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Anti-inflammatory compounds from leaves and root bark of *Alchornea cordifolia* (Schumach. & Thonn.) Müll. Arg.

H. Mavar-Manga [a], M. Haddad [a], L. Pieters [b], C. Baccelli [a], A. Penge [c], J. Quetin-Leclercq [a,*]

[a] *Laboratoire de Pharmacognosie, Unité CHAM 72.30, Ecole de Pharmacie, Université catholique de Louvain, Av. E. Mounier, 72, 1200 Bruxelles, Belgium*
[b] *Department of Pharmaceutical Sciences, Laboratory of Pharmacognosy and Phytochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*
[c] *Faculté de Pharmacie, Université de Kinshasa, BP 212, Kinshasa XI, Democratic Republic of Congo*

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Abstract

Alchornea cordifolia is one of the most widely-used plants in traditional medicine throughout Africa, principally for inflammatory, antimicrobial and parasitic diseases. In continuation of our investigations on its anti-inflammatory activity, we fractionated the leaf and root bark extracts and isolated six compounds which exhibited significant topical anti-inflammatory activity in the mouse ear oedema model using croton oil at a dose of 90 μg/cm^2. Daucosterol (**2**), acetyl aleuritolic acid (**4**), *N1*,*N2*-diisopentenyl guanidine (**5**) and *N1*,*N2*,*N3*-triisopentenyl guanidine (**6**) were shown to be more active than indomethacin, while β-sitosterol (**1**) and di(2-ethylhexyl) phthalate (**3**) were less effective. This is the first report on the presence of compounds **1**, **2**, **3** and **4** in this plant and of the anti-inflammatory activity of **3**, **5** and **6**. These compounds may account, at least in part, for the use of *A. cordifolia* in folk medicine to treat inflammation.

Keywords: *Alchornea cordifolia*; Anti-inflammatory activity; Mouse ear oedema test; Triterpenoids; Guanidine alkaloids; Di(2-ethylhexyl) phthalate; OPLC; Euphorbiaceae

1. Introduction

Studies on *A. cordifolia* were stimulated by its wide range of medicinal folk uses throughout Africa to treat diseases such as dermatitis, asthma, hepatitis, splenomegaly, vaginitis, metritis and colitis (Mavar-Manga et al., 2004). As some of these uses could be related to anti-inflammatory properties, we decided to analyse this activity.

In 2003, a Nigerian team reported that the ethanol fraction from hexane extract of *A. cordifolia* leaves exhibited potent anti-inflammatory activity (AIA) using egg–albumin-induced hind paw oedema model in rats (Osadebe and Okoye, 2003). We have recently reported the topical anti-inflammatory property of the methanolic leaf extract (MeOH-S) in the ear mouse oedema model (Mavar-Manga et al., 2004). This extract was further fractionated by liquid–liquid extraction and the most active fraction (hexane) gave 42% inhibition at 0.7 μg/cm^2. An alkaloid rich extract also showed interesting activity (57.3% inhibition at 289.3 μg/cm^2) and showed that the polyphenolic compounds present in the methanol extract (tannins and flavonoids) did not account completely for the observed activity. More recently, a methanol extract from *A. cordifolia* seeds was reported to exhibit AIA (86% inhibition for 250 μg/pellet) by HET-CAM (Hen's Egg Test—Chorio-Allantoic Membrane) assay and also to possess anti-oxidant activity in the DPPH assay (IC_{50} = 0.68 μg/ml) and inhibit angiogenesis, with a good score of 0.9, with no toxicity being observed on the eggs (Nia et al., 2005).

To obtain good yields of lipophilic extracts, which were shown previously to possess activity (Mavar-Manga et al., 2004), new extracts from the leaves and the root barks were prepared of the plant parts frequently used in traditional medicine with

Abbreviations: AIA, anti-inflammatory activity; DEHP, di(2-ethylhexyl) phthalate.

* Corresponding author. Tel.: +32 2 764 72 30; fax: +32 2 764 72 53.
E-mail address: Joelle.Leclercq@uclouvain.be (J. Quetin-Leclercq).

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a view to isolating the compounds responsible for the activity. This is the work reported in this paper.

2. Materials and methods

2.1. Plant material

Fresh green leaves of *A. cordifolia* (Schumach. & Thonn.) Müll. Arg. (Euphorbiaceae) were collected in Kinshasa and identified at INERA (Institut National pour l'Etude et la Recherche Agronomique of University of Kinshasa). A voucher specimen is deposited at the National Botanic Garden of Belgium (BR) bearing the number SP 848103.

2.2. Extraction and isolation

All solvents used were of analytical grade.

2.2.1. Identification of components of the more active hexane fraction

Eight hundred milligram of the highly active hexane fraction (F2) obtained from the methanol Soxhlet extract (Mavar-Manga et al., 2004) was submitted to an OPLC (Optimum Performance Laminar Chromatography, Bionisis, Le Plessis Robinson, France) separation on HTsorb™ preparative Silica gel plate F_{254} (sorbent tickness: 500 μm; particle size: 11 μm) with the following successive solvents: hexane, toluene, toluene–EtOAc (1:1), EtOAc and MeOH (each 40 ml, flow rate 1.25 ml/min, 0.625 ml/tube). Fractions 31–60 (19–38 ml) contained **3** (9.1 mg), while toluene–EtOAc fraction (80–120 ml) was shown to contain **1** and **2** which were purified from another fraction (AL-H, hereafter).

2.2.2. Preparation of other crude lipophilic extracts

Powdered dried leaves (200 g) was moistened with a 10% aqueous Na_2CO_3 (500 ml) overnight and then extracted successively in a Soxhlet with EtOAc (1.5 l) and $CHCl_3$ (1.5 l). These extracts were combined and evaporated under reduced pressure. The concentrated extract (1.372 g) was partitioned between hexane and MeOH–H_2O (8:2) to give two fractions and a residue. After evaporation of the solvents, lipophilic (AL-H: 0.715 g) and a polar (AL-M: 0.479 g) fractions were obtained.

The extraction of root bark powder (200 g) was performed in the same way as described above for leaves and gave 1.873 g of total extract and fractions AR-H (0.292 g) and AR-M (0.767 g).

2.2.3. Isolation of anti-inflammatory constituents

AL-H and AR-H extracts, possessing significant AIA, were subject to column chromatography (Omnifit® 75 cm × 1.5 cm, Filter Service, Eupen, Belgium) on silica gel (LiChroprep® 15–25 μm, Merck Darmstadt, Germany) using a gradient of toluene–EtOAc (100:0, 95:5, 90:10, 85:15, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9; each 320 ml) followed by an EtOAc–MeOH gradient (1:0, 1:1, 0:1; each 300 ml). Compound **1** was isolated from both AL-H and AR-H, yielding respectively 45.3 mg (368–464 ml) and 35.2 mg (564–620 ml). AR-H fraction also gave **2** (6.7 mg; 1920–1968 ml) and **4** (46.1 mg; 160–220 ml). **5** and **6**, as well as compound **3**, were isolated from AL-M fraction as previously described (Mavar-Manga et al., 2006).

2.2.4. General experimental procedure

MS analyses were achieved using ESI or APCI sources in positive mode by direct injection into LCQ mass spectrometer (Finnigan, San Jose, CA). 1D and 2D NMR spectra were recorded in $CDCl_3$ at 400 MHz (^1H) and 100 MHz (^{13}C) on a Bruker DRX-400 spectrometer.

LC–MS analysis was performed on a MAT-LCQ® Advantage ion-trap mass spectrometer equipped with an APCI source (Thermo-Finnigan, San Jose, CA). LC separations were performed on a LiChroCart RP18 column (250 mm × 4 mm i.d., particle size: 5 μm) (Merck Darmstadt, Germany), using a gradient elution program: mobile phase A was ammonium acetate 0.01 M (adjusted at pH 6 with AcOH) and mobile phase B was 100% acetonitrile. The gradient program was 60% A and 40% B linearly increasing to 90% A and 10% B over 16 min, held for 14 min and then returned to initial conditions (60% A and 40% B) over 2 min. The injection volume was 20 μl of a 12 mg/ml solution in methanol (HPLC grade) and the mobile phase flow rate was 0.2 ml/min. The chromatogram was monitored at 275 nm. Mass spectra were acquired in the positive ion mode. The following APCI inlet conditions were applied: heated vaporization temperature: 470 °C, heated capillary temperature: 200 °C, sheath gas flow: 70 psi, auxiliary gas: 20 psi and discharge current: 5 μA. Collision induced dissociation (CID) spectra were recorded at relative collision energy of 33%.

2.3. Anti-inflammatory activity (AIA)

The experimental design was approved by the ethical committee for animal experimentation of Faculty of Medicine (Université catholique de Louvain) bearing the number 2003/03/FMD/UCL/013 and has the agreement of the Belgian Ministry of Public Health under the number LA1230522.

28–33 g male albino Swiss mice (Cd-1) were used (Charles River company, Iffa-Credo, Belgium). Animal quarters were maintained at 22 °C and 60% humidity with 12 h light–dark cycle. Cutaneous inflammation was induced as described previously (Tubaro et al., 1985; Mavar-Manga et al., 2004).

At least two experimental groups of five animals were tested at a unique dose level of 90 μg/cm^2 for fractions, compounds, and the reference drug indomethacin, which gave about 50% of inhibition at this concentration (Mavar-Manga et al., 2004). Solutions and suspensions were made in absolute ethanol and/or acetone.

Because of the small variation of the indomethacin inhibition value between different sets of tests, the anti-inflammatory activity was also expressed using the effect of indomethacin as 100% (Table 1) to allow comparisons more easily, since this reference compound was used as a positive control in all sets of tests.

Croton oil and indomethacin were Sigma–Aldrich products (Steinheim, Germany); ketamine hydrochloride was Ketalar® (Pfizer).

Table 1
Anti-inflammatory activity of extracts and compounds isolated from *A. cordifolia* leaves and root barks (at 90 μg/cm^2)

Extracts/compounds	N[a]	Oedema (mg) mean ± S.E.	Inhibition (%)	Inhibition vs. indomethacin (%)
Control	32	7.9 ± 0.3	–	–
F2	16	2.9 ± 0.7*	63*	162
AL-H	8	4.0 ± 0.4*	49*	126
AR-H	15	5.2 ± 0.3*	34*	88
1	8	5.7 ± 0.4*	28*	71
2	8	3.9 ± 0.3*	50*	109
3	16	5.0 ± 0.4*	36*	92
4	8	3.6 ± 0.9*	54*	138
5	8	4.2 ± 0.5*	46*	119
6	8	3.7 ± 0.8*	53*	135
Indomethacin	31	4.8 ± 0.2*	39*	100

[a] Number of animals.
* Significant (student's *t*-test $P \leq 0.05$).

The pharmacological data were analysed by Student's *t*-test and significance was assumed for *P*-values lower than 0.05.

3. Results and discussion

As the hexane fraction of the methanol Soxhlet extract of leaves of *A. cordifolia* (F2) was previously shown to possess a very high AIA (Mavar-Manga et al., 2004), we decided to identify its major components. Unfortunately, the yield of this extract was very low, so it was decided to isolate them from other *A. cordifolia* extracts from leaves and root bark, which contained the same compounds as could be seen from their chromatographic profiles. The alkaloids recently isolated from the root barks were also tested since, in our previous work, the alkaloid rich fraction also gave interesting anti-inflammatory activity (Mavar-Manga et al., 2004, 2006) (Fig. 1).

From the results (Table 1), it can be observed that the topical application of the F2 fraction from a new batch showed less activity than that observed for the F2 fraction from the previous work i.e. 42% inhibition at 0.7 μg/cm^2 (Mavar-Manga et al., 2004). However, the AIA was still greater than that of indomethacin, as was the hexane fraction from the leaves (AL-H) (162% and 126% inhibition respectively compared to indomethacin considered as 100%) while the hexane fraction from the root barks (AR-H) was less effective (88% inhibition compared to indomethacin 100%).

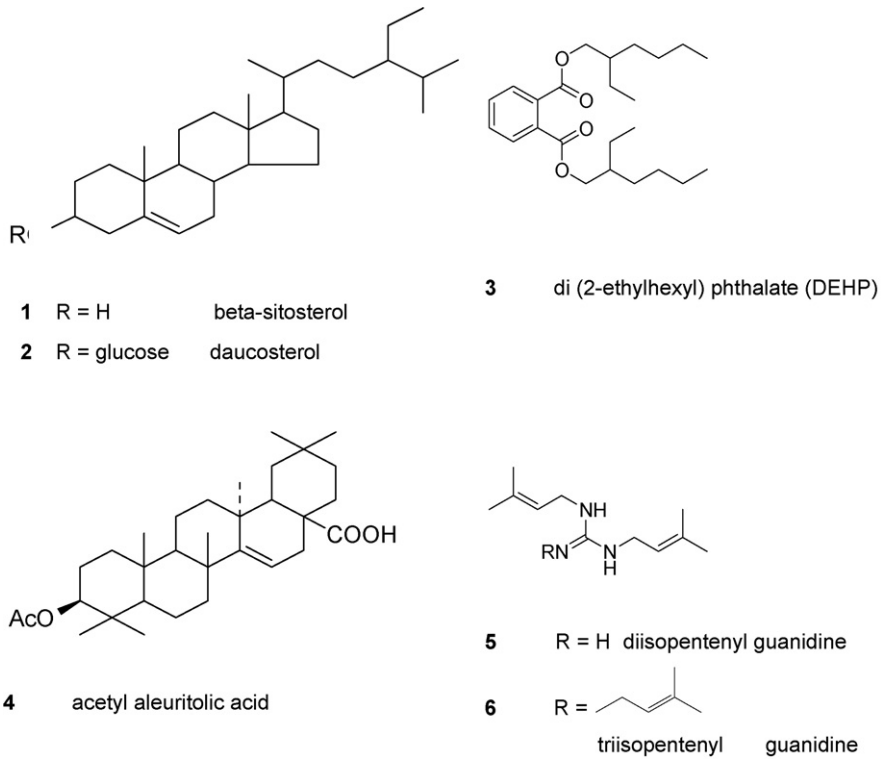

Fig. 1. Structures of isolated compounds.

We isolated and/or identified from these three extracts β-sitosterol (**1**), daucosterol (**2**) and DEHP (di-(2-ethylhexyl) phthalate) **3**, also known as di-octyl phthalate.

DEHP (**3**) is a well known synthetic plasticizer, already reported to be present in latex and nectar secretion of two Euphorbia species *E. cyparissias* L. and *E. seguieriana* Necker (Toth-Soma et al., 1993) and may have a taxonomic significance. The effective presence of **3** in leaves, not as a contaminant from solvents, was further confirmed by different experiments. Firstly DEHP was isolated from the hexane extracts from the leaves (AL-H) and not from the root bark extract (AR-H) treated with the same batch of solvent; secondly, an HPLC–MS analysis (Tr DEHP: 23.9 min) of the crude hexane fraction of a methanol extract of the leaves compared to a blank prepared with the same procedure and solvent batch showed that *A. cordifolia* extract contained DEHP which was not detected in the blank. The plant leaves were not conserved in plastic bags, so these could be discounted as a source of DEHP. The present study could not determine if DEHP is synthesised by the plant, absorbed by the roots or adsorbed from external atmosphere by the sticky medium of the plant's nectaries, but this compound, whatever its origin, appears likely to be present in preparations made by the local population.

DEHP showed an AIA similar to indomethacin (92% of indomethacin activity) in the test reported here, although it is considered as pro inflammatory in other studies (Oie et al., 1997; Gourlay et al., 2003). Nevertheless, the Scientific Committee on Medicinal Products and Medical Device (SCMPMD) of European Union concluded that there is no clear indication or evidence that DEHP is a sensitizer in animals or humans nor causes respiratory symptoms related to sensitisation. The Scientific Committee emphasised that DEHP has a low toxic potential by oral ($LD_{50} > 25$ g/kg on rats and mice) or dermal routes (SCMPMD, 2002). It can also be noted that this compound is more effective than β-sitosterol (**1**), whose anti-inflammatory properties, and of its glucoside are widely reported in literature (Navarro et al., 2001; Aguilar-Guadarrama and Rios, 2004). **1** was reported to be the anti-inflammatory principle of *Opuntia ficus – indica* Mill. (cactus) (Park et al., 2001) and *Culcasia scandens* P. Beauv. (Okoli and Akah, 2004). A mixture of **1** and **2** was also reported to be patented for an immunomodulating activity (Bouic et al., 1997; Bouic, 2001). We also noted that the β-sitosterol glucoside (**2**) was more active than its aglycone (28% versus 50% of inhibition respectively) in our test (Table 1).

The presence of these three active compounds cannot fully explain the activity of F2, since they were all less active than the crude fraction, unless synergy is considered as a possibility.

The column fractionation of AR-H also afforded acetyl aleuritolic acid (**4**) which was shown to possess the highest AIA (Table 1). This compound has been already reported to be effective at 50 mg/kg on rat paw oedema induced by carrageenin, dextrin and histamine, after intraperitoneal injection, while it also increased histamine-induced vascular permeability (Maciel et al., 2000). This triterpene **4** has been found in many Euphorbiaceae roots and barks e.g. *Aparisthmium cordatum* (Juss.) Baill. (Souza et al., 2004) an American species which is often confused with *A. cordifolia* and its anti-inflammatory activity is also reported in other studies (Santos and Mukherjee, 1992; Peres et al., 1998).

All the isolated compounds were identified by comparison of their MS and NMR spectra compared with data from the literature (McLean et al., 1987; Akihisa et al., 1991; Pouchert and Behnke, 1993; Faizi et al., 2001). Copies of the original spectra are obtainable from the author for correspondence.

As the alkaloid rich fraction was also previously shown to have AIA (Mavar-Manga et al., 2004), we also tested the two alkaloids, diisopentenyl guanidine (**5**) and triisopentenyl guanidine (**6**), recently isolated from the plant (Mavar-Manga et al., 2006). They exhibited a higher AIA than indomethacin, with the better activity for **6** (135% and 119% respectively of the activity of indomethacin) (Table 1). **6**, in contrast to **5**, was cytotoxic on several cell lines (Mavar-Manga et al., 2006), so the possibility that the toxicity is related to the anti-inflammatory effect is reduced because **5** shows little cytotoxcity and may be a promising lead compound.

This paper reports for the first time the presence of β-sitosterol (**1**), daucosterol (**2**), di(2-ethylhexyl) phthalate (**3**) in *A. cordifolia* leaves and their topical AIA. From the root bark extract, β-sitosterol (**1**), daucosterol (**2**), acetyl aleuritolic acid (**4**), and two guanidine alkaloids (**5** and **6**) were isolated. It is also the first report on the AIA of **3** and of the two guanidine alkaloids, **5** and **6**. All these compounds could not totally account for the topical AIA of *A. cordifolia* so the possibility of synergistic action, as reported for **1** and **2**, cannot be ruled out (Bouic et al., 1997). Furthermore, in crude extracts used in traditional medicine, these compounds might also act additionally or synergistically with the polyphenols (tannins and flavonoids) which are also known to be present (Mavar-Manga et al., 2004). This points out the interest of using crude extracts which contain several types of molecules acting with different mechanisms. Nevertheless, our studies show that *A. cordifolia* leaves and root barks contain many anti-inflammatory compounds which may account, at least in part for their application in folk medicine.

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