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Der Pharmacia Lettre, 2016, 8 (3):173-181
(http://scholarsresearchlibrary.com/archive.html)

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ISSN 0975-5071
USA CODEN: DPLEB4

Anthostema aubryanum Baill: Its Anti-inflammatory and Antioxidant Activities of the root bark extract

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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, 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, the crude aqueous-alcohol 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. The extract showed a good anti-inflammatory activity (ED_{50} = 5.294±0.020 mg/Kg BDW) compared to diclofenac (ED_{50}=1.994±0.001). The root bark also showed a high total phenolic content (74.53±0.004) vitamin E equivalent (VEE)/g) and free radical scavenging activity (IC_{50} = 8.841 μg/ml) compared to the standard vitamin E (8.605±0.01). The observed activities may be attributed to the presence of alkaloids, terpenoids, flavonoids, coumarins, anthraquinones, fatty acids, reducing sugars and tannins found in the phytochemicals screening of the plant. The present study for the first time has established the scientific basis for the traditional uses of A. aubryanum for treating inflammation, edema, boil and wounds.

Keywords: Anthostema aubryanum, Euphorbiaceae, phytoconstituents, anti-inflammatory, anti-oxidant, free radicals.

INTRODUCTION

Anthostema aubryanum (Baill, family, Euphorbiaceae) is an evergreen monoecious shrub to medium-sized tree up to 30 m high with succulent white latex in all parts [1]. The genus is native to Africa and consists of only three species: *Anthostema aubryanum* (Baill), *Anthostema senegalense* (A. juss) and *Anthostema madagascariense* (Baill) [2]. The genus is closely related to Dichostema. 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 DRC 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, pain, malaria, wounds (especially post abortion or after delivery) [3]. In Democratic Republic of Congo, it is used to treat infections of the gastrointestinal tract, constipation, dyspepsia, diarrhoea and dysentery [4,5]. In Senegal, a bark maceration is drunk to treat and manage intestinal infection, kidney problems, edema, impotence and as a laxative [6]. 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 [7]. 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 genus is generally known to predominantly contain phorbol esters, the chemistry and pharmacology of the root bark are yet to be investigated. In the present study, an investigation into the anti-inflammatory and antioxidant activities along with phytochemical analysis of the crude methanol is discussed.

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 Folin-Ciocalteau reagent, vitamin E, anhydrous sodium carbonate 99%, Diphenyl-2-picrylhydrazyl (DPPH) and all other chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

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.

Preparation of Extract

Powdered air-dried root bark of *A. aubryanum* (200 g), were extracted with 400 ml of 70% methanol using Soxhlet apparatus for 3 h. The extractive solvent was evaporated in vacuum yielding corresponding dried 70% methanol extract (10.20 g, yield of 5.10% w/w).

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 29^0C 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). 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

Determination of anti-inflammatory activity

The carrageenan foot edema model in rats was used to evaluate the anti-inflammatory properties of test samples [8]. 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 [9]. The foot volumes were then measured every hour for a total period of 5 or 6 h. For the anti-inflammatory activity measurements, crude extracts (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 [10].

In vitro antioxidant assay

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

For the DPPH assay, the antioxidant activity of the crude methanol extract was assessed in terms of the hydrogen donating or radical scavenging abilities of the extract using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method of Chandrasekar et al [11]. Aliquots of the crude 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 [12].

Total phenolic content assay

The total phenolic content (TPC) of crude methanol extract was determined using the modified Folin-Ciocalteau method [13]. In this method, I ml of the extract solution (1.0 mg/ml) in distilled water was introduced into a test tube followed by 1 ml of Folin-Ciocalteau reagent and I ml of 2.0% sodium carbonate. The content of the test tube was mixed thoroughly and the reaction mixture was allowed to stand for 2 h with shaking at 25^0C in an incubator. The mixture was then centrifuged at 3000 rpm for 10 minutes before measuring the absorbance of the resulting complexes at 760 nm using UV-VIS spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). Quantification of total phenolic was based on a vitamin E standard curve generated by preparing 0-100 µg $L^{-1}$of vitamin E. The TPC were expresses as milligrams of vitamin E equivalents (VEE)/100 g extract.

Total antioxidant capacity assay

This assay is based on the reduction of molybdenum, Mo^{+6} to Mo^{+5} by the extract and the subsequent formation of a green phosphate-molybdate complex at acidic pH. The modified phosphomolybdenum method of Prieto et al. [14] was used. Test tubes containing 1 ml each of the extract (1.0 mg/ml) and 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM disodium phosphate and 4 mM ammonium molybdate) were incubated at 95^0C for 90 minutes. After the mixture has cooled to room temperature, the absorbance of the resulting complexes was measured at 695 nm using a spectrophotometer. A standard curve was constructed for vitamin E and the TAC values were expressed as vitamin equivalents (VEE) in mg/100g extract. Different concentrations of vitamin E (0.015-0.25 mg/ml) were prepared in methanol and used to obtain the calibration curve.

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

Phytochemicals analysis

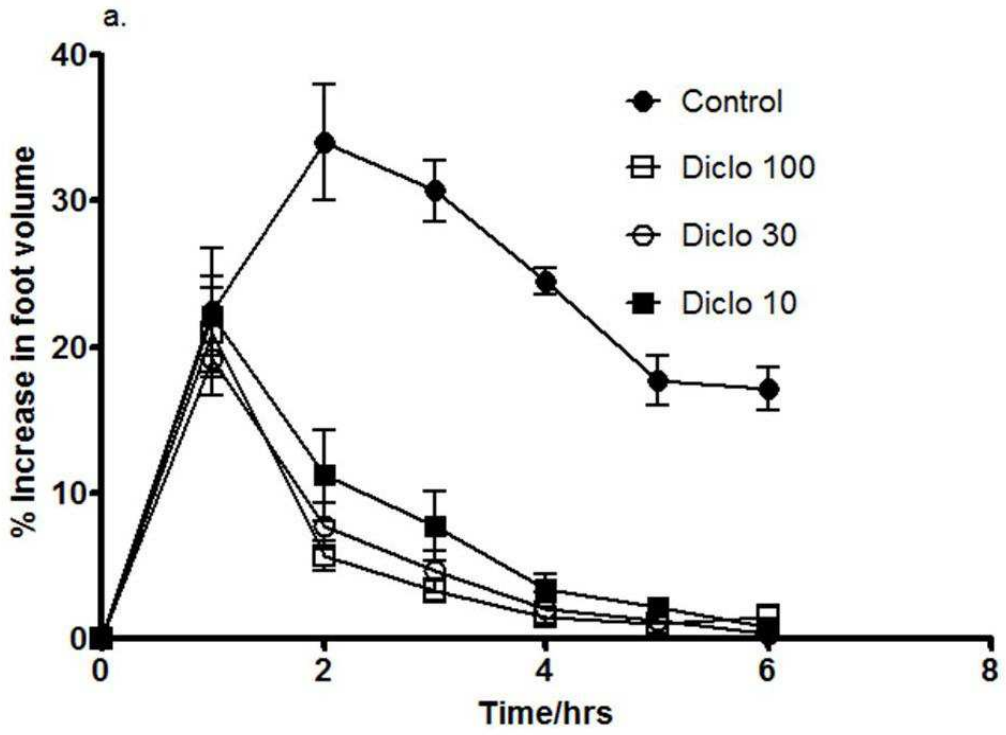
The crude methanol extract was screened for alkaloids, flavonoids, terpenoids, steroids, tannins, anthraquinones, cardiac glycosides, phenols and saponins. This was performed using different reagents and mobile phases described in the literature [15-16] to identify the major chemical groups. The presence of alkaloids were confirmed using Dragendorff's reagent and 3% Ce $(NH_4)_2SO_4$ in 85% H_3PO_4. Froth test and Stiasny's reagent were used to detect saponins and tannins respectively.

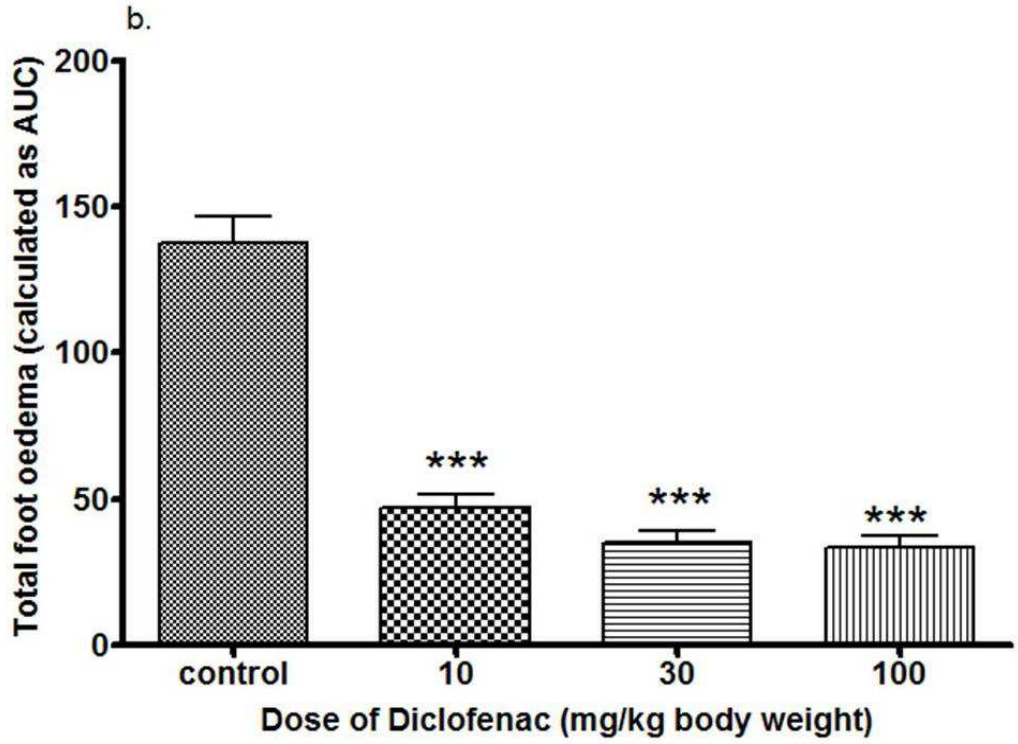
STATISTICAL ANALYSIS

Doses and concentrations responsible for 50 % of the maximal effect (EC_{50} and IC_{50}) for the extract 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.


a.
% Increase in foot volume
40
30
20
10
0
0
2
4
6
8
Time/hrs
Control
Diclo 100
Diclo 30
Diclo 10


b.
Total foot oedema (calculated as AUC)
200
150
100
50
0
***
***
***
control
10
30
100
Dose of Diclofenac (mg/kg body weight)

RESULTS AND DISCUSSION

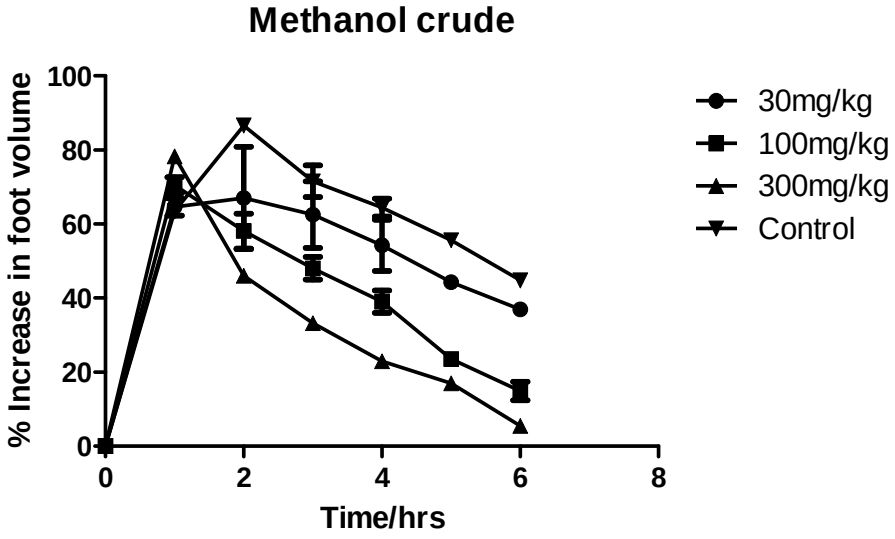
Anti-inflammatory activity

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 [17]. This model of inflammation has been shown to be attributed to the rapid release of acute phase mediators such as histamine and bradykinins followed by cyclooxygenase products including prostaglandins [17] .

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 crude extract of the root bark of *A. aubryanum* similarly suppressed the carrageenan-induced inflammation in a dose-and time-dependent manner. The percentage inhibition of edema was calculated by the following equation:

$$\%\ \text{inhibition of oedema} = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

AUC is the Area under the curve for the test samples and control



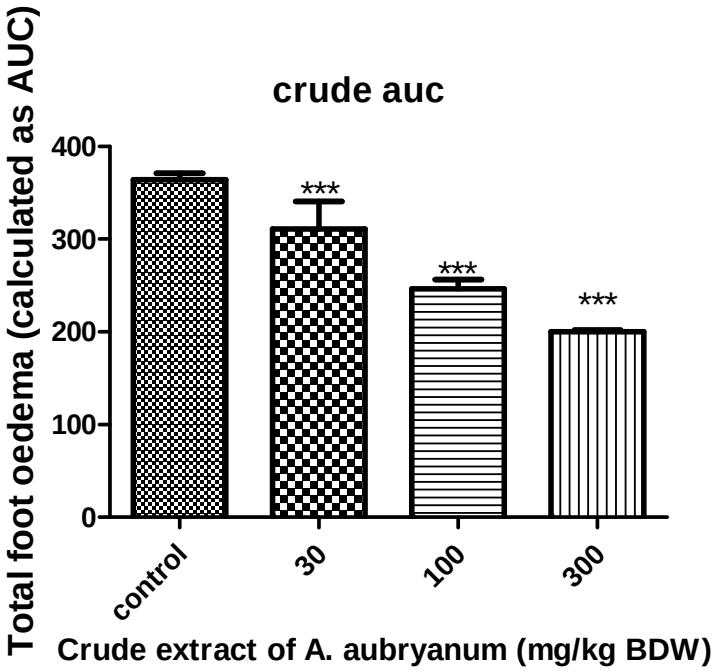


Fig.1 Time-course edema development following carrageenan injection into rat paws and dose (mg/Kg)-dependent anti-inflammatory effect of the standard positive control diclofenac (A) and the crude root bark extract of A. aubryanum (B). All data from 2 to 6 h of the treated groups are significantly different ($p < 0.05$) from the negative control group at each time point

Antioxidant activity

The antioxidant activity of *Anthostema aubryanum* (Baill) was evaluated by the DPPH method. It has a high antioxidant activity. The concentration that provided 50% radical scavenging (IC_{50}) was determined as 8.841±0.024 compared to the vitamin E standard of 8.605±0.002. 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. Antioxidants scavenge the DPPH radical by donating a proton. Different authors use different initial radical concentrations and different reaction times.

Total Phenolic Contents (TPC)

Total phenolic content (TPC) is expressed as vitamin E equivalent/100 g extract. The root bark extract has a higher value of TPC (74.53±0.004 mg VEE/100 g extract).

Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) is expressed as Vitamin E equivalent/100 g extract. The root bark extract has a very high TAC value (95.57 ± 2.309 mg VEE/100 g extract). These values are very significant ($p<0.001$) and are quite comparable

The relationship between the antioxidant capacity and total phenolic content analysis was highly significant (R^2 = 0.8926).

A high TPC value is often correlated with high antioxidant activity, though not all plant extracts exhibit the same pattern due to their different antioxidant mechanisms [18] and also Folin-Ciocalteu not being specific to just phenolic contents but to any other substances that could also be oxidized by the reagent [19].

The results indicate that the phenolic compounds could be the main cause of antioxidant power of plant samples, in accordance with the previous findings that many phenolic compounds in plants are good sources of natural antioxidants [20].

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 [21].

It has been established that compounds with high antioxidant activities may also contribute toward the inhibition of tyrosinase, cholinesterase (AChE) and nitric oxide (NO) production in cells. Inflammatory conditions may enhance the production of reactive oxygen/nitrogen species (ROS/NOS), which leads to oxidative stress that can damage important organic substrates. Antioxidants can scavenge free radicals and protect organisms from ROS/NOS-induced damage, leading to a reduction in inflammation [22,23]. Antioxidants can also prevent major degenerative diseases and aging and might have protective effects toward Alzheimer's disease [24]. 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 [25,26]. 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 [25, 26]. Oxidative –related processes coupled with tyrosinase activity can also trigger melanogenesis, which causes skin pigmentation [22, 27]. 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 [18].

Thus, the high levels of antioxidant activity found in the plant extract may also result in a higher inhibition of tyrosinase and cholinesterase activities as well as nitric oxide production. Phenolic compounds are widely distributed in plants and have gained much attention due to their antioxidant activities and free radical scavenging abilities which have potential beneficial implications for human health. Hence, *Anthostema aubryanum* has some therapeutic potential.

Phytochemical analysis

The preliminary phytochemical analysis has revealed that methanolic extract of *Anthostema aubryanum* is characterized by the presence of alkaloids, steroids, flavonoids, coumarins, anthraquinones, fatty acids, reducing sugars, cyanogenic glycosides, tannins and saponins. Carotenoids and glucosides were not detected in our experimental conditions. The presence mainly of alkaloids, flavonoids, steroids and terpenoids may largely contribute to the observed pharmacological activity because more chemicals belonging to these phytochemicals present in other medicinal plants had previously been reported to exhibit such pharmacological activity at different extents [28-33].

It has been established that flavonoids are the major anti-inflammatory agents and they can inhibit both cyclooxygenase and lipoxygenase pathways of the arachidonic metabolism depending on their chemical structures [34, 35]. Also, different flavonoids belonging to the C-glucosylflavonoids group mainly isolated from *Ficus thonningii*, *Trollius ledebouri*, *Linum usitatissimum* and *Aspalathus linearis* are well established good antioxidants which scavenge and reduce free radical formation [28].

Table 1 Phytochemical Analysis of *A. aubryanum*

Constituents	Observation
Alkaloids	+
Terpenoids	+
Flavonoid aglycones	+
Coumarins	+
Anthraquinone aglycones	+
Fatty acids	+
Reducing sugars	+
Tannins	+
Anthraquinone glycosides	+
Flavonoid glycosides	+
Carotenoids	-
Glucosides	-

+ = Present, - = Absent

Alkaloids in asserted skeletal type based on pyridine ring system have very good anti-inflammatory activity [36]. Terpenoids significantly inhibit the development of chronic joint swelling and may affect different mechanism relevant to inflammations arising in response to varied etiological factors [37]. Also, diterpenes (e.g. Phytol) found in *F. thonningii* is an anti-inflammatory agent and a potential therapeutic candidate for the treatment of rheumatoid arthritis and other chronic inflammatory diseases like asthma [33].

In conclusion, the present study for the first time has established the scientific basis for the ethnomedicinal uses of the root bark of *A. aubryanum* for treating inflammatory pain and wounds.

Isolation and characterization of the active compounds as possible therapeutic agents are currently underway in our laboratory

Acknowledgements
We gratefully acknowledge the technical assistance of the staff at the Department of Biomedical and Forensic Sciences, UCC, Cape Coast and the Department of Pharmacognosy, KNUST, Kumasi.

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