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Topical anti-inflammatory constituents of lipophilic leaf fractions of *Alchornea floribunda* and *Alchornea cordifolia*

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The leaves of *Alchornea floribunda* and *Alchornea cordifolia* are used traditionally as topical anti-inflammatory agents. In this study, two highly lipophilic fractions AFLF and ACLF isolated from *A. floribunda* and *A. cordifolia* leaves respectively were investigated for topical anti-inflammatory effects using xylene-induced mice ear oedema as a model of inflammation. AFLF and ACLF at 5 mg per ear showed significant ($p < 0.01$) topical anti-inflammatory effect with oedema inhibitions of 64.0% and 79.0% at 2 h, respectively. When compared to indomethacin (5 mg per ear), these fractions showed significantly ($p < 0.05$) higher topical anti-inflammatory effect. Gas chromatography–mass spectrometry analysis revealed that AFLF is composed mainly of long chain saturated and unsaturated hydrocarbons (18.78%) and their oxygenated derivatives (1.89%); while ACLF is rich in volatile oils eugenol (21.26%) and cadinol (4.76%), and other constituents like, nanocosaine (36.86%) and steroid derivatives, ethyl iso-allocholate (4.59%) and 3-acetoxy-7,8-epoxylanostan-1-ol (15.86%). Analysis of the volatile oil (ACV) extracted from the fresh leaves of *A. cordifolia* revealed the presence of high concentrations of eugenol (41.7%), cadinol (2.46%), Caryophylene (1.04%), Linalool (30.59%) and (E)-α-bergamotene (4.54%). These compounds could be contributing to the topical anti-inflammatory effects of *A. floribunda* and *A. cordifolia* leaf extracts.

Keywords: topical anti-inflammatory; GC–MS; lipophilic constituents; volatile oils; *Alchornea floribunda*; *Alchornea cordifolia*

1. Introduction

Anti-inflammatory corticosteroids and non-steroidal anti-inflammatory agents have been the mainstays for the topical treatment of cutaneous disorders or disruptions

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characterised by skin inflammation or hyperproliferative epidermal activity (David, Marvel, & James, 1986). For these conditions, topical application of these agents is preferred over systemic use to avoid unwanted effects, especially gastro-intestinal erosions which could be debilitating. These drugs are formulated to penetrate the stratum corneum in amounts sufficient to exert therapeutic activity without attaining high or toxic plasma levels. Incorporating botanical anti-inflammatory ingredients into topical medications is, generally, more acceptable to the public and is not associated with the usual scepticism and concerns of toxicity normally associated with some herbal oral medications.

The general acceptability and marketability of topical medications with botanical ingredients are high and there are many commercial products of proven efficacy (Glaser, 2005). These herbal ingredients are incorporated into various skin care products to achieve one or more of some therapeutic or cosmetic benefits. They are often used to reduce wrinkles, to lighten uneven pigmentation, as antimicrobial agents, or as anti-inflammatory agents (Glaser, 2005). Several lipophilic constituents with anti-inflammatory properties have been isolated from plants. The lipophilic nature of these agents promotes their penetration into the stratum corneum of skin and makes them suitable for use in topical medications (Grace, Roger, Skeith, & Anderson, 1999). These ingredients formulated alone or in combination with other steroidal or non-steroidal anti-inflammatory drugs have great potential and they need to be harnessed against many inflammatory skin conditions.

Alchornea floribunda (Müll. Arg.) and *Alchornea cordifolia* (Shum. Thon) (Euphorbiaceae) are applied locally as traditional remedy for arthritis, muscle pain and other inflammatory disorders (Duke, Mary, & Judi, 2002). They are claimed to possess remarkable anti-inflammatory and pain-relieving properties when applied topically. The crushed leaves are usually rubbed on painful joints or made into paste and applied to painful stingray wounds. The local as well as the systemic anti-inflammatory properties of the various extracts and fractions of *A. floribunda* and *A. cordifolia* have been validated by the results of some pharmacological studies (Mavar-Manga, Brkic, Marie, & Quetin-Leclercq, 2004; Mavar-Manga, et al., 2008; Okoye & Osadebe, 2009, 2010; Okoye et al., 2010; Osadebe & Okoye, 2003; Osadebe, Ebi, & Okoye, 2008). In one of the studies, it was shown that the topical anti-inflammatory effect of *A. cordifolia* is due to highly lipophilic constituents of the *n*-hexane fraction of the leaf extract (Mavar-Manga et al., 2004). Some constituents isolated from the leaf extracts of *A. cordifolia* were shown to possess topical anti-inflammatory effect in croton oil-induced ear oedema in mice (Mavar-Manga et al., 2008). Three stigmastane steroids isolated from *A. floribunda* leaves have also been shown to exhibit significant anti-inflammatory activity in xylene-induced ear oedema in mice (Okoye et al., 2010).

In this study, we report the isolation and identification of some lipophilic constituents of the *n*-hexane fractions of *A. floribunda* and *A. cordifolia* leaves with topical anti-inflammatory properties. The chemical constituents of these fractions as well as the volatile oils extracted from the fresh leaves of *A. cordifolia*, as deduced from the gas chromatography–mass spectrometry (GC–MS) analysis, are reported.

2. Results and discussion

Hexane extracts from *A. cordifolia* and *A. floribunda* leaves were shown in previous studies to possess significant anti-inflammatory activities (Okoye & Osadebe, 2009; Okoye et al., 2010; Osadebe & Okoye, 2003). In another study, Mavar-Manga et al. (2004) reported that the topical anti-inflammatory effect of *A. cordifolia* leaves may be due to the presence of highly lipophilic hexane fraction from the methanol extract. In this study, we decided to carry out column chromatographic separation of the hexane extracts of both *A. cordifolia* and *A. floribunda* leaves and isolated two highly lipophilic fractions ACLF and AFLF, respectively. The anti-inflammatory effect of these isolates is shown in Table 1.

AFLF and ACLF were found to exhibit significant ($p<0.01$) topical anti-inflammatory activity. Lipophilic and volatile constituents from plants have been shown in several studies to possess anti-inflammatory activity (Kavimani, Karpagam, Jaykar, & Ilango, 1997; Kavimani, Vetrichelvan, Ilango, & Jaykar, 1996; Mariea, Dejanb, & Quetin-Leclercq, 2007; Martins et al., 2008; Shimizu et al., 1990; Souza, Siani, Ramos, Menezes-de-Lima, & Henriques 2003). The topical anti-inflammatory activity may be related to the ability of the lipophilic constituent to permeate lipoidal skin layers. Some volatile oils and lipophilic compounds have also been shown to enhance skin permeability of non-steroidal anti-inflammatory drugs. For example, limonene (Priborsky, Takayama, Obata, Priborska, & Nagai, 1992) is reported to promote percutaneous absorption of non-steroidal anti-inflammatory drugs (NSAIDs) in rats while nerolidol has been shown to increase the skin permeation of naproxen® (Ray & Ghosal, 2003).

The highly lipophylic fractions AFLF and ACLF isolated from the hexane extracts of *A. floribunda* and *A. cordifolia* leaves, respectively, were analysed using GC–MS apparatus. The result of the analysis is given in Table 2. AFLF was found to be a mixture of long chain saturated and unsaturated hydrocarbons and their oxygenated derivatives as well as long chain carboxylic (fatty) acids, primary alcohol and their esters. ACLF is, however, rich in volatile oils euginol (**1**) (21.26%) and cadinol (**2**) (4.76%). Other constituents are long chain primary alcohols (4.78%) and long chain saturated hydrocarbon, nanocosane (36.86%) and two steroid derivatives ethyl iso-allocholate (**3**) (4.59%) and 3-acetoxy-7,8-epoxylanostan-1-ol (**4**) (15.86%).

None of the constituents of AFLF has been reported to possess anti-inflammatory effect. Some long chain saturated and unsaturated carboxylic acids

Table 1. Effect of ACLF and AFLF on xylene-induced ear oedema in mice.

Treatment	Dose (mg per ear)	Oedema (mean ± SEM) at 2 h	Inhibition (%)
ACLF	5	$1.50 \pm 0.50^{**,\#}$	79.0
AFLF	5	$2.57 \pm 0.57^{**}$	64.0
Indomethacin	5	$3.71 \pm 0.60^{*}$	48
Vehicle	0.05 mL	7.14 ± 1.03	–

Notes: $^{*}p<0.05$; $^{**}p<0.01$, $n=7$, values significantly lower than the negative control; $^{\#}p<0.05$, $n=7$, significantly lower than that of AFLF.

Table 2. Chemical composition of ACLF, AFLF and ACV.

	Compounds identified in ACLF		Compounds identified in AFLF		Compounds identified in ACV	
	Compound	%	Compound	%	Compound	%
1	3-Allyl-6-methoxyphenol (Eugenol)	21.26	2,6,10-Trimethyldodecane	0.75	1-Octen-3-ol	0.28
2	2-Isopropyl-5-metyl-9-methylene-5-hydroxy-Bicyclo[4.4.0]dec-1-ene (Cadinol)	4.76	2,6,10-Trimethyltetradecane	2.67	D-Limonene	0.13
3	3,7,11,-Trimethyldodecanol	2.2	E-2-Methyl-3-tetradecene-1-ol acetate	1.04	Eucalyptol	0.4
4	1-Heptatriacotanol	2.58	7-Methyl-Z-tetradecen-1-ol acetate	3.5	L-Fenchone	0.71
5	Nonacosane	36.86	3-Octyl-, *cis*-oxiraneoctanoic acid	1.12	Linalool	30.59
6	Ethyl iso-allocholate	4.59	9-Hexadecenoic acid	1.6	Borneol	0.21
7	3-(Octadecyloxy)propyl oleate	11.89	3-Ethyl-5-(2-ethylbutyl)-octadecane	3.13	4-Methyl-1-isopripyl-(R)-3-cyclohexene-1-ol	1.88
8	3-Acetoxy-7,8-epoxylanostan-1-ol	15.86	2,6,10,14-Tetramethylpentadecane	7.39	Caryophylene	1.04
9			1,1-*Bis*(dodecyloxy)-hexadecane	1.89	(E)-α-bergamotene	4.54
10			9-Hexylheptadecane	2.93	Eugenol	41.7
11			17-Pentatriacotene	1.91	Cubenol	0.48
12			3-(Octadecyloxy)propyl oleate	0.99	Cadinol	2.46

Notes: ACLF, highly lipophilic fraction isolated from *A. cordifolia* leaves; AFLF, highly lipophilic fraction isolated from *A. floribunda* leaves; ACV, volatile oil extracted from *A. cordifolia* leaves.

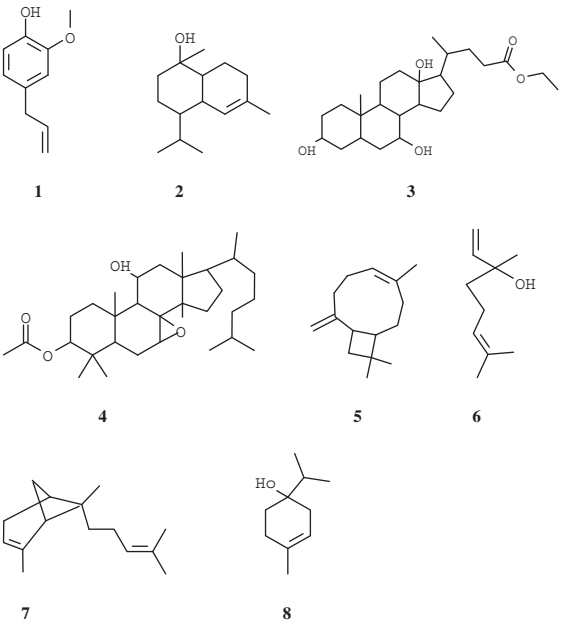

Figure 1. Structures of some major constituents identified in ACLF and ACV.

are, however, reputed to possess anti-inflammatory activity (Li et al., 2004; Pompéia et al., 2000). The anti-inflammatory effect of ACLF could be imputed to the presence of the steroid derivatives, ethyl iso-allocholate (**3**) and 3-acetoxy-7,8-epoxylanostan-1-ol (**4**), and the high concentration of cadinol (**2**) (4.76%) and eugenol (**1**) (21.26%) (Figure 1). Steroids have been severally reported to possess anti-inflammatory effect (Mavar-Manga et al., 2008; Okoli & Akah, 2004; Okoye et al., 2010). Also, the volatile oils, cadinol and eugenol, have been shown in previous studies to possess anti-inflammatory activity (Jadhav, Khandelwal, Ketkar, & Pisal, 2004; Mariea et al., 2007). The high concentration of some of these constituents with established anti-inflammatory effect in ACFL may explain why this fraction exhibited significantly higher ($p<0.05$) anti-inflammatory activity than AFLF. The presence of the two volatile oils (cadinol and eugenol) in such high concentration in a lipophilic solvent fraction from the dry leaf of *A. cordifolia* suggested that the plant material could be rich in volatile constituents. Consequently, we subjected the fresh leaves of *A. cordifolia* to hydrodistillation with the view to extracting the volatile constituents. The GC–MS analysis of the extracted essential oil showed the presence of high concentration of eugenol (**1**) (41.7%), cadinol (**2**) (2.46%), caryophylene (**5**) (1.04%), linalool (**6**) (30.59), (E)-α-bergamotene (**7**) (4.54%) and 4-methyl-1-isopropyl-(R)-3-cyclohexene-1-ol (**8**) (1.88%). Other constituents in moderate concentrations are D-limonene (0.13%), Eucalyptol (0.4%), L-fenchone (0.7%), Borneol (0.21) and Cubenol (0.48). Some of these volatile oils e.g. eugenol, limonene, linalool and caryophylene, have been shown in previous studies to possess anti-inflammatory activity (Jadhav et al., 2004; Kavimani et al., 1996, 1997; Mariea et al., 2007; Shimizu et al., 1990; Souza et al., 2003). These constituents may thus contribute significantly to the anti-inflammatory activity of *A. cordifolia* leaves.

In conclusion, this study further validates the claimed ethnomedicinal use of crushed leaves of *A. cordifolia and A. floribunda* in topical management of arthritis and other inflammatory diseases. The isolated fractions are composed of highly lipophilic constituents and their ability to permeate the lipoidal skin layers makes them suitable, either alone or in combination with NSAIDs, for formulation as topical anti-inflammatory agents. More so, the golden yellow colour of these isolates will add to the aesthetics and patient acceptability of such topical preparations.

3. Experimental

3.1. *Collection and preparation of plant material*

The leaves of *A. cordifolia* and *A. floribunda* were collected in August 2006 from Orba in Nsukka, Enugu State, Nigeria. The plant materials were authenticated by Mr. Alfred Ozioko of Bioresources Development and Conservation Programme, Nsukka, Enugu State, Nigeria. Voucher specimens, *A. cordifolia* (0012) and *A. floribunda* (06/085), have been deposited at the herbarium of the Department of Pharmacognosy, University of Nigeria, Nsukka, Enugu State, Nigeria. The leaves were cleaned and air-dried for 10 days and milled into powder.

3.2. *Chemicals*

For extraction and chromatographic separation, *n*-hexane (Fluka®), of analytical grade, was used. Silica gel (60–120 mesh; Merck, England); pre-coated silica gel GF_{254} (May and Baker, England) was used. Xylene (Fluka®) was used as phlogistic agent, while indomethacin (Sigma–Aldrich) was used as the reference anti-inflammatory drug.

3.3. *Extraction and chromatographic separation of the lipophilic constituents*

About 1 kg each of the pulverised leaves of *A. floribunda* and *A. cordifolia* were macerated in 5 L of *n*-hexane at room temperature for 48 h. The extracts were filtered and concentrated *in vacuo* using rotary evaporator to obtain 7.18 g dark green mass of HEF (hexane extract of *A. floribunda*) and 13.16 g dark green mass of HEC (hexane extract of *A. cordifolia*).

HEF (6 g) was chromatographed on silica gel (60–120 mesh, 450 g) packed into a glass column ($3.5 \times 75\,cm^2$) with the bed of height 50 cm and eluted with 2 L of *n*-hexane. All the hexane eluates were combined and concentrated to obtain 1 mL of yellow-red volatile liquid (*Alchornea floribunda* lipophilic fraction, AFLF). About 10 g of HEC was similarly separated to obtain 1.2 mL of golden yellow volatile liquid (*Alchornea cordifolia* lipophilic fraction, ACLF). The isolated fractions were stored in the refrigerator at 0–4°C until used.

3.4. *Extraction of volatile oils from fresh leaves of* A. cordifolia

Fresh leaves (400 g) of *A. cordifolia* were milled and the volatile constituents isolated by steam distillation. Briefly, the plant material was placed in a round bottom flask

and 400 mL of water added. The flask was connected to Clevenger apparatus and heated in a water bath for 4 h. The volatile oil fraction of *A. cordifolia* (ACV) was recovered and stored in refrigerator (0°C) before conducting analysis of the constituents with GC–MS apparatus.

3.5. *Animals*

Albino mice (20 ± 5 g), of both sexes obtained from the Faculty of Veterinary Medicine University of Nigeria, Nsukka, Enugu State, Nigeria, were used. Animals were allowed free access to standard livestock pellets (Guinea feed Nigeria) and water. The protocols on the use and handling of the animals conformed to internationally acceptable best practices and were approved by the local Ethics Committee of our institution.

3.6. *Topical anti-inflammatory tests*

The effect of AFLF and ACLF on acute topical oedema was evaluated by a modification of previously reported methods (Okoli & Akah, 2004; Tubaro, Dri, Delbello, Zilli, & Della, 1985). Adult albino mice (20 ± 5 g) of either sex were divided into groups of seven animals. The treatment groups received AFLF and ACLF dissolved in xylene (5 mg per ear) applied on the anterior surface of the right ear. Control animals received either equivalent volume of the phlogistic agent (xylene) or indomethacine dissolved in xylene (5 mg per ear). Two hours after application, the mice were sacrificed and both ears removed. Circular sections (5 mm) of both the right (treated) and left (untreated) ears were punched out using a cork borer, and weighed. Oedema was quantified as weight differences between the two earplugs. The anti-inflammatory activity was evaluated as per cent oedema inhibition in the treated animals relative to the control animals (Okoli & Akah, 2004; Tubaro, Dri, Delbello, Zilli, & Della, 1985) using the relation:

$$\%\ \text{Oedema reduction} = \left[1 - \frac{\text{Rt} - \text{Lt}}{\text{Rc} - \text{Lc}}\right] \times 100,$$

where Rt is the mean weight of right earplug of treated animals, Lt the mean weight of left earplug of treated animals, Rc the mean weight of the right earplug of control (vehicle treated) animals and Lc the mean weight of the left earplug of control (vehicle treated) animals.

3.7. *GC–MS analysis of AFLF, ACLF and ACV*

AFLF, ACLF and ACV were analysed by GC–MS using Agilent 5973N mass selective detector coupled to Agilent 6890N gas chromatograph, equipped with a cross-linked 5% PH-ME siloxane HP5-MS capillary column (30 m × 0.25 mm, film thickness of 0.25 μm). Operating conditions were as follows: carrier gas, helium with a flow rate of 2 mL min^{-1}; column temperature, 60–275°C at 4°C min^{-1}; injector and detector temperatures, 280°C; injected volume 2 μL; split ratio, 1 : 50. The MS operating parameters were as follows: ionisation potential, 70 eV; ionisation current, 1 A; ion source temperature, 200°C; and resolution of 1000. Identification of

components in AFLF, ACLF and ACV were based on comparison of the retention times and computer matching of MS fragments with the NISTOL2.L library.

3.8. *Statistical analysis*

Results of anti-inflammatory effect obtained were analysed by SPSS v.11 using one-way ANOVA and subjected to Fischer least significant difference (LSD) *post hoc* tests and expressed as mean ± SEM. Differences between means were considered significant at $p < 0.05$.

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