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J. Nat. Prod. Plant Resour., 2016, 6 (2):13-24
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ISSN : 2231 – 3184
CODEN (USA): JNPPB7

Anti-inflammatory and Antioxidant Activities of Canthinone Alkaloids from Anthostema aubryanum (Baill)

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ABSTRACT

The stem and root bark of Anthostema aubryanum (Baill, family, Euphorbiaceae) are extensively used in African ethnomedicine for treating and managing a number of disease conditions which include inflammation, pain, wounds, boil, dyspepsia and a variety of other disease conditions. Although the chemistry and pharmacology of many phytoconstituents isolated from the family are fairly established, the plant has not yet been investigated. In the present study, an alkaloidal extract of the root bark was demonstrated to display a time- and dose (30-300 mg/Kg p.o.)-dependent anti-inflammatory effect in rats (ED_{50}=13.84±0.011) compared to diclofenac (ED_{50}=1.994±0.002). Comprehensive chromatographic and spectroscopic analyses have led to the isolation and characterization of two major anti-inflammatory and antioxidant alkaloids as 5-methoxycanthin-6-one and canthin-6-one. The dose (10-100 mg/Kg p.o.)-dependent anti-inflammatory effects of the 5-methoxycanthin-6-one (ED_{50}=60.84±0.010) and canthin-6-one (ED_{50}=96.64±0.012) were either comparable or significant as the positive control, diclofenac. The radical scavenging activity of the alkaloidal extract (IC_{50}=23.12±0.010), 5-methoxycanthin-6-one (IC_{50}=27.62±0.090) and canthin-6-one (IC_{50}=33.60±0.011) was also significantly comparable to Vitamin E (IC_{50}=8.605±0.002) used as positive control. Canthinone alkaloids are well-known constituents of the Simaroubaceae and Rutaceae and display a wide range of biological activities. However, they are being reported as constituents of the Euphorbiaceae here for the first time.

Keywords: Anthostema aubryanum, Euphorbiaceae, free radicals, anti-inflammatory, antioxidant, 5-methoxycanthin-6-one, canthin-6-one

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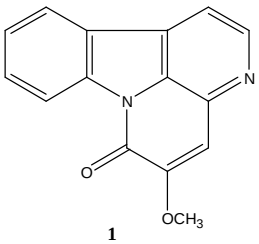
INTRODUCTION

Pain and inflammation are the major conditions associated with various diseases. Typical inflammatory diseases such as meningitis, rheumatoid arthritis, asthma, colitis and hepatitis are the leading cause of disability and death [1] and chronic inflammation has been implicated in the pathogenesis of cancer, cardiovascular, pulmonary and neurodegenerative diseases [2]. Inflammation activates neutrophils and macrophages to produce reactive oxygen species and reactive nitrogen species as well nitric oxide which deregulate cellular function causing tissue damage leading to chronic inflammatory diseases [3] and also inhibit wound healing.

Anthostema aubryanum (Baill, Euphorbiaceae- the Spurge family) is an evergreen monoecious shrub to medium-sized tree up to 30 m high with succulent white latex in all parts [4]. The genus is native to Africa and consists of only three species: *Anthostema aubryanum* (Baill), *Anthostema senegalense* (A. juss) and *Anthostema madagascariense* (Baill) [5]. The genus is closely related to *Dichostema* and *Anthostema* was first described as a genus in 1824 [5]. Geographically it is distributed in the tropical regions of Western Africa ranging from Guinea-Bissau to the Congo basin in the DR Congo in Central Africa and Madagascar. The plant is known in native countries by its local names such as 'Assogo" among the people of the Congo basin in DR Congo and "Sese" among the Akans in Ghana and Anyi people in Cote D'Ivoire and is used extensively in Ethnomedicinal and ethnoveterinary practice. The stem bark and roots of *A. aubryanum* are widely employed in treating edema, boils, pain, malaria, wounds (especially post abortion or after delivery) and pregnancy related problems [6]. In Democratic Republic of Congo, it is used to treat infections of the gastrointestinal tract, veneral diseases, constipation, dyspepsia, diarrhoea and dysentery [7,8]. In Senegal, a bark maceration is drunk to treat and manage intestinal infection, kidney problems, edema, impotence and as a laxative [9]. The bark is also used as a fish poison to catch small fish in Senegal. Its other species, *Anthostema senegalense* is used to treat leprosy, menstrual problems and helps with the expulsion of the afterbirth [10]. The latex is toxic, acrid and vesicant and can cause blindness. The latex is used as a drastic purgative and is applied externally to sores. The latex is used in traditional medicine as glue and the smoke from the wood is reportedly used to drive away animals. Like many woody trees, *A. aubryanum* is commonly used in many homes for fencing, firewood and construction. Although the family is generally known to predominantly contain terpenoids, the chemistry and pharmacology of the root bark of *A. aubryanum* are yet to be investigated. In the present study, an investigation into the anti-inflammatory and antioxidant activities of the root bark along with phytochemical analysis has resulted in the identification of two alkaloids as 5-methoxycanthin-6-one **(1)** and canthin-6-one **(2)** with significant pharmacological activity.



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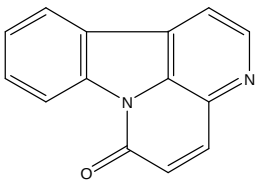

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MATERIALS AND METHODS

Chemicals

All organic solvents used for the experiments were of analytical grade and obtained from BDH Laboratory Supplies (Merck Ltd, Lutterworth, UK). The standard reference drug, diclofenac was purchased from Troge (Hamburg, Germany). The vitamin E, anhydrous sodium carbonate 99%, Diphenyl-2-picrylhydrazyl (DPPH) and all other chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

General experimental procedures

^{1}H and ^{13}C NMR were obtained on a JEOL 500 MHz instrument. Chemical shifts were reported in δ (ppm) using the solvent (CDCl3 or methanol-D4), standard and coupling constants (J) were measured in Hz. The high resolution (Q-ToF) mass spectroscopy instrument, SYNAPTG2-Si#UGA333 (Thermo Fisher Scientific, UK), with an electrospray ionization probe was used for accurate mass measurement over the full mass range of m/z 50-2000. Nanospray analyses were performed in positive ionization mode. Column chromatography was performed with aluminum oxide neutral gel (grade II, 70-230 mesh) and TLC with Merck precoated silica gel F_{254}. Compounds were visualized by UV light and by spraying with Dragendorff's reagent, Mayer's reagent and 3% Ce $(NH_4)_2SO_4$ in 85% H_3PO_4. Melting points were determined using electrochemical melting point-9100 apparatus.

Plant material collection and identification

The root bark of *Anthostema aubryanum* (Euphorbiaceae) was harvested from Adukrom in the Nzema East Metropolis in the Western region of Ghana, in December, 2014 and was identified by curators of the University of Cape Coast Herbarium (Ghana). A voucher specimen with reference number (HBS/Antho/2014/2895R) has been deposited at the Herbarium of the Department of Botany, School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, Ghana.

Isolation of the anti-inflammatory compounds

The dried and powdered root bark of *Anthostema aubryanum* (1.20 Kg) was alkalinized and extracted by Soxhlet in 70% MeOH (2x2.5 L) for 48 h. The combined extracts were concentrated under reduced pressure to afford a brownish crude extract (32.20 g). The crude extract was dissolved in 5% acetic acid, refrigerated for 24 h and filtered. The clear acidic solution was extracted with Hexane (3x200 mL). The Hexane layer was discarded and the aqueous phase basified with 10% $NH_{3\ (aq)}$ (25%), extracted with CH_2Cl_2 (3x150 mL). The organic layer was dried using $MgSO_4$ and evaporated under reduced pressure to dryness, light brownish crude (0.680 g, yield= 0.1%) were obtained. The screening of this extract using Dragendorff's reagent, Mayer's reagent and 3% Ce $(NH_4)_2SO_4$ in 85% H_3PO revealed the presence of alkaloids. The alkaloidal fraction was subjected to aluminum oxide gel (70-230 mesh) column chromatography and eluted with a gradient of CH_2Cl_2-EtOAc and EtOAc-MeOH to give three fractions: 1(CH_2Cl_2-EtOAc, 2:1, 220 mg), 11 (CH_2Cl_2-EtOAC, 1:1, 170 mg) and 111 (EtOAc-MeOH, 2:1, 210 mg). Fraction 1 was purified by preparative TLC on aluminum oxide gel 60 F_{254} (0.25 mm thickness) and crystallized in acetone to give compound 1 as light yellow needles (160 mg, R_f 0.70 in toluene-EtOAc 3:1). The fractions 11 and 111 were combined based on their TLC analyses and subjected to repeated smaller column chromatography as above, eluting with CH_2Cl_2-EtOAc mixtures of 2:1 and 1:1. The fractions eluted with CH_2Cl_2-EtOAc 1:1 were combined, concentrated under reduced pressure, purified by preparative TLC and crystallized in acetone to give compound 2 as light yellow needles (170 mg, R_f 0.40 in toluene-EtOAc 3:1). All the compounds were soluble in chloroform.

5-methoxy-canthin-6-one (1): Light yellow needles with a bright yellow-green fluorescence at 360 nm; m.p 223-234^{0}C; V_{max}/cm^{-1}1670, 1635 (C=O, conjugated), 1610, 1575 (arom).

Elemental analysis: Found: C, 72.03; H, 3.92; N, 11.08. $C_{15}H_{10}N_2O_2$ requires C. 71.99; H, 4.03, N, 11.19 %; δ_H (500MHz, MeOD, J/Hz) 7.57 (1H,d, H-1, J=5.0), 8.68 (1H,d,H-2, J=5.0), 7.80 (1H,d, H-4, J=10), 8.20 (1H,d, H-8, J=7.7), 7.7 (1H,t, H-9, J=7.7), 7.5 (1H,t, H-10, J=7.7), 8.00 (1H,d, H-11, J=7.7), 4.06 (s, 3H). HR-MS (m/z) 251.0898 [M+H] - (calc. for $C_{15}H_{10}N_2O_2$).

Canthin-6-one (2): Light yellow needles with a light blue fluorescence at 360 nm; m.p 156-157^{0}C, (Lit. 155-156); V_{max}/Cm^{-1}1670, 1630 (C=O, conjugated); 1600 (arom).
Elemental analysis: Found: C, 76.32; H, 3.63; N 12.78. $C_{14}H_8N_2O$ requires C, 76.35; H, 3.66; N, 12.72%; δ_H (500MHz, MeOD, J/Hz) 8.0 (1H,d, H-1,J=5.0Hz), 8.7 (1H,d, H-2, J=5.0), 8.1 (1H, H-4, J=10.0), 8.4 (1H,d, H-8, J=10.0), 7.7 (1H,t, H-9, J=8.5), 7.5 (1H,t, H-10, J=8.5), 8.2 (1H,d, H-11,J=8.5). HR-MS (m/z) 221.0755 [M+H]- (calc. for $C_{14}H_8N_2O$).

In vitro anti-inflammatory activity

Experimental animals

Sprague Dawley rats aged six weeks and weighing 100-150 g were obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana, and were housed in standard stainless steel cages (30 × 47 × 20 cm) at a population density of 5 rats per cage. Food (Cheletin diet, from GAFCO Tema, Ghana) and water were available *ad libitum* through 1-quart gravity-fed feeders and water trough at 29^{0}C with a 12 h light: dark cycle at the animal house of Department of Biomedical and Forensic Science, UCC throughout the experimental period. The animals were then randomly divided into 5 groups (n= 5).

Determination of anti-inflammatory activity

The carrageenan foot edema model in rats was used to evaluate the anti-inflammatory properties of the test samples [11]. Six weeks-old rats (weighing between 100 and 150 g) were put into groups of five animals. After carrageenan (2% w/v) was injected intraplantar into right footpads, the initial foot volume (time zero) were taken by water displacement plethysmography using an electronic Von Frey plethysmometer (Model 2888, HTC Life Science Inc., Ca 91367, Canada) as described by Feridoni et al [12]. The foot volumes were then measured every hour for a total period of 5 or 6 h. For the anti-inflammatory activity measurements, alkaloid fraction (30, 100 and 300 mg/Kg) and diclofenac (10, 30, 100 mg/Kg) were administered orally 1 h prior to carrageenan injection. Animals receiving 2 mL/kg normal saline served as control. The foot volumes were individually normalized as percentage of change from their values at time zero and then averaged for each treatment group. The total inflammation during the entire observation period for each treatment was also calculated in arbitrary unit as the area under the curve (AUC) and compared with the untreated group [13]. All experimental protocols were in compliance with the National Institute of Health guidelines for the care

and use of laboratory animals and were approved by the Department of Biomedical and Forensic Science, College of Agriculture and Natural Sciences, University of Cape Coast, Ghana, Ethics Committee

In vitro antioxidant assay

2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

For the DPPH assay, the antioxidant activities of the alkaloidal extract and the isolated compounds were assessed in terms of the hydrogen donating or radical scavenging abilities of the extract and the compounds using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method of Chandrasekar et al [14]. Aliquots of the extract (0.25-2.0 mg/ml) and vitamin E (standard) (0.04-1.28 mg/ml) were mixed with 100 mM Tris-HCl buffer (800 µL, pH= 7.4). Then 1 ml of freshly prepared 500 µM DPPH in methanol was added to the mixture. The mixture was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm with a spectrophotometer, (Cecil CE 7200 spectrophotometer, Cecil instrument Ltd, Milton Technical Centre, England) for triplicate measurements. All samples were analyzed in triplicate. Pure methanol was used as a blank. The actual decrease in absorption induced by the test sample was compared with the positive control, vitamin E. The amount of remaining DPPH against the sample concentration was plotted to obtain the amount of antioxidant (µg) necessary to decrease free radicals by 50% (IC_{50}). A smaller IC_{50} value corresponds to a higher antioxidant activity [15].

DPPH scavenging effect (%) = (Absorbance of the control-absorbance of the test sample)/Absorbance of the control) x 100

Statistical Analysis

Doses and concentrations responsible for 50 % of the maximal effect (EC_{50} and IC_{50}) for the test samples were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y= \frac{a+(b-a)}{\left(1+10^{(LogEC50-X)}\right)}$$

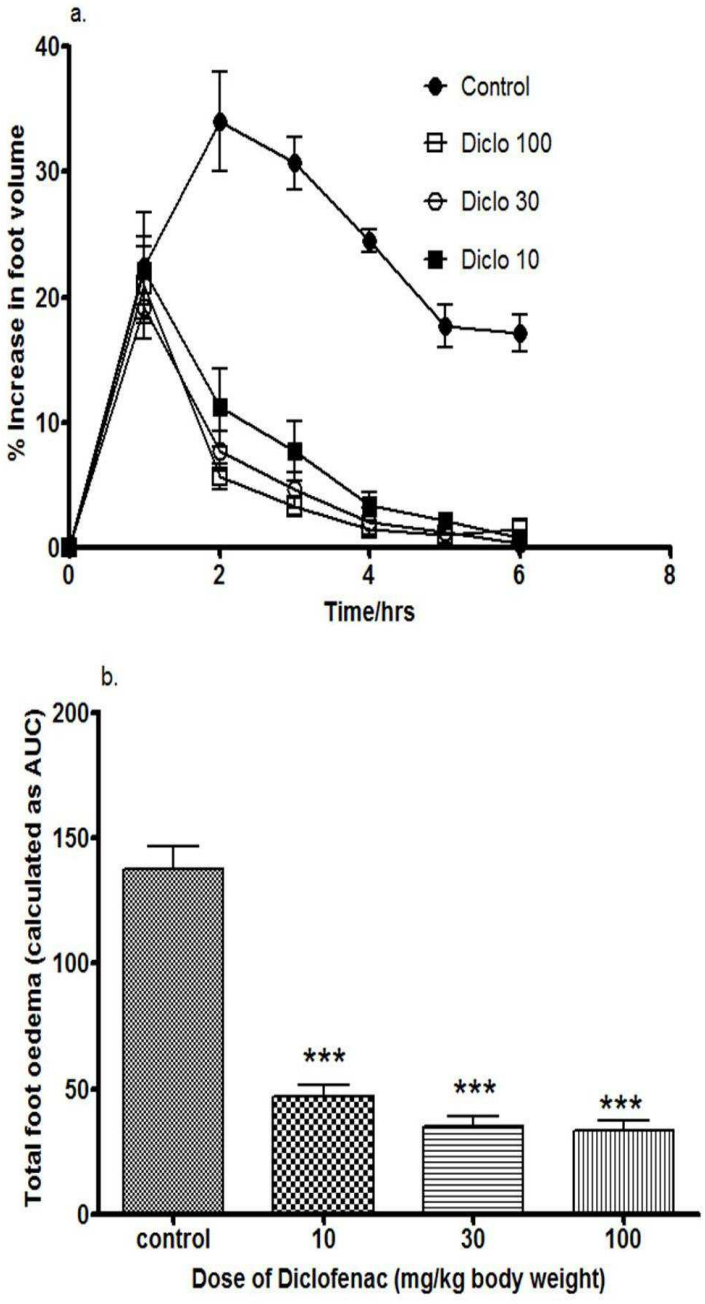
Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape. The fitted midpoints (ED_{50}/IC_{50} values) of the curves were compared statistically using *F* test. Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Anti-inflammatory activity

The stem and root bark of *Anthostema aubryanum* are routinely employed in traditional medicine to treat a variety of disease conditions including inflammatory pain, wounds, boil and edema. Many compounds with numerous pharmacological activities have been isolated from Euphorbiaceae but little is known about the pharmacology of the root bark of *Anthostema aubryanum*. The carrageenan-induced edema model in rodents is based on the principle of release of various inflammatory mediators by carrageenan and is the most accepted *in vitro* experimental model for anti-rheumatic activities in laboratory animals [16].

In our experimental conditions, we first used a positive control diclofenac which showed a time-dependent anti-inflammatory effect at all hours (Fig. 1). The AUC calculation showed that the three tested doses (10, 30 and 100 mg/Kg BDW) of diclofenac suppressed the carrageenan-induced edema under the experimental condition by 36.16 ± 2.4, 48.94 ± 2.2 and 59.20 ± 2.6 respectively. From Fig.1, it can be seen that oral administration of the alkaloidal extract of the root bark of *A. aubryanum* similarly suppressed the carrageenan-induced inflammation in a dose-and time-dependent manner.


a.
% Increase in foot volume
Time/hrs
Control
Diclo 100
Diclo 30
Diclo 10
b.
Total foot oedema (calculated as AUC)
***
***
***
control
10
30
100
Dose of Diclofenac (mg/kg body weight)

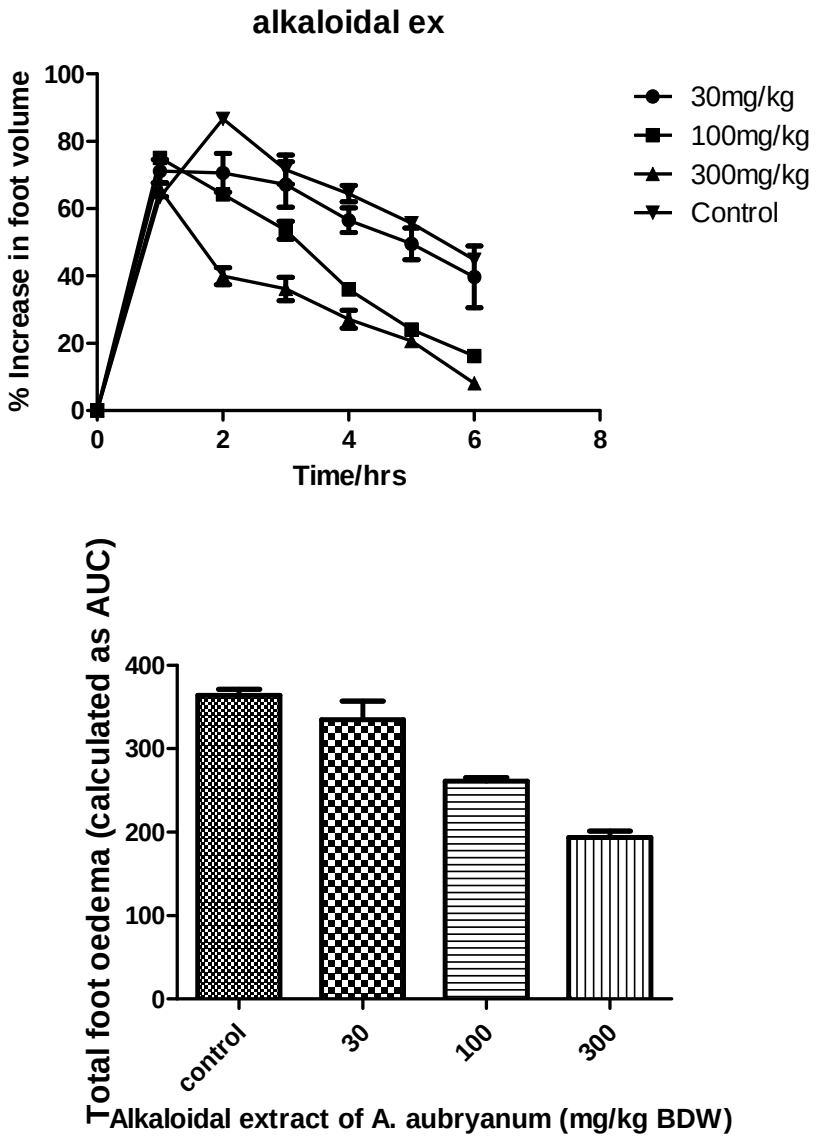


Figure1: Time-course edema development following carrageenan injection into rat paws and dose (mg/Kg)-dependent anti-inflammatory effect of the standard positive control diclofenac (A) and the alkaloidal extract of A. aubryanum (B). All data from 2 to 6 h of the treated groups are significantly different ($p < 0.05$) from the negative control group at each time point

The anti-oedematogenic activity was quantified using the ED_{50}. This is the dose required to reduce the inflammation by 50%. The crude and alkaloidal extracts of *A. aubryanum* showed significant activity (ED_{50}=5.294 ± 0.020 and 13.84 ± 0.011mg/Kg BDW resp.) compared to diclofenac (ED_{50}=1.994 ± 0.001 mg/Kg BDW) (table1).

The bioactivity-guided fractionation of the alkaloidal extract led to the isolation of two compounds (**1-2**), the major compound (**1**) showing the best pharmacological activity. The higher pharmacological activity of compound **1** is due to the presence of the methoxy group which makes it more lipophilic and is able to cross the membranes or the blood brain barriers. The identification of the two compounds as 5-methoxycanthin-6-one and canthin-6-one was based on comparison of spectroscopic data (UV, IR, NMR, MS and elemental analysis) with those published before [17-22] and authentic samples.

Although canthinone alkaloids are well-known constituents of the Simaroubaceae and Rutaceae and display a wide range of biological activities, they are being reported as constituents of the Euphorbiaceae here for the first time.

The time course study amply shows that the two compounds displayed anti-inflammatory activity in a dose dependent manner (Figure 2).

From the AUC analysis with due comparison with the positive control, diclofenac, all doses (3-100 mg/Kg) of **1-2** and diclofenac displayed significant ($p<0.001$) inflammatory reduction when compared with the untreated control group. The AUC analysis shows that all doses of the isolated compounds administered through the same oral route displayed moderate activity as diclofenac (table2).

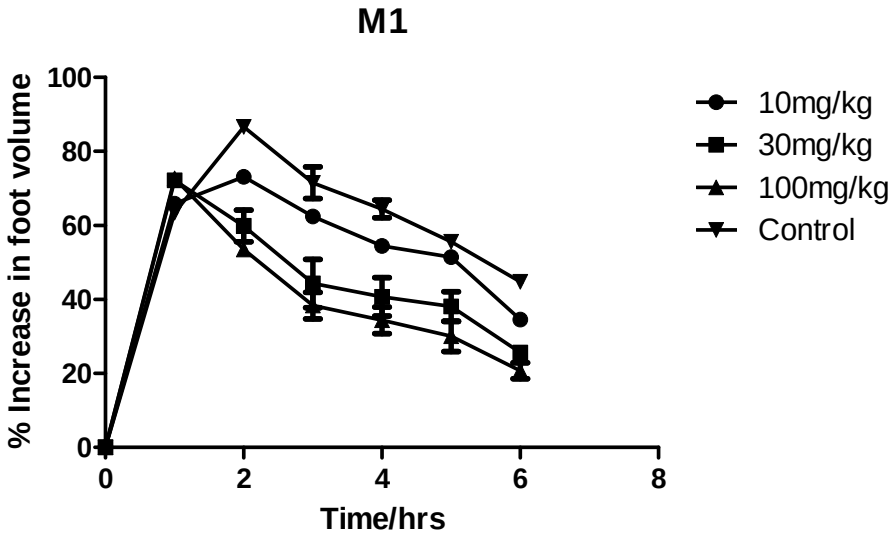
Although the synergistic effects of other minor constituents with a similar pharmacological effect are possible, 5-methoxycanthin-6-one (**1**) and canthin-6-one (**2**) as major constituents of the root bark of *Anthostema aubryanum* are likely to play a major role in the reported ethnomedicinal uses of the plant. The overall anti-inflammatory, as measured by the ED_{50} (5.294 ± 0.020) was lower than that of Diclofenac (1.994 ± 0.001) used as positive control (Figure 3).

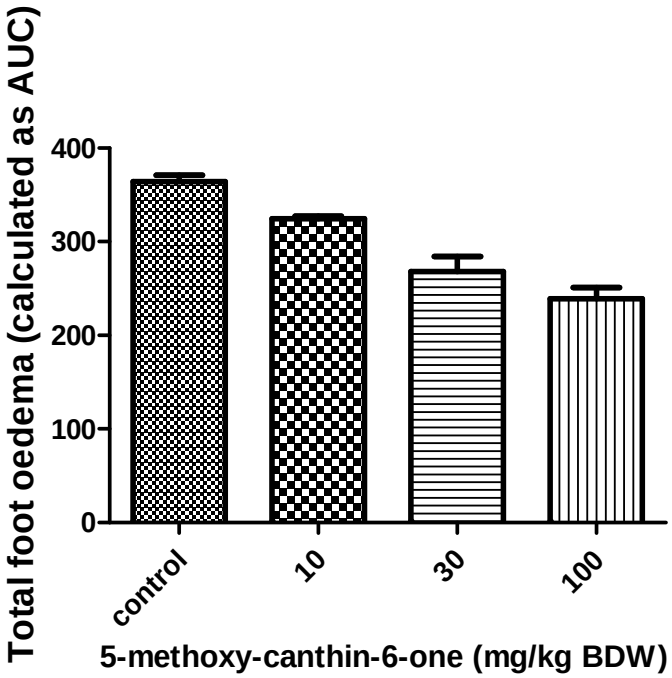
Table 1 Effect of crude extracts and standard drug on carrageenan-induced oedema

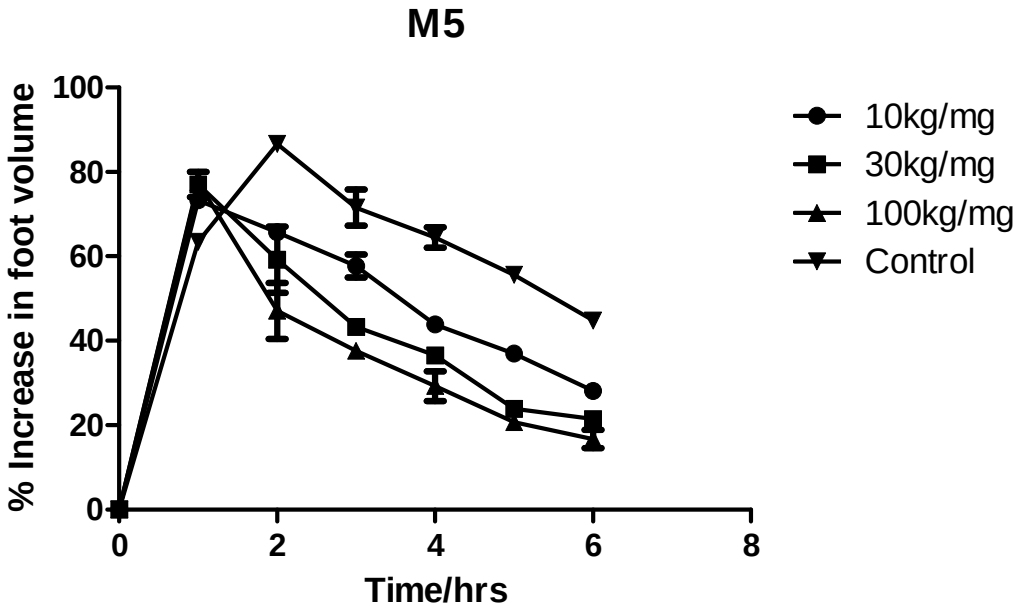
Extracts/Drug	**ED_{50} (mg/Kg) ±SEM**
Total Crude	5.294 ± 0.020
Alkaloidal crude	13.84 ± 0.011
Diclofenac	1.994 ± 0.001

Table 2 Effect of canthinone alkaloids and standard drug on carrageenan-induced oedema

Alkaloids/Drug	**ED_{50} mg/Kg ± SEM**
5-methoxy-canthin-6-one	60.84 ± 0.010
Canthin-6-one	96.64 ± 0.012
Diclofenac	1.994 ± 0.002




Total foot oedema (calculated as AUC)
400
300
200
100
0
control
10
30
100
5-methoxy-canthin-6-one (mg/kg BDW)


M5
% Increase in foot volume
100
80
60
40
20
0
0
2
4
6
8
Time/hrs
10kg/mg
30kg/mg
100kg/mg
Control

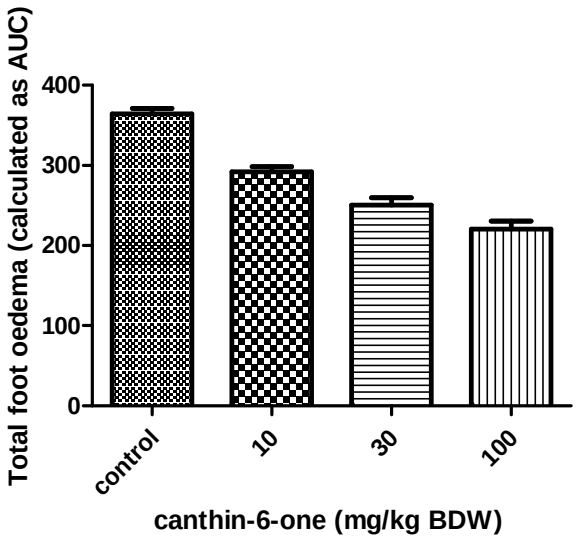


Figure 2: Time course edema development following carrageenan injection into rat paws and dose (mg/Kg body weight)-dependent anti-inflammatory effect of 5-methoxycanthin-6-one and canthin-6-one at time 2-5 h. Data for all doses of the isolated compounds were significantly different (p<0.001) from untreated control group.

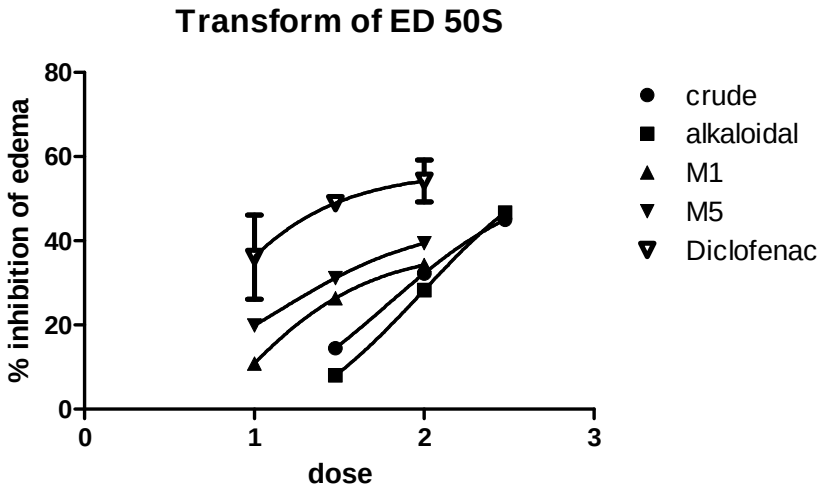


Figure 3: Dose response curves for crude, alkaloidal extract, compounds (10-100mg/kg *p.o*) and diclofenac (10-100mg/kg *i.p*) on carrageenan - induced foot oedema in rats

Antioxidant activity

The DPPH assay is a valid and simplest assay to evaluate scavenging activity of antioxidant, since the radical compound is stable and does not have to be generated as in other radical scavenging assays [23]

The extracts as well as the isolated compounds showed a concentration dependent DPPH radical scavenging activity (fig 4). The methanolic extract showed the highest activity followed by the alkaloidal extract, 5-methoxycanthin-6-one and canthin-6-one. The decrease in the absorbance of DPPH was due to phytoconstituents in the plant extracts. Phenolic compounds are widely distributed in plants and have gained much attention due to their antioxidant activities and free radical scavenging abilities which have beneficial implications for human health [24].

The phenolic compounds may contribute directly toward the observed high antioxidant activity through different mechanisms exerted by different phenolic compounds or through synergistic effects with other non phenolic compounds [25].

It has been observed that compounds with high antioxidant activities may also contribute toward the inhibition of tyrosinase, cholinesterase (AChE) and nitric oxide (NO) production in cells [26]. Inflammation activates neutrophils and macrophages to produce reactive oxygen/nitrogen species (ROS/NOS) and nitric oxide (NO) which leads to oxidative stress that can damage important organic substrates and also inhibit wound healing. Oxidative-related processes coupled with tyrosinase activity can also trigger melanogenesis, which causes skin pigmentation [27]. Antioxidants can scavenge free radicals and protect organisms from NO and ROS/NOS-induced damage, leading to a reduction in inflammation [27,28].

Antioxidants can also prevent major degenerative diseases and aging and might have protective effects toward Alzheimer's disease [29]. The inhibition of cholinesterase is suggested to be quite useful in the treatment of Alzheimer's disease and other diseases including senile dementia, ataxia and Parkinson's disease. Alzheimer's disease is the result of a deficiency in the cholinergic system due to the rapid hydrolysis of acetylcholine. Hence, nerve impulse transmission is terminated at the cholinergic synapses. By suppressing cholinesterase, cholinergic neurotransmission can be restored [30,31]. Tacrine is one of the synthetic drugs used for treating the symptoms of cognitive dysfunction or memory loss associated with Alzheimer's disease. However, adverse effects have been reported for these synthetic drugs, including gastrointestinal disturbances and suppression of bioavailability [30, 31]. There is therefore the need for continuous search especially from natural sources for alternative agents which are safe, relatively inexpensive, highly tolerated and convenient for many patients. There are no reports of the cholinesterase inhibition properties of any *Anthostema* species. However, *Anthostema* species are expected to have cholinesterase (AChE) inhibition properties because it has been reported that plants belonging to the Euphorbiaceae family have AChE inhibitory potential [30].
Thus, the high levels of antioxidant activity found in the plant extracts and the isolated compounds may also result in a higher inhibition of tyrosinase and cholinesterase activities as well as nitric oxide production.

Hence, *Anthostema aubryanum* has some therapeutic potential.

Table 2 Free radical scavenging activity of extracts of and standard drug

Extracts/Drug	**IC_{50} (µg/ml) ± SEM**
Methanolic extract	8.841 ± 0.024
Alkaloidal extract	23.12 ±0.010
Vitamin E	8.605 ± 0.002

Table 3 Free radical scavenging activity of isolated compounds and standard drug

Compounds/Drug	IC_{50} µg/ml ± SEM
5-methoxy-canthin-6-one	27.62 ± 0.090
Canthin-6-one	33.60 ± 0.011
Vitamin E	8.605 ± 0.002

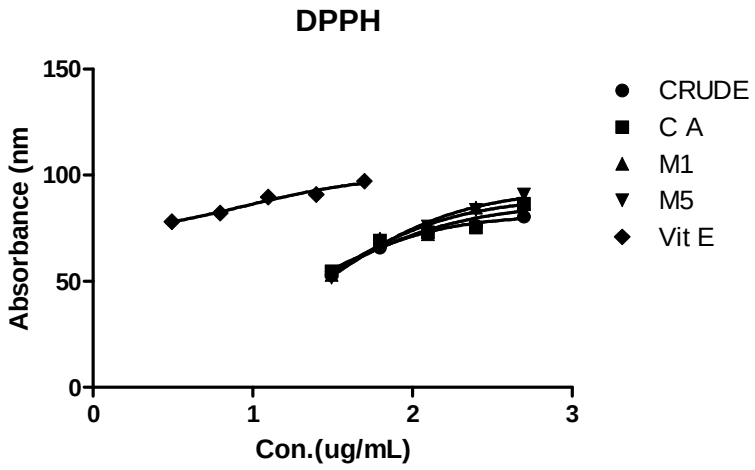


Fig. 4: Free radical scavenging activity of A. aubryanum and Vitamin E

In view of the present findings and other pharmacological activities of compounds **1** and **2**, the ethnomedicinal uses of *A. aubryanum* for treating and managing inflammatory conditions, wounds, pain and as antimicrobial agent seems to be justified.

CONCLUSION

The present study for the first time has established the scientific basis of the traditional uses of *A. aubryanum* root bark as an anti-inflammatory and antioxidant agent. Bioassay-guided fractionation of the alkaloidal extract of the root bark resulted in the isolation of two known compounds identified as 5-methoxycanthin-6-one and canthin-6-one (**1**,**2**) that displayed anti-inflammatory and antioxidant activities comparable with the positive controls diclofenac and tocopherol respectively. The investigations of the anti-inflammatory and antioxidant activities of canthin-6-one alkaloids continue in our laboratory to determine *in vivo* activities towards inflammation and wound healing and to evaluate their toxicity.

Acknowledgements
We gratefully acknowledge the technical assistance of staff at the Greenwich NMR and icipe-BCED Q-ToF Mass spectrometry laboratories, Department of Biomedical and Forensic Sciences, UCC, Cape Coast and the Department of Pharmacognosy, KNUST, Kumasi.

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