value in parenthesis represent inhibition of edema (%) relative to control; NI = no inhibition; CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction.

phase. Also, the duration of paw licking was affected more than the number in both phases (Table 7).

Antimicrobial effects of extract and fractions

The extracts and fractions inhibited the growth of the tested microorganisms to varying extents (Tables 8 and 9). The CCE inhibited the growth of *P. aeruginosa*, *S. aureus*, *B. subtilis*, *A. niger* and *C. albicans* with MIC of 2.50, 1.25, 0.625, 2.50 and 1.25 mg/ml, respectively. The HF inhibited the growth of *P. aeruginosa* with MIC of 1.25 mg/ml, while DF was active against *S. aureus* and *B. subtilis* with MIC of 2.50 and 1.25 mg/ml, respectively. The MF inhibited *S. aureus* and *B. subtilis* and *A. niger* with MIC of 1.25, 0.625 and 2.50 mg/ml, respectively (Table 9). The MBC and MFC values are also shown (Table 9).

DISCUSSION

Experimental evaluation of the anti-inflammatory, analgesic and antimicrobial effects of *C. calobotrys* leaves showed that the extract and fractions suppressed topical and systemic acute and chronic inflammation and inhibited chemically-induced pain and microbial growth. Studies on acute inflammation revealed that topical as well as systemic application of the extract and fractions potently suppressed the acute inflammatory response. Topical application of xylene causes instant irritation of the mouse ear, which leads to fluid accumulation and edema characteristic of the acute inflammatory response. Carrageenan-induced paw and xylene-induced ear edema, respectively are mediated by inflammatory mediators such as histamine, serotonin, and bradykinin. Thus, the likely inhibition of the action of these mediators of acute inflammation may account for the anti-inflammatory activity of the extract and fractions in both tests. However, the magnitude of inhibition obtained in both tests showed that the extract and fractions elicited more effective and consistent effect in the topical than systemic acute inflammation.

Table 4. Effect of extract and fractions on global edematous response to formaldehyde arthritis.

Treatment	Dose (mg/kg)	AUC	Inhibition (%)
CCE	200	3.55±0.92	10.13
	400	2.59±0.86	34.43
HF	200	2.08±0.28*	47.34
	400	2.49±0.40	36.96
DF	200	2.30±0.44*	41.77
	400	2.22±0.35*	43.80
MF	200	2.49±0.44	36.96
	400	1.44±0.28*	63.54
Piroxicam	20	1.1±0.14*	72.15
Control	-	3.95±0.21	-

n = 5; * $P<0.05$ compared to control (one way ANOVA; LSD post hoc); Values of area under the curve (AUC) shown are mean ± SEM; inhibition (%) of AUC was calculated relative to the control. CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction.

Table 5. Effect of extract and fractions on granuloma tissue growth.

Treatment	Dose (mg/kg)	Granuloma tissue weight (mg)	Inhibition (%)
CCE	200	67.6±7.98	25.30
	400	56.00±3.78*	38.12
HF	200	59.13±5.87*	34.66
	400	74.9±8.69	17.24
DF	200	84.3±8.47	6.85
	400	81.5±8.38	9.94
MF	200	61.1±7.99*	32.49
	400	64±3.63*	29.28
Piroxicam	10 mg/kg	49.87±5.49*	44..9
Control	-	90.5±18.29	-

n = 5; * $P<0.05$ compared to control (one way ANOVA; LSD post hoc); Values of granuloma tissue weight shown are mean ± SEM; inhibition (%) of granuloma tissue growth was calculated relative to the control. CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction.

It is not clear to us why single oral administration of the extract and fractions evoked an irregular and inconsistent effect in the systemic acute anti-inflammatory activity test. However, we may be persuaded to suggest that issues bordering on absorption of the constituents of extracts and fractions from the gastrointestinal tract may have played a role in limiting their actions on the acute inflammatory response.

Chronic administration of the extract and fractions inhibited the global edematous response to formaldehyde arthritis as well as granuloma tissue growth. Chronic inflammation usually arises as a consequence of incomplete elimination or containment of the pro-inflammatory stimuli by acute inflammation and is usually marked by neutrophil infiltration, fluid exudation and fibro-blast proliferation amongst others. These mechanisms operate in conjunction with various mediators including those associated with acute inflammation as well as the development of proliferative cells which can either spread or form granuloma. Granuloma tissue formed on an inert foreign body in a dead space comprises an accumulation of modified macrophages (Whaley and Burt, 1996) and other cells and tissues (Whaley and Burt, 1996; Bairy and Rao, 2001). Although the precise mechanisms of anti-inflammatory effect of the extract and fractions are yet to be elucidated, suppression of the formaldehyde arthritis and granuloma tissue growth is a clear indication of inhibition of the proliferative phase of inflammation and interference with cellular migration. Inhibition of cellular migration reduces the severity or magnitude of the inflammatory response, and may also contribute to the amelioration of acute inflammation by extract and fractions.

Since the inflammatory response is commonly/typically associated with pain production, we also investigated the analgesic activity of the extract and fractions. The results showed that the extract and fractions inhibited the abdominal writhing response and reduced the number and duration of formalin-induced paw licking in mice. The abdominal constrictive response induced by acetic acid is a sensitive procedure used to evaluate peripherally acting analgesics (Collier et al., 1968; Gene et al., 1998) as acetic acid is an abdominal irritant that stimulates local receptors within the peritoneum to induce pain (Vogel and Vogel, 2002). Inhibition of acetic acid-induced writhing by the extract and fractions indicates peripherally mediated analgesic activity. Evaluation of possible involvement of central mechanisms in the analgesic activity of the extract and fractions using the formalin paw licking test revealed reduced number and duration of paw licking in the two phases of the test.

Pain induced by formalin occurs in a biphasic manner, consisting of an early neurogenic component followed by a late tissue-mediated response (Wheeler-Aceto and Cowan, 1991). The early response is believed to repre-sent direct effect of formalin on pain fibers, particularly

Table 6. Effect of extract and fractions on abdominal writhing in mice.

Treatment	Dose (mg/kg)	No. of writhing	Inhibition of writhing (%)
CCE	200	98.4±19.71	19.08
	400	45.75±3.83*	62.38
HF	200	61.8±12.63*	49.18
	400	83.0±8.55	31.74
DF	200	75.4±5.68*	37.99
	400	70.6±15.54*	41.94
MF	200	87.6±12.38	27.96
	400	51.0±7.74*	58.06
ASA	200	75.4±27.61*	37.99
Control	-	121.6±13.45	-

n = 5; *Significant at $P < 0.05$ (ANOVA; LSD post hoc test) relative to control; CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction, ASA = acetylsalicylic acid.

Table 7. Effect of extract and fractions on licking duration and number of formalin injected rat paw.

Treatment	Dose (mg/kg)	Early phase (0-5 min)		Late phase (20 to 30 min)	
		Licking duration (s)	No. of licking	Licking duration (s)	No. of Licking
CCE	200	39.8±11.01 (26.3)	8.8±2.31 (24.1)	42.4±6.19 (27.9)	12.8±1.02 (13.5)
	400	33.0±7.16 (38.9)	8.8±1.59 (24.1)	54.0±8.58 (8.2)	19.4±2.25 (NI)
HF	200	44.6±9.76 (17.4)	9.0±1.82 (22.4)	47.6±8.49 (19.0)	13.6±3.53 (8.1)
	400	33.2±8.36 (38.5)	9.8±1.74 (15.5)	45.6±4.80 (22.4)	17.8±2.42 (NI)
DF	200	39.4±7.42 (27)	12±2.02 (NI)	45.4±5.80 (22.8)	16.4±2.58 (NI)
	400	22.4±5.28* (58.5)	7.6±1.47 (34.5)	43.4±7.30 (26.2)	14.6±2.09 (1.35)
MF	200	25.8±8.30* (52.2)	7.8±2.20 (32.8)	28.8±6.18* (51.0)	10.8±2.52 (27.0)
	400	22.0±2.02* (59.3)	7.6±1.17 (34.5)	43.0±3.42 (26.8)	14.4±1.03 (2.7)
ASA	200	48.2±10.29 (10.7)	9.4±2.01 (19.0)	35.2±8.99* (40.1)	12.4±2.89 (16.2)
Control	-	54.0±8.84	11.6±1.33	58.8±10.89	14.8±1.24

n = 5; *$P < 0.05$ (ANOVA; LSD post hoc test); values in parentheses represent inhibition (%) relative to control; NI= no inhibition; CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction, ASA = acetylsalicylic acid.

particularly C fibers, causing release of bradykinin and tachykinin (Shibata et al., 1989). The late phase is due to inflammatory reaction caused by prostaglandins, bradykinin and excitatory amino acids (Damas and Liegeois, 1999). Centrally acting analgesics inhibit both phases, while peripherally acting agents inhibit the late phase (Shibata et al., 1989). The extract and fractions inhibited the number and duration of paw licking in both phases, with greater effect on the early phase, suggesting they may act through central mechanisms augmented by peripheral actions.

Antimicrobial activity assay showed that the extract and fractions inhibited microbial growth. The range of MIC obtained suggests that the extract and fractions may possess potent antimicrobial activity. Usually, there is an inflammatory response to microbial infection; hence, *C. calobotrys* leaf may offer added benefit of addressing inflammation as well as exerting antimicrobial activity in diseases due to susceptible organisms.

Solvent-guided fractionation of the extract was performed

Table 8. Sensitivity of microorganisms to extract and fractions.

Microorganism	Extract (5 mg/ml)											
	CCE			HF			DF			MF		
P. aeruginosa	-	-	-	-	-	-	+	+	+	+	+	+
S. typhi	+	+	+	+	+	+	+	+	+	+	+	+
K. pneumoniae	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	-	-	-	+	+	+	-	-	-	-	-	-
B. subtilis	-	-	-	+	+	+	-	-	-	-	-	-
E. coli	+	+	+	+	+	+	+	+	+	+	+	+
A. niger	-	-	-	NT	NT	NT	+	+	+	-	-	-
C. albicans	-	-	-	NT	NT	NT	+	+	+	+	+	+

n = 3 (results of three determinations are shown); CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction; NT = not tested; - = no microbial growth, + = microbial growth.

Table 9. Antimicrobial potency of extract and fractions.

Microorganism	MIC (mg/ml)				MBC (mg/ml)				MFC (mg/ml)			
	CCE	HF	DF	MF	CCE	HF	DF	MF	CCE	HF	DF	MF
P. aeruginosa	2.50	1.25	NS	NS	2.50	2.5	NS	NS	- [a]	- [a]	- [a]	- [a]
S. aureus	1.25	NS	2.50	1.25	2.50	NS	5.0	2.50	- [a]	- [a]	- [a]	- [a]
B. subtilis	0.625	NS	1.25	0.625	2.50	NS	2.50	1.25	- [a]	- [a]	- [a]	- [a]
A.niger	2.50	NT	NS	2.50	- [a]	- [a]	- [a]	- [a]	5.00	NT	NS	2.50
C. albicans	1.25	NT	NS	NS	- [a]	- [a]	- [a]	- [a]	2.50	NT	NS	NS

n = 3 (results of three determinations are shown); CCE = crude extract, HF = hexane fraction, DF = dichloromethane fraction, MF = methanol fraction, MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, MFC = minimum fungicidal concentration, NS = not sensitive; NT = not tested; [a] = not applicable.

to relate activity to phytoconstituents. Although the extent of phytochemical studies may not permit the assignment of biological activity to any specific constituent, flavonoids, saponins, tannins, phenolic compounds and glycosides have all been associated with various degrees of anti-inflammatory and analgesic activities (Garcia-Leme et al., 1973; Wang et al., 2008; De Melo et al., 2009). Furthermore, various flavones, including quercetin and rutin isolated from various plants have been demonstrated to produce significant amelioration of acetic acid and formalin induced nociceptive responses (Calixto et al., 2000). Further studies are on-going to identify the bio-active constituent(s) of the extracts.

Conclusion

C. combretum leaf possesses topical, systemic and chronic anti-inflammatory, in addition to antimicrobial activities. Also, the plant exhibited antinociceptive activity, acting possibly via central mechanisms augmented by peripheral actions. These provide a pharmacological basis for the ethnomedicinal use of the plant for the management of painful, arthritic and other inflammatory

conditions.

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