Antiinflammatory and Analgesic Effects in Rodent Models of Ethanol Extract of *Clausena anisata* Roots and their Chemical Constituents

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The *in vivo* antiinflammatory and analgesic activities of the crude ethanol extract and chemical constituents of *Clausena anisata* roots were investigated. The crude extract, which was devoid of any visible acute toxicity, displayed significant antiinflammatory effect at the dose of 1000 mg/kg (p.o.) when assessed using the carrageenan-induced oedema model. In the acetic acid-induced writhing and hot plate tests, it produced a very significant (p < 0.001), dose-dependent analgesic effect, with maximum analgesic activity of 72.1% at 1000 mg/kg (p.o.). Phytochemical analysis of the crude extract resulted in the isolation of four coumarins (anisocoumarin B, osthole, imperatorin and xanthotoxol) and a carbazole alkaloid, heptaphylline. Among the isolated compounds, osthole and anisocoumarin B produced the highest antiinflammatory activity at 9 mg/kg (p.o.): slightly better than the positive control, indomethacin. Except for xanthotoxol, all the isolated compounds administered at 6 mg/kg (p.o.) produced significant analgesic activity and higher than diclofenac; with heptaphylline being the most potent (48.7%). The analgesic activity of anisocoumarin B (50.4%) was the highest among the isolates tested and the standard, tramadol, in the hot plate test. The nonselective opioid receptor antagonist, naloxone, abolished the analgesic effect of the crude extract and the tested isolates (anisocoumarin B and xanthotoxol) in the hot plate test suggesting an effect via the central opioidergic system. These findings provide the scientific basis for the use of *C. anisata* roots in traditional medicine as antiinflammatory and analgesic agents.

**Keywords:** *Clausena anisata* roots, Antiinflammatory, Analgesic, Coumarins, Carbazole alkaloid, Antinociception, Opioid antagonist.

While self-limiting acute inflammatory response is critical for maintaining homeostasis and defense against a range of pathogens, unregulated inflammation is a feature of many disease conditions from inflammatory pain and allergies to cancer, cardiovascular diseases, and autoimmune disorders. To date, a number of drugs belonging both to the small molecular weight and protein-based modulators are available to combat inflammation and the associated diseases. Due to various limitations (e.g. poor efficacy and unwanted side effects), especially for chronic diseases such as arthritis and inflammatory bowel diseases, the world-wide effort on the search for safe and effective drugs continues in many academic and pharmaceutical industry laboratories [1a,b]. In addition to suppression of inflammatory responses through action like the steroidal or non-steroidal mechanisms, drug candidates targeting inflammatory pain through the opiate mechanism of action, like that of tramadol, are highly valuable.

*Clausena anisata* (Wild.) Hook f. ex. Benth (Rutaceae) is an evergreen shrub or tree that grows up to 10 m high. The plant is widely distributed in higher-rainfall regions of tropical West Africa, as well as Asia and Australia [2a]. The plant is characterised by its distinctive pinnate compound leaves, and by flowers which are highly scented [2a]. In West Africa, the leaves, stems and roots are used to treat countless diseases such as oral candidiasis, fungal infections, epilepsy, convulsion, toothache, gut disturbances, malaria and high blood pressure [2b-f]. Furthermore, the roots serve as major ingredients in a decoction known as ‘Asena’ in Ghana, which is commonly used to treat arthritis, general bodily pains and other inflammatory conditions at the WHO collaborative Centre for Plant Medicine Research (CPMR) at Mampong-Akwapim. Antiinflammatory, antipyretic, anti-plasmodial and analgesic activities of its leaves extracts and hypoglycemic effect of the roots so far have not been investigated. This work was therefore designed to investigate these pharmacological properties and isolate and characterize the active principles of *C. anisata* roots (1-5).
The acute toxicity study of the crude ethanolic extract of *C. anisata* roots over a period of 12 days was conducted at exceedingly high doses of either 2500 or 5000 mg/kg p.o., which are 2.5 and 5-times higher than the maximum therapeutic concentrations used in the study respectively. All extract-treated animals neither showed any sign of toxicity nor died during the observation period.

The carrageenan-induced edema in rat’s paw that serves as the gold standard in vivo model of antiinflammatory evaluations was employed. The dose regime and protocols were based on our preliminary experiments and previous reports where we have employed the method for the identification of several novel anti-inflammatory agents from medicinal plants [4a-c]. As shown in Figure 1, the maximum carrageenan-induced oedema was attained within 3 h of injection and was sustained during the 5 h observation period. As expected, and in line with our previous report [4c], the positive control indomethacin displayed a time- and dose-dependent antiinflammatory effect within the dose range of 9 and 30 mg/kg (p.o.) (Figure 1).

Even though the analgesic activities of *C. anisata* leaves were demonstrated previously [3a], the roots have not yet been studied for such activities. The acetic acid-induced writhing response in animals is one of the commonest methods of analgesic activity evaluation for experimental agents. As expected, the standard positive control, diclofenac, caused a dose-dependent analgesic effect, as evidenced by the reduction in the righting response (Figure 2). Similarly, the crude extract displayed dose-dependent analgesic effects, with all the tested concentrations (10, 100 and 1000 mg/kg, p.o.) inducing significant activities. Hence, the analgesic activity of the crude extract appears to be more pronounced than the antiinflammatory activity as only the 1000 mg/kg dose produced a suppressive effect against the carrageenan-induced oedema (Figure 1).

The acetic acid-induced writhing test is a visceral pain model [6a] used in evaluating both central and peripheral analgesic activities of substances [6b]. Intraperitoneal injection of acetic acid into the abdominal cavity of rats or mice results in elevated levels of prostaglandins in the peritoneal exudates after about 30 min [6c]. The stretching and abdominal contractions obtained in the acetic acid-induced writhing test therefore correlates with sensitization of nociceptors to the production of prostaglandins [6b]. Our data on the analgesic effect of the crude extract of *C. anisata* roots are therefore consistent with those obtained in the carrageenan-induced oedema assay, where possible inhibition of prostaglandins production and/or action could serve as the main mechanism of action.

The analgesic activity of the crude extract was further assayed using the hot plate method where the standard tramadol was employed as a positive control. In untreated animals, there was a tendency for the percent mean PTI to reduce over time as the animals appear to be more sensitive to the hot plate stimuli. This sensation was however suppressed in a time-dependent manner when animals were treated by the positive control, tramadol (Figure 3a). The overall analgesic effect, as evidenced from the percent OPTI of over 20% (Figure 3b), was recorded for the two doses of tramadol. As shown in Figure 3c, the crude extract also showed a dose-dependent analgesic effect that appears to be gradually reversed over a period of 5 h. Furthermore, the percent OPTI response showed a remarkable dose-dependent analgesic effect that ranged from about 25 to just over...
75% (far superior to that of the positive control) for the concentration range tested (Figure 3d). It is worth noting that the hot plate test, as a thermally-induced nociception model, is routinely employed to evaluate the central analgesic effect of pharmacological agents [66]. The dose-dependent antinociception of the crude extract in the hot plate test, similar to that of a known analgesic with an opioidergic mechanism of action, tramadol (p < 0.001), may suggest a central mechanism of action.

In order to establish the possible central mechanism of the crude extract in the observed analgesic/antinociceptive effect, the hot plate assay was further reassessed with naloxone co-administration. As shown in Figure 3 (e,f), the activity of the crude extract was completely abolished by the non-selective opioid antagonist, naloxone. This indicates that the analgesic effect of the crude extract was likely to be mediated via the central opioidergic system.

Having the antiinflammatory and analgesic activity of the crude extract of C. anisata roots established, it was worthwhile to investigate the possible active principles of the roots. Preliminary phytochemical screening of the crude extract using the standard procedure [7a] revealed the presence of coumarins, alkaloids, terpenoids and free reducing sugars. When the crude extract was suspended in aqueous medium and re-extracted with light petroleum, the presence of coumarins, alkaloids and terpenoids was still evident. Hence, the light petroleum fraction was taken for further purification through repeated column chromatography over silica gel and recrystallization to yield five compounds (1-5). The detection and identification of all of the isolated compounds in the various parts of C. anisata have been described previously [3c-g, 7b-c]. In the present study, comprehensive spectroscopic analysis including 2D NMR studies (COSY, HMQC, HMBC and NOESY) and accurate mass analyses were employed for unambiguous chemical shift assignments and structural analysis.

As with the crude extract, the isolated compounds were tested for their in vivo antiinflammatory activities using the same carrageenan-induced oedema model. The dose-dependent effect of these compounds in an overall total inflammatory effect analysis during the entire observation period is shown in Figure 4. All compounds showed significant antiinflammatory activity at 9 mg/kg (p.o.) suggesting their potent antiinflammatory effects in vivo. Furthermore, all compounds, except for compound 2 (heptaphylline) and 5 (imperatorin) showed comparable activity with the positive control indomethacin when tested at the dose of 9 mg/kg (p > 0.05).

Osthole (4) is one the best characterised anti-inflammatory natural products with known effect as an inhibitor of the carrageenan-induced hind paw edema in rats [8a]; carrageenan-induced lung inflammation in rats [8b]; hepatic injury in a rodent model of trauma-hemorrhage [8c]; inhibition of inflammatory reaction following permanent middle cerebral artery occlusion in rats [8d]; and iNOS protein expression at 10 µM [8e], among others. Imperatorin (5) has been shown to suppress the degranulation and eicosanoid generation in activated bone marrow-derived mast cells [8f]; inhibit the release of pro-inflammatory cytokines production, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α in LPS-stimulated RAW 264.7 cells [8g-8h]; block the protein expression of iNOS and cyclooxygenase-2 (COX-2) in LPS-stimulated RAW264.7 macrophages [8i]; inhibit acute lung injury induced by lipopolysaccharide in mice [8j] and anti-inflammatory activity on TPA (12-O-tetradecanoylphorbol-13-acetate) mice ear model [8k]. While xanthotoxol (3) is known to exert neuroprotective effects via suppression of the inflammatory response in a rat model of focal cerebral ischemia [8l] its antiinflammatory effect is largely unknown. On the other hand, heptaphylline (2) has been known for some time to have an in vitro antiinflammatory effect such as suppression of inflammation mediators (iNOS, TNF-alpha, and COX-2) expression in the mouse macrophage RAW 264.7 cell line [8f], while anisocoumarin B (1)
has not previously been shown to display anti-inflammatory activity. The identification of compounds 1-5 from the roots of *C. anisata* is, therefore, in good agreement with the observed *in vivo* anti-inflammatory effect of the crude extract and the proposed mechanism of action, including the prostaglandins system.

Since the yield of the isolated compounds did not permit the undertaking of a comprehensive dose range analysis, they, along with the positive control, were screened for analgesic activity at one fixed dose of 6 mg/kg (p.o.). As shown in Table 1, only compound 1 (xanthotoxol) failed to show analgesic activity at the tested dose. The observed analgesic activity of the active compounds, which was comparable in many cases with the standard diclofenac, was in good agreement with the activity profile of our data for the crude extract in the same assay. It is, therefore, reasonable to conclude that the isolated active compounds as active principles could contribute to the analgesic activity of the acclaimed *C. anisata* roots in the traditional medicine.

### Table 1: Analgesic activity of the isolated compounds and diclofenac on acetic acid - induced writhing pain in mice. *

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% Righting Response Vs Control</th>
<th>% Analgesic Activity</th>
</tr>
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<tbody>
<tr>
<td>1 (Anisocoumarin B)</td>
<td>61.4 ± 16.0</td>
<td>38.6</td>
</tr>
<tr>
<td>2 (Heptaphylline)</td>
<td>49.1 ± 12.6</td>
<td>50.9</td>
</tr>
<tr>
<td>3 (Xanthotoxol)</td>
<td>112.1 ± 3.3</td>
<td>No effect</td>
</tr>
<tr>
<td>4 (Osthole)</td>
<td>53.6 ± 6.7</td>
<td>46.4</td>
</tr>
<tr>
<td>5 (Imperatorin)</td>
<td>61.9 ± 12.7</td>
<td>38.1</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>53.9 ± 7.5</td>
<td>46.1</td>
</tr>
</tbody>
</table>

* Tested at 6 mg/kg (p.o.)

The analgesic activity of osthole (4) was reported before [8l], including in sciatica induced by the lumber disc herniation model [9a-b], while the rest of the isolated compounds appear to be reported here for the first time. Due to the small yields of the compounds, it was not possible to repeat all of the experiments in the hot plate assay and hence data were provided in Figure 4 only for anisocoumarin B (1) and xanthotoxol (3). While both compounds appear to show significant activity, the effect of anisocoumarin B (1) was higher than that of the positive control, tramadol. As with the analgesic effect of the crude extract, the antinociceptive effects of these compounds were suppressed by the non-selective opioid antagonist, naloxone, suggesting a similar central effect (Figure 4).

In conclusion, the present study established that the roots of *C. anisata* possess significant antiinflammatory and analgesic effects similar to NSAIDs and/or opioids and could be safely used to manage some inflammatory and painful disorders. This study also validates the use of *C. anisata* roots in traditional medicine as an anti-inflammatory and analgesic agent. Phytochemical analysis of the crude extract resulted in the isolation of prenyl derivative compounds: four coumarins (anisocoumarin B, osthole, imperatorin and xanthotoxol) and a carbazole alkaloid, heptaphylline. In a further validation of the traditional uses of the plant, the isolated compounds also possess potent antiinflammatory and analgesic activities.

### Experimental

#### Materials:
Carrageenan, naloxone hydrochloride dehydrate, and acetic acid were products of Sigma Chemical Co. (St. Louis USA). Indomethacin, diclofenac sodium, tramadol hydrochloride and Tween 80 were purchased from Sigma Chemical Company, Bliss GVS (India), Bristol Laboratories Ltd and VWR International, PROLABO (CE) respectively. Sodium chloride was from Timstar Laboratory Suppliers Ltd., Cheshire, England. All organic solvents and other reagents used for extraction, isolation and purification of the compounds were either analytical or HPLC grade.

#### Plant material:
The roots of *C. anisata* were harvested from the arboretum of CPMR at Ayikumah in the Eastern Region of Ghana in March, 2012. After authentication by Mr H. R. Blaggoge, a voucher specimen (CPM0312) was deposited at the herbarium of CPMR.

#### Extraction and isolation:
The crude extract (140 g) was suspended in 80% ethanol-water (2.4 L) and extracted 4-times with light petroleum (2.4 L). The combined fractions were dried in a rotary evaporator at 40°C to obtain 16.8 g of a dark brown syrup. A portion of this fraction (13.0 g) was subjected to column chromatography (CC) over normal phase silica gel (400 g) and elution made with light petroleum, light petroleum containing increasing amount of ethyl acetate, and finally a gradient of EtOAc containing ethanol. Aliquots of fractions from the column were collected and grouped by similarity in their TLC profiles to give 7 main fractions (P1-P7). Fraction P1 eluted with light petroleum/ethyl acetate (95:5) gave colorless crystals, which were filtered, washed (light petroleum/chloroform 6:1) and recrystallized from light petroleum/ethyl acetate (70:30) to give compound 1 (40.5 mg). Fraction P2 (light petroleum/ethyl acetate 80:20) gave a yellow ppt, which appeared to be a mixture when assessed by TLC (solvent, light petroleum/chloroform 2:3; anisaldehyde spray reagent). Further CC over 40 g of silica and elution with light petroleum/chloroform gave 3 sub-fractions. The sub-fraction eluted with light petroleum/chloroform (90:10) gave compound 2 (10 mg) as bright yellow crystals. Fraction P4 (solvent, eluent, light petroleum/ethyl acetate 60:40) was subjected to further fractionation with a stepwise gradient elution with light petroleum/chloroform/ethyl acetate. A total of 87 fractions of 30 mL aliquots was obtained and grouped into 4 sub-fractions. Compound 3 (35.0 mg) was obtained from the fraction eluted with light petroleum/chloroform/ethyl acetate (40:40:20) as brown solid particles. Fraction P5, eluted with light petroleum/ethyl acetate (60:40) gave a colorless ppt, which was subjected to repeated recrystallization from light petroleum/chloroform (4:1) to obtain compound 5 (20.5 mg). Fraction P6, eluted with light petroleum/ethyl acetate (30:70) yielded white crystals, which were recrystallized from light petroleum/ethyl acetate (5:1) to obtain compound 6 (30.0 mg).

#### Animals:
All animals used in the study were bred at the Animal House Unit of CPMR, Ghana. The animals were fed on palette feed purchased from Agricare Limited located in Kumasi, Ghana; and were allowed free access to sterile water and feed *ad libitum*. Animals were housed in aluminum cages under standard temperature and pressure. Animals were cared for and handled according to the guidelines and procedures of the Foundation for Biomedical Research on the use of animals in research (F.B.R., 1987).

#### Acute toxicity studies:
The acute toxicity of the crude extract was assessed in groups of 6 male Sprague-Dawley rats, Swiss albino mice and C57/BL6 mice. For each animal group, 2 doses of the crude extract (2500 and 5000 mg/kg p.o.) and a vehicle control (2% Tween 80 aqueous solutions) were administered at 10 mL/kg body weight per animal. They were observed for signs of toxicity such as pilo-erection, motor impairment, sedation, salivation, hyperexcitability and death within 24 h and for an extra 16 days.

#### Antiinflammatory activity assay:
Our carrageenan-induced paw oedema model of antiinflammatory study [5a] has been described.
previously [4a-c]. Briefly, Sprague–Dawley rats of either sex were divided into groups and received orally either the vehicle (2% Tween 80 in water) or various doses of test agents (extracts, isolated compounds or the reference drug, indomethacin). After 1 h of drug treatment, inflammation was induced by injection of 0.1 mL of 1%, w/v, carrageenan in 0.9% normal saline into the sub plantar area of the right hind paw of rats. Paw volumes were measured by volume displacement using a Plethysmometer (Ugo Basile 7140) before and at hourly intervals from 1-5 h after carrageenan injection. The mean hourly oedema for each group and the total mean oedema for the 5 h observation period were calculated as described previously [4b].

Analgesic activity assay: Acetic acid-induced writhing assay: The analgesic effect of the test agents was assessed using the acetic acid-induced writhing model as described previously [10a]. Swiss albino mice were divided into 7 groups (n = 5) and treated (p.o.) with various doses of the crude extract, isolated compounds and the standard, diclofenac sodium. Animals receiving 2% Tween 80 in water served as the untreated vehicle control group. Following drug or vehicle treatment (45 min), each mouse was injected with aqueous acetic acid (1%, v/v) at 1 mL/100 g (i.p) and isolated into separate plastic cages. The number of writhing movements and stomach contractions was counted for 20 min. The inhibition of writhing movements in the treated group(s) compared with the control group was taken as the percentage analgesic effect (% A.E.), which was calculated using the formula:

\[
\%\text{ Analgesic effect} = \left(\frac{\text{MRc} - \text{MRt}}{\text{MRc}}\right) \times 100
\]

Where: MRc = mean writhing count of the control; MRt = mean writhing count of extract or other drug treated group.

Analgesic activity assay: Hot plate assay: The mouse hot plate analgesic activity evaluations were performed as described previously [10b]. C57BL/6 mice of either sex were divided into 7 groups, each containing 6 animals. The mice were separately placed on an electric hot plate (Ugo Basile hot/cold plate 35 100) maintained at 55 ± 0.5°C and the time taken to lick, lift, shake or stamp any of the hind limbs or jump constitute latency time and was recorded. Baseline latencies (T0) were obtained as the mean of 2 determinations prior to any treatment. Only mice with baseline latency of 3.5–9 s were used. Test samples of either the crude extract, purified compounds or standard reference, tramadol (3, 9 and 15 mg/kg, Group 4-6) or vehicle control (2% Tween 80 in water control) were then administered orally. Latency of each mouse was measured at 1 h (T1) intervals for 5 h after each treatment. The analgesic activity was expressed as percentage of pain threshold inhibition (%PTI) [6b] calculated as:

\[
\%\text{PTI} = \left(\frac{T0 - T1}{T0}\right) \times 100
\]

Where T0 and T1 refer to treatment time and time zero respectively. Overall analgesic response (% OPTI) for each group was obtained as the sum of mean % PTI of all animals per group over the experimental period.

Mechanism of antinociceptive action: The effect of the nonselective opioid antagonist, naloxone, on the extract or purified compounds- induced analgesia was evaluated in the hot plate assay using 3 groups (n=6) of C57BL/6 mice. Drug administration was made as follows: CRE (at 10 mg/kg p.o.) was administered and 15 min later naloxone 2 mg/kg (i.p) (in distilled water) at 10 mL/100 g was given (Group 1). The procedure was repeated for CRE (10 mg/kg p.o.) alone (Group 2) and 2% Tween 80 aqueous solution as vehicle control (Group 3). The analgesic activity was then assessed as described above in the hot plate test. The same procedure was repeated for compounds 1 and 3 at 9 mg/kg p.o. each.

Statistical analysis: All statistical analyses were performed using Graph Pad Prism Version 6 with the level of significance set at 95 % confidence interval of difference. The overall mean oedema and overall analgesic response (%OPTI) were calculated as sum of the oedema volume or latency in each group.

Spectroscopic analysis: 1H NMR, 13C NMR and 2D-NMR (COSY, NOESY, HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument, as described previously [10c].

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