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Evaluation of the Anti-inflammatory, Antimicrobial and Antioxidant activities of the stem bark extract of *Croton penduliflorus* Hutch (Euphorbiaceae)

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Abstract

Croton penduliflorus is used in folklore medicine for the treatment of dysmenorrhea, fever, wounds, gastro-intestinal and others. The study evaluated the anti-inflammatory, antimicrobial and antioxidant activities of a hydro-ethanolic extract of stem bark of *Croton penduliflorus*. Carrageenan-induced oedema model in chicks was used to evaluate the anti-inflammatory activity of the extract. Total phenol content (TPC), DPPH radical scavenging and total antioxidant capacity (TAC) assays were used for assessment of antioxidant capacities. Agar well diffusion and micro-dilution methods were used to assess the antimicrobial activity of the extract. The extract caused a dose-dependent decrease ($P < 0.05$) in foot oedema with the highest inhibition of $69.23 \pm 1.05\%$ at a dose of 300 mg/kg whereas diclofenac and dexamethasone gave $92.46 \pm 1.15\%$ and 89.77 ± 0.95 at 100 mg/kg and 3 mg/kg body weight of the chicks respectively. The TPC and TAC were 1238 mg TAE/g and 362.33 mg AAE/g respectively. The MICs of the extracts ranged between 25-50 mg/ml for the test organisms. The hydro-ethanol extract of the stem bark of *Croton penduliflorus* shows considerable anti-inflammatory, antimicrobial and antioxidant activities.

Keywords: Agar-well diffusion, Minimum inhibitory concentration, DPPH radical scavenging, Total antioxidant capacity, *Croton penduliflorus*

1. Introduction

Inflammation is a complex biological process including several chemical mediators which are induced by the vascular tissue of the body when it comes in contact with harmful stimuli such as pollens, irritants, pathogens and damaged cells^[1]. These mediators evoke a complementary response against external stimuli which involves the release of reactive oxygen and nitrogen species by phagocytic leukocytes. However, upon prolonged activation *in-vivo*, such as the presence of pathogens like *Staphylococcus aureus*, the deleterious effect of reactive oxygen species takes upper hand, leading to the destruction of the tissues by affecting the structure-function model of macromolecules. Lipid peroxidation mediated by free radicals might yield a large number of reactive aldehydes and also lipid peroxides which are involved in pathophysiological changes associated with oxidative stress^[2-4].

Agents with anti-inflammatory, antioxidant and anti-microbial activities would be useful in mitigating the inflammatory reaction cascade especially if a microorganism is the offending agent. Antioxidants are substances which slow down or stop oxidation reactions and thereby protect the tissues by neutralizing the free radicals and other oxidants which are toxic by-products of cell metabolism^[5].

Synthetic drugs are mainly used in conventional medicine for the treatment of inflammation, but various chemical studies have shown these drugs not to be very safe. It is reported that about 90 % of these drugs are associated with drug related toxicities and other diverse effects complicating the treatment process^[1]. Hence new treatment regimens are needed as alternatives to these drugs.

In this quest, medicinal plants represent a viable option as they are reported to be devoid of such effects^[1]. The hydrosols and extracts of leaves, fruit peels, propolis, of some plants have exhibited varying degrees of antimicrobial activity^[3,7,8]. The stem bark extract and fractions of *Ficus exasperate* Vahl. (Moraceae) exhibited anti-inflammatory, antioxidant and antimicrobial Activities^[2]. Some parts of plants such as stems, fruits and leaves have been found to possess antioxidants^[9-12]. Natural antioxidants include phenolic compounds (flavonoids, phenolic acids, and tannins), nitrogen-containing compounds (alkaloids, amino acids etc), tocopherols etc^[13].

The diverse array of secondary metabolites contained in plants make them the preferred choice in researching for compounds with antioxidant, anti-inflammatory and antimicrobial properties. Therefore, this research sought to evaluate the hydro-ethanolic extract of the stem bark of *Croton penduliflorus* for its antimicrobial, anti-inflammatory and antioxidant potential.

Materials and Method

Plant material collection and processing

The stem bark of *Croton penduliflorus* was harvested in the month of November, 2015, at Mpraeso in the Kwahu South District in the Eastern Region of Ghana. It was authenticated by Dr. George Sam of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology, Kumasi, where a specimen has been kept (KNUST/X/2012/SX). It was then air-dried for 7 days and milled into a coarse powder. 300 g of the powdered stem bark of *Croton penduliflorus* was dissolved in 1 litre of 70% ethanol and extraction was done using the soxhlet apparatus. The extract was concentrated to a smaller volume by using a rotary evaporator (R-114, Buchi, Switzerland) at 40°C and evaporated to dryness on a water bath to give an extract referred to as ECP (yield = 26 %^{w/w}).

Drugs and chemicals used

All chemicals, with the exception of the reference drugs were purchased from Sigma Aldrich Co Ltd. Irvine, UK. Organic solvents were of analytical grade and purchased from BDH Laboratory Supplies (England). Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively.

Experimental animals

Cockerels (*Gallus gallus*) were obtained from Akati farms, Kumasi, Ghana as 1-day post-hatch and were housed in stainless steel cages (34 x 57 x 40 cm³) at a population density of 12-13 chicks per cage. Feed (Chick Mach, GAFCO) and water were available *ad libitum* through 1-quart gravity-fed feeders and water trough. Room temperature was maintained at 29°C and overhead incandescent illumination was maintained on a 12 hour light-dark cycle. Daily maintenance of the chicks was done till the seventh day when experiment was conducted. Sample size of 5 chicks per cage was used throughout the experiment. Permission was sought from Medical School, KNUST.

Determination of the anti-inflammatory activity

The carrageenan foot-pad oedema experimental model of inflammation in the chick previously reported by Woode *et al.* [14], was used to evaluate the anti-inflammatory properties of ECP. Seven day-old chicks (weighing between 30-55 g) were put into 10 groups (n=5). The initial foot volumes were measured and measurement repeated one hour after the carrageenan challenge (2%). The extract was then dosed orally at 30, 100, and 300 mg/kg to the first three groups respectively. Dexamethasone and diclofenac injected intraperitoneally at 0.3, 1, 3 mg/kg and 10, 30, 100 mg/kg respectively, were administered to 4th to 9th groups of chicks. The foot volumes were then measured at hourly intervals for 5 hours by water displacement plethysmography as described by Fereidoni *et al.* [15] using an electronic Von Frey plethysmometer (Model 2888, IITC Life Science Inc, Ca 91367 Canada). The control chicks (Group 10) were given 2 mL/kg normal saline. The oedema part of the inflammation

was quantified by measuring the difference in foot-volume before carrageenan injection and at the various time intervals. The foot volumes were individually normalized as percentage of change from their values at time zero then averaged for each treatment group. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\% \text{ inhibition} = \left[\frac{AUC_{\text{control}} - AUC_{\text{treatment}}}{AUC_{\text{control}}} \right] \times 100$$

The protocol was in compliance with the National Institute of Health guideline for the care and use of laboratory animals and was approved by the Department of Pharmacology ethics Committee.

Determination of Antioxidant Capacity

DPPH radical scavenging assay

The free radical scavenging assay was used to determine the antioxidant activity as described by Govindarajan [16], with few modifications. 1 mL quantity of Extract (500, 250, 125, 62.5 and 31.25 µg/mL) was added to 3 ml of methanol solution of DPPH in a test tube and incubated at 25°C for 30 minutes. The absorbance of the mixture was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Center, England). Ascorbic acid (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/mL) was used as the standard free radical scavenger. A solution of 1 mL methanol and 3 mL DPPH was prepared, incubated at 25°C for 30 minutes and used as control. The result for the scavenging activity was expressed as percentages of the control. The EC₅₀, which is the concentration required to scavenge 50 % of the DPPH molecule, was calculated. Each test was carried out in triplicates.

The % DPPH scavenging activity (% of control) of the antioxidant was calculated as follows.

$$\% \text{ DPPH scavenging effect} = \left[\frac{A_c - A_t}{A_c} \right] \times 100$$

Where: A_c = Absorbance of control, A_t = Absorbance of the test drug/ extract

Concentrations responsible for the 50 % of the maximal effect in the DPPH assay (EC₅₀) for each drug/extract were determined using an interactive computer least square method, with the following nonlinear regression (three-parameter logistic) equation.

$$y = \frac{a + (b - a)}{(1 + 10^{(\log_{10} X - X_0) / \Delta X})}$$

Where, X is the logarithm of dose and Y is the response. Y starts at *a* (the bottom) and goes to *b* (the bottom) with a sigmoid shape.

Total antioxidant capacity (TAC) assay

The total antioxidant capacity was determined as described by Prieto *et al.* [17], using ascorbic acid as the standard control. The assay is based on the reduction of molybdenum, Mo⁺⁶ to Mo⁺⁵, by the extracts and subsequent formation of a green phosphate-molybdate complex at an acidic pH. 1mL of the extract (31.25-500 µg/mL) was delivered into a test tube containing 3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM disodium phosphate and 4 mM ammonium

molybdate). The mixture was incubated at 95°C for 90 minutes, cooled to room temperature, and absorbance measured at 695 nm against a blank (a solution of every other solution excluding the extract and Ascorbic acid). Antioxidant capacity was expressed as ascorbic acid equivalent (mg/g of extract).

Total phenol content (TPC) assay

Total phenol was evaluated using Folin Ciocalteu reagent [18]. Various concentrations of the extract (0.1 mL of 31.25-500 µg/mL) and tannic acid (1 mL of 1.5625-200 µg/mL) was mixed with 0.5 mL of Folin Ciocalteu's and incubated for 15 minutes. Afterwards, 2.5 mL aqueous Na₂CO₃ (2% solution) was added and also incubated for 15 minutes. The mixture was centrifuged, and absorbance was recorded at 760 nm. The standard curve was prepared using tannic acid solutions (0.03-0.1 mg/mL) in methanol. Total phenol content was expressed in terms of tannic acid equivalent (mg/g of extract).

Antimicrobial Assay

Microorganisms used

The antibacterial activities of the *C. penduliflorus* stem bark extract was evaluated against some bacterial species which included both Gram positive (*Enterococcus faecalis*, *Staphylococcus aureus*, *K. pneumonia* and *Bacillus subtilis*) and Gram negative (*Escherichia coli*, *N. gonorrhoea* and *Pseudomonas aeruginosa*) bacteria and fungus *C. albicans*. The organisms were provided by the Department of Pharmaceutical microbiology, Faculty of Pharmacy, KNUST.

Agar well diffusion assay

The extract was tested for antimicrobial activity using the agar well diffusion as described by Berghe and Vlietinck [19]. Wells of 9 mm diameter were made in 20 mL nutrient agar with 2 loopfuls of a suspension of organisms (10⁵ CFU/mL) aseptically. The extract was tested at 100 µL of 50 mg/mL solution in 10 % aqueous DMSO. The plates were incubated at 37 °C for 24 hours after which zones of inhibition were measured.

Micro dilution assay

Minimum inhibitory concentration (MIC) was determined using the 96-well micro titre-plate-based serial dilution method [20]. 20 µL of a bacterial suspension (10⁵ CFU/mL) was dispensed into each well containing 100 µL of double strength nutrient broth. The extract was incorporated in 10 % aqueous DMSO to obtain a stock solution of 1 mg/mL. To each well was added 100 µL of the test extract at various concentrations. Ciprofloxacin was used as a positive control. The microplates were incubated for 24 hours at 37 °C. 20 µL of a 5% solution of p-iodonitrotetrazolium was incorporated as an indicator of cell growth. The test was carried out in triplicates.

Phytochemical analysis

The presence of plant secondary metabolites in the hydro-ethanolic extract of *Croton penduliflorus* were determined by simple standard qualitative phytochemical methods [21].

Statistical analysis

The statistical analysis was done using graph pad prism for windows version 5.0 (Graph Pad Software, San Diego, CA, USA). The differences in AUC's were analyzed by one way analysis of variance (ANOVA) followed by student-Newman-

Keuls' *post hoc* test, $P \leq 0.05$ was considered statistically significant.

Results and Discussion

Anti-inflammatory activity

Inflammation is frequently associated with pain and involves physiologic effects such as increase in vascular permeability, protein denaturation and membrane alteration. During the inflammatory process, high levels of reactive oxygen species (ROS) are produced to provide a defense against invaders. Scavenging of these ROS is therefore conceivably important in alleviating the inflammatory reaction.

From the study, the hydro-ethanolic extract of *C. penduliflorus* stem bark and the standard drugs significantly decreased the foot pad oedema of the chicks. This is shown as the time-course curve and area under the curve (Figure 1b). The extract caused a dose dependent decrease in oedema (Figure 1b) with the highest percentage inhibition of 69.23 ± 4.607 at 300 mg/kg body weight. The positive control drugs diclofenac (an NSAID) and dexamethasone (steroidal anti-inflammatory drug) also showed a significant dose dependent decrease in oedema (Figure 1c-f) with percentage inhibitions of 92.46 ± 0.992 and 89.77 ± 0.969 respectively. Induction of oedema by carrageenan is due to the synthesis and release of histamine, serotonin, kinins, prostaglandins and cyclooxygenase-2 [22] which began from the first hour through to the second hour. The extract inhibited the increase in oedema from the second hour. These mediators increase vascular permeability and subsequently results in the observed oedema [23]. The anti-oedematous effect observed suggest an inhibitory effect of the extract on the synthesis and/or release of any one of the inflammatory mediators. Hence, the study has shown that the stem bark extract of *C. penduliflorus* has a considerable anti-inflammatory activity.

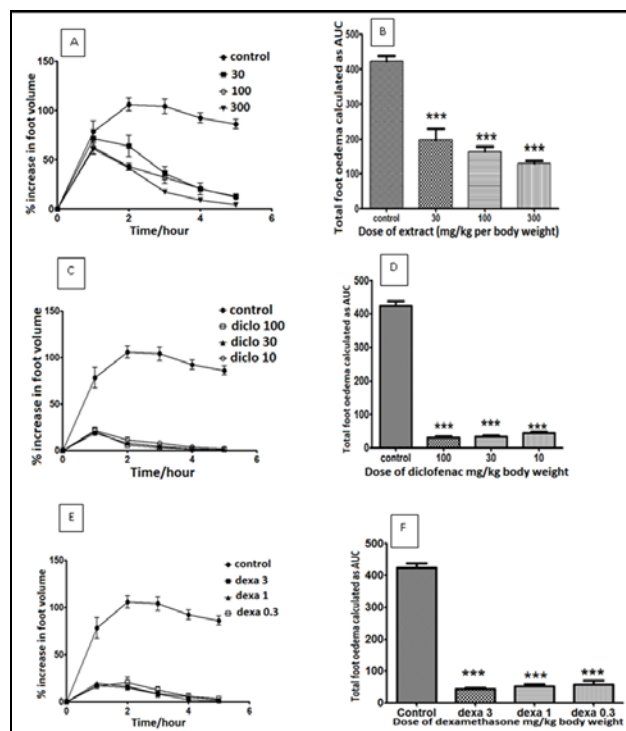


Fig 1: Effect of *Croton penduliflorus* stem bark extract, diclofenac and dexamethasone on foot volume. *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

Table 1: Percentage inhibition of oedema in carrageenan anti-inflammatory bioassay

Drug/Extract	% Inhibition of oedema
Extract (EAA)	69.23 ± 4.607
Diclofenac	92.46 ± 0.992
Dexamethasone	89.77 ± 0.969

Antioxidant activity

Ascorbic acid was used as the standard drug in the DPPH radical scavenging activity. The extract and ascorbic acid showed a concentration dependent radical scavenging activity (Figure 2A) recording an EC₅₀ values of 315.7 and 11.40 µg/ml respectively. From the phosphomolybdate total antioxidant assay (TAC), the total antioxidant capacity was calculated as mg of ascorbic acid equivalent per gram of extract (AAE). Ascorbic acid showed a good linearity in the range 200-3.125 µg/ml with a correlation coefficient (r²) of 0.9805. The extract showed antioxidant activity with a value of 362.333 mg AAE/g. Again it showed a high phenolic content of 271 mg of tannic acid (TAE) per gram of the extract. Thus, it has been demonstrated in this study that hydro-ethanolic extract of the stem bark of *C. penduliflorus* has considerable antioxidant activity.

Antioxidants are the first line of defense against free radical damage, and are critical for maintaining optimum health and well-being. The need for antioxidants becomes even more critical with increased exposure to free radicals. Endogenous antioxidants and the body's protective defenses mitigate inflammation and hence reduce tissue damage. However, during oxidative stress, these endogenous antioxidants are overwhelmed by the surge of reactive oxygen species. The reactive oxygen species are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. Oxidative damage to DNA, proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, including inflammation, heart diseases and cancer [24]. Thus the extract, through its antioxidant activity may exert a protective effect by decreasing oxidative damage to DNA. The antioxidants are needed to curb the upsurge of reactive oxygen species leading to gradual return of the cells state of redox homeostasis, thereby preventing ailments [25]. This may notably help mitigate inflammatory diseases. Hence it can be said that the antioxidant activity of the stem bark of *C. penduliflorus* may play a role in its anti-inflammatory activity.

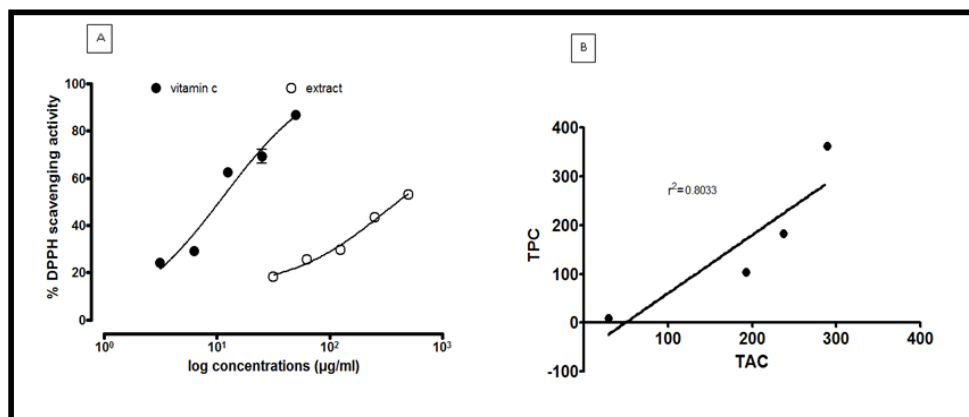


Fig 2: (a) DPPH radical scavenging activity of extract and Vitamin C and (b) correlation between total antioxidant activity and Total phenol content.

There was a strong positive correlation existing between the total antioxidant and the total phenol content with r² of 0.8033 (Figure 2B). This means that about 80.33% of the antioxidant activity of *C. penduliflorus* was due to its phenol constituents, with the remaining percentage accruing from the other phytochemical compounds.

The phytochemical analysis of the extract revealed the presence of glycosides, flavonoids, triterpenoids, and saponins (Table 2). Flavonoids are noted for their antioxidant, antiviral, antiplatelet, antitumor, antiallergic and anti-inflammatory activities [26, 27]. Anti-inflammatory activity of flavonoids has been linked to a number of activities including their antioxidant and radical scavenging activity, regulation of cellular activities of inflammation-related cells, modulation of activities of arachidonic acid metabolism enzymes (phospholipase A2, cyclooxygenase, lipoxygenase), nitric oxide synthase, modulation of the production of other pro-inflammatory molecules and modulation of pro-inflammatory gene expression [21]. Therefore, the presence of flavonoids in *C. penduliflorus* may be responsible for its antioxidants and anti-inflammatory activities. This is the first report of the antioxidant and anti-inflammatory activities of *C. penduliflorus*.

Table 2: Phytochemical screening on the extract

Test	Results
Alkaloids	-
Coumarins	-
Flavonoids	+
Glycosides	+
Phytosterols	-
Saponins	+
Tannins	-
Triterpenoids	+

Key: - absent, + present

Antimicrobial assay

The stem bark extract of *C. penduliflorus* showed activity against all the Gram-positive bacteria (*S. aureus*, *K. pneumoniae*, *E. faecalis* and *B. subtilis*). It was active against the Gram-negative bacterium *P. aeruginosa* but not *E. coli* (Table 3) in the agar-well diffusion technique. The standard reference ciprofloxacin, however, showed broad spectrum antibacterial activity. *Staphylococcus aureus* and *Streptococcus pyrogenes* were the most susceptible organisms to the extract. The minimum inhibitory concentration observed was 25 µg/mL for *Staphylococcus*

aureus. These microorganisms are commonly implicated in wounds. *C. penduliflorus*, thus may be useful as a wound healing agent as it epitomizes a single agent with anti-inflammatory, antioxidant and antimicrobial activities necessary for wound healing and also to ward off infections. It also showed some activity against *N. gonorrhoea* and *C. albicans* and thus may be useful in the treatment of sexually transmitted infections.

Table 3: Mean zones of growth inhibition of ECP and the reference drug against test organisms

Organisms	Mean zones of inhibition (mm) ± SEM	
	ECP	Ciprofloxacin
<i>S. pyrogenes</i>	20.5±0.5	27.5±0.5
<i>B. subtilis</i>	19.5±0.5	31±0.0
<i>S. aureus</i>	20.0±0.0	17.5±0.5
<i>K. pneumoniae</i>	15.0±0.5	21.5±1.5
<i>N. gonorrhoea</i>	15.5±0.5	21.5±1.5
<i>P. aeruginosa</i>	16.5±0.5	19.5±0.5
<i>E. coli</i>	-	23.5±0.5
<i>E. faecalis</i>	17.5±0.5	31.5±0.5
<i>C. albicans</i>	15.5±0.5	30.0±0.0

ECP: Extract of *Croton penduliflorus* stem bark.

Conclusion

The study has shown that the hydro-ethanolic extract of the stem bark of *C. penduliflorus* has anti-inflammatory, antioxidant and antimicrobial activities and thus gives credence to its use in folklore medicine. This is the first report of the anti-inflammatory, antioxidant and antimicrobial properties of the stem bark extract of *Croton penduliflorus*.

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