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Antioxidant and *in vivo* Wound Healing Activities of *Clausena anisata*

Nicholas Agyepong[1], Christian Agyare[1*], Paul Poku Sampene Ossei[2] and Yaw Duah Boakye[1]

[1]Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[2]Department of Pathology, Komfo Anokye Teaching Hospital, Kumasi, Ghana.

Authors' contributions

This work was carried out in collaboration between all authors. Author CA conceived and designed the study and wrote the first draft. Author NA performed the in vivo wound healing and antioxidant works and managed literature search and analysis of data. Author PPSO performed the histological studies and author YDB did the statistical analysis and wrote the protocol. All authors read and approved the final manuscript.

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ABSTRACT

Leaves of *Clausena anisata* are used in many parts of West Africa including Ghana for management of wounds and other skin infections. The study was to evaluate the *in vivo* wound healing and antioxidant properties of ethanol leaf extract of *C. anisata.* The wound healing activity of ethanol leaf extract of the *C. anisata* was investigated using excision wound model. The antioxidant activity was determined using 2, 2-diphenyl-1 picrylhydrazyl (DPPH) free radical scavenging method. For wound healing activity, concentrations of 7 and 10% w/w aqueous cream of the extract were used. The progression of wound healing was determined by the periodic assessment of the contraction of excision wounds and histological studies. The leaf extract cream (7% w/w) was found to significantly increase the rate of wound contraction (p<0.001) at days 13 to 19 compared to the untreated. The n-propyl gallate used as the reference antioxidant agent had IC_{50} of 4.19 µg/mL and that of the ethanol extract was 32.9 µg/mL. These findings may justify the medicinal uses of *C. anisata* for the management of wounds.

**Corresponding author: E-mail: cagyare.pharm@knust.edu.gh;*

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1. INTRODUCTION

A wound may be considered as a disruption of anatomical and functional integrity of living tissue or the torn in the protective function of the skin, which may arise as a result of chemical, physical or microbial agent [1]. There are different types of wounds, including injuries, cuts and bites, diabetic, gastric and duodenal ulcers. These wounds can be classified as acute or chronic depending on the time it takes to heal. Without complications, most wounds or acute wounds tend to heal within few weeks. Chronic wounds in contrast, require a prolonged time to heal, do not heal, or recur frequently. These wounds tend to occur when the normal wound healing process has been compromised, due to microbial infection, metabolic disturbances, or an underlying disease [2].

Wound healing is complex and dynamic process involving the reconstruction and regeneration of devitalized and damaged cellular structures and tissue layers as closely as possible to the original state [3]. The process comprises of several critical biochemical events that can be grouped into four sequential, but overlapping and distinct phases of hemostasis, inflammation, proliferation or granulation and tissue remodeling. Any delay in the process may lead to a prolonged time of healing or non-healing chronic wound [4]. The large number of patients with serious chronic wounds is now a worldwide concern and approximately 6 million patients globally have chronic wounds of various types [5].

The contraction of wound occurs throughout the healing process, commencing in the fibroblastic stage where the area of the wound undergoes shrinkage [6]. This process may be aided by antioxidant agents, which are metabolites and naturally found in the body and in plants, play an important role in the progression of wound healing due to their properties to inhibit oxidative stress on wound due to free radicals such as superoxides, hydroxyl radicals and singlet oxygen species [7]. Plants produce wide array of antioxidant compounds which includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols, that mob up the free radicals that mostly occur in the hemostasis and inflammation phases during the healing process [8,9].

Clausena anisata (Willd) Hooke. (Rutaceae) is a shrub widely used in many parts of West Africa including Ghana as therapeutic alternatives for the management of wounds and treatment of other bacterial and fungal infections due to their broad array of phytochemicals [10,11].

Furanocoumarins, imperatorin, oxypeucedanine and chalepin have been isolated from *C. anisata* [12,13]. Clausenol, coumarins and limonoids [12], β-pinene and sabinene chemotypes [14] have been isolated from the plant. *C. anisata* has been found to possess antimicrobial [10,12], antiparasitic, central nervous depressant [15] and hypoglycemic properties [16]. We have already reported the presence of secondary metabolites including flavonoids, tannins, saponins, alkaloids, steroids, phenolics and glycosides in the ethanol extract of *C. anisata.* Also, the high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) profiles of the ethanol extract of *C. anisata* have been determined [10]. The study was designed to evaluate the antioxidant and *in vivo* wound healing properties of ethanol leaf extract of *C. anisata*.

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves of C*. anisata* were collected from the Physique Garden at the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana between the months October and November, 2010. The plant was authenticated by Dr. G.H. Sam of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana, with the voucher specimen number, KNUST/HML/2012/L 061.

2.2 Extraction Procedure

Fresh leaves of *C. anisata* were air dried (30 to 38ºC) and milled into powder. Two hundred (200) grams of the powdered leaves was weighed and extracted with 1000 mL of 70% ethanol by cold maceration for 72 h. This was subsequently filtered through Whatman filter paper (number 10). The filtrates were put together and concentrated using rotary evaporator at 40ºC and lyophilized. The dried powdered extract was kept in desiccator.

2.3 Determination of Antioxidant Activity

The determination of the free radical scavenging activity of the crude extract and solvent fractions were carried out using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay as described by Mensor et al. [17]. Four concentrations (0.1, 0.3, 1.0 and 3.0 mg/mL) of the ethanol extract were prepared. One (1) milliliter of 0.3 mM DPPH in methanol was added to 3.0 mL of both the extract and standard. The concentrations (0.01, 0.03, 0.1 and 0.3 mg/mL) of extract were prepared and allowed to stand at room temperature in a dark chamber for 30 min. The UV absorbance of the resulting solution was measured at 517 nm. The procedure was repeated using n- propylgallate as reference compound. The decrease in absorbance was then converted to percentage inhibition using the formula;

$$\text{Percentage inhibition (\%)} = (A_0 - A_1)/ A_{0\,X}100\%$$

A_0 is the absorbance of DPPH; A_1 is the absorbance of the extract/sample + DPPH.

2.4 Ethical Protocol for Animal Work

Thirty healthy Sprague-Dawley rats (170 to 195 g), of either sex of comparable age were used. The animals were housed in clean metal cages and maintained on normal commercial pellet diet. The animals were given water *ad libitum* and maintained under laboratory conditions (room temperature 28ºC, relative humidity of 60 to 70% and 12 h light to dark cycle). The animals were kept in the animal house of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana for a week prior to the start of the experiment. This was done to enable animals adapt to the new environment. Techniques and methods used in this study were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health (NIH), US, Department of Health Services Publication no. 83-23, revised 1985). The protocols for the study were approved (Pharm/EthC/X8122013) by the Department of Pharmacology Ethics Committee, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

2.5 Excision Wound Model

The animals were anaesthetized with ketamine (50 mg/kg). The dorsal fur of the animals were shaved to a circular diameter of 40 mm by means of razor blades and the anticipated area of the wound to be created was outlined on the shaved skin of the animals with ammonium oxalate crystal violet paint. The shaved area was cleaned with 70% v/v ethanol before excision wounds were created using slightly modified method as described by Bhakta et al. [18]. A full thickness of the skin with circular diameter of 20 mm and (an approximate area of 314 mm^2) were created along the markings using toothed forceps, surgical blades and pointed scissors. Different concentrations of the *C. anisata* extract (7 and 10% w/w) were prepared using aqueous cream BP as the base (vehicle) since aqueous creams are suitable dosage forms for delivering wound healing agents as topical preparations [19]. The entire wound was left open for 24 h and the animals divided into six groups of five animals each.

Group 1 animals were topically treated with silver sulphadiazine (1% w/w) cream; Group 2 animals were treated with aqueous cream (vehicle) and Group 3 animals were left untreated. Groups 4 and 5 animals were treated with concentrations of 7 and 10% w/w of *C. anisata* ethanol extract aqueous creams, respectively.

Wound treatment commenced on the second day of wound excision. The extract and reference drugs were then topically applied to the wounds 24 hourly for 19 days. During the course of treatment, scaled photographs of the wound areas were taken (by means of a high resolution Digital Camera, alongside a millimeter scale every 48 h starting from the 1st day of wound treatment. The wound areas were then determined with the aid of a computer program (Graph pad prism, San Diego, CA, USA).

2.6 Wound Healing Properties Assessment Parameters

The process of wound healing and the quality of the regenerated tissues at the wounded site were assessed by determining the following parameters:

2.6.1 Wound size / contraction

Wound size measurement can be used to monitor the progress of healing through changes in the area of the wound with time. The size of the wound was measured at a regular interval of 72 h. An excision wound area was measured by vernier calipers and millimeter ruler and

expressed in percentage of healed wound area [20].

2.6.2 Histological studies

Wound tissue specimens from untreated and treated animals were taken during healing process at day15 to assess microscopic changes in wound bed. The cross sectional full thickness wound scar of about 6 mm thick sections from each group were collected at the end of the experiment to evaluate the histological alterations using the slightly modified method of Sadaf et al. [21]. Samples were fixed in 10% buffered formalin for 24 h and dehydrated with a sequence of ethanol-xylene series of solutions, processed and blocked with paraffin at 40 to 60ºC and then sectioned into 5 to 6 µm thick sections. The sectioned wound tissues were deparaffinized and stained with hematoxylin and eosin stain (HE) and observed under a light microscope at x600 magnification.

2.7 Statistical Analysis of the Data

Graph Pad prism version 5.0 windows (Graph Pad Software, San Diego, CA, USA) were used for all the statistical analysis. The data was analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The values of $p<0.05$ were considered to be statistically significant. The graphs were plotted using Sigma plot for Windows Version 11.0 (Sytsat software Inc., Germany).

3. RESULTS

3.1 Antioxidant Activity

The ethanol leaf extract of *C. anisata* was found to exhibit antioxidant activity. Though the antioxidant property of the extract was not as potent as the reference compound, the half maximal inhibitory concentration (IC_{50}) was found to be 32.9 µg/mL with the reference compound (n-propyl gallate) under the same conditions determined to be 4.19 µg/mL (Fig. 1).

3.2 Influence of Ethanol Leaf Extract on Wound Contraction

Wound contraction was assessed throughout the healing process, commencing from the third day, where the area of the wound undergoes shrinkage. The progression of wound healing was determined by the periodic assessment of the contraction of excision wounds. The aqueous cream extract at 7% w/w concentration was found to significantly increase the rate of wound contraction ($p<0.001$) at days 13 to 19 compared to the untreated wounds. There was no significant difference between the 7 and 10% w/w ethanol extracts (Table 1).

3.3 Histological Investigations

Histological studies of treated and untreated sections of the wound tissues showed varying degree of fibrosis. Specimen showed 70 to 80% dense and thickened fibrosis, late stage atrophy of the epidermis and also orthohyperkeratosis in the dermoepidermal junction for 7 and 10% w/w extract cream. The untreated showed a thin layer of fibrosis of the epidermis. However, the positive control (1% w/w silver sulphadiazine) showed 60 to 70% fibrosis (Fig. 2).

4. DISCUSSION

Since ancient times, natural substances including products, extracts and parts of medicinal plants have been used to treat or manage various acute and chronic injuries, wounds and other skin disorders [22] and the secondary metabolites such as alkaloids, flavonoids, tannins and other phenolic compounds present in these plants and their extracts have been found to be responsible for these pharmacological or biological activities [10].

The phenolic compounds have been found to be capable of regenerating endogenous tocopherol, in the phospholipid bilayer of lipoprotein particles, back to its active antioxidant form and are also known to inhibit various types of oxidizing enzymes and free radicals [9]. These potential mechanisms of antioxidant action make the diverse group of phenolic compounds the target in the search for health beneficial phytochemicals [9,23].

The ethanol leaf extract of *C. anisata* had antioxidant activity with the IC_{50} of 32.9 µg/mL and the n-propyl gallate (reference antioxidant) under the same conditions was found to be 4.19 µg/mL (Fig. 1). Though the antioxidant property of the extract was not as potent as the reference antioxidant, this may likely protect the cells against oxidative stress. Antioxidant property of the *C. anisata* may suggest that external application of the extract on the wounds may trap free radicals liberated in the inflammation phase from the cells surrounding the wounds and also

have the ability to protect cells from microbial infections. The antioxidant activity may be due to phenolic compounds because of their redox properties, which play a determining role in the progression of wound healing [24]. Migration of fibroblasts may be as a result of antioxidants properties in the herbal extract that may have the ability to stimulate the early expression of growth factors [25].

Wound repair involves fibroblasts migration from the wound edges to the wound site, proliferation and subsequently production of collagen, the main component in the extracellular matrix. Stimulation of fibroblasts is one mechanism by which herbal extracts might enhance the wound repair process even though, keratinocytes also need to migrate from the wound edge to provide a provisional matrix for the fibroblasts to migrate on, this might be accelerated secondary to a mature dermal matrix [25,26]. The 7 and 10% w/w leaf extract-treated wounds showed significant (p< 0.001) increase in the rate of wound contraction from days 9 to 19 compared to the untreated wounds (Table 1). The improved wound contraction rate may suggest that the extract enhance wound healing by stimulating fibroblast proliferation, keratinocytes proliferation and differentiation as well as collagen biosynthesis.

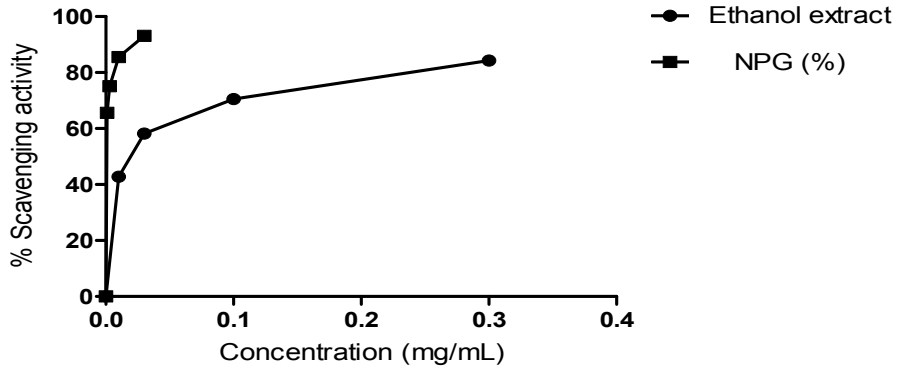

Fig. 1. Percentage DPPH radical scavenging activity of ethanol leaf extract of *C. anisata* and n-propyl gallate

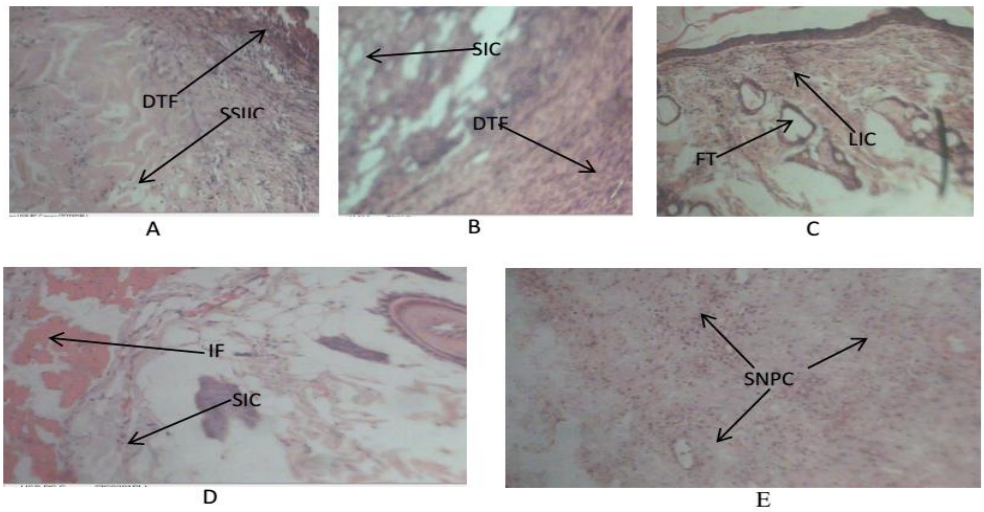

Fig. 2. Microscopic histological section of excised wound tissues. Histological section stained with hematoxylin and eosin, (A) 10% w/w extract, (B) 7% w/w extract, (C) untreated, (D) 1% w/w silver sulphadiazine and (E) vehicle only

DTF: Dense thickened fibrosis; SSIIC: Slightly scanty inflammatory infiltrate cells; SIC: Scanty inflammatory cells; IF: Incomplete fibrosis MF: Moderate/mild fibrosis; SNPC: Scattered neutrophils with plasma cells; LIC: Localized inflammation cells; TF: Thin fibrosis; FT: Fatty tissues/cells; magnification: ×600

Table 1. Influence of *C. anisata* ethanol leaf extract on rate of wound contraction

Days	Wound area contraction (%)				
	Untreated	Vehicle	SS (1% w/w)	7% w/w	10% w/w
1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
3	12.1±0.14	8.0±0.10	14.8±0.11	12.2±0.22	11.2±0.26
5	25.7±0.22	18.6±0.12	23.9±0.56	32.4±0.16	24.7±0.16
7	36.1±0.10	25.7±0.12	40.2±0.22	44.7±0.10	44.0±0.22
9	39.5±0.18	30.3±0.18	42.1±0.12	49.1±0.11	48.5±0.44
13	54.9±0.11	59.8±0.16	76.5±0.33***	75.9±0.57***	76.7±0.21***
15	63.0±0.10	70.0±0.26	82.4±0.41***	81.6±0.22***	83.8±0.10***
17	73.7±0.16	75.0±0.33	89.1±0.34***	87.2±0.16***	90.0±0.14***
19	76.4±0.22	80.8±0.11	91.5±0.61***	90.2±0.14***	93.3±0.10***

*Values were expressed as mean±SEM (n=5). Data was analyzed using Two-way ANOVA followed by Bonferroni's post hoc test (***p< 0.001) were considered statistically significant compared with the untreated wounds.*
SS= Silver sulphadiazine

Histological observations of the sections of wound tissue treated with 7 and 10% w/w extract cream, showed 70 to 80% dense and thickened fibrosis compared with silver sulphadiazine (1% w/w) showing 60 to 70%, which was characterized by the accumulation of fibroblasts for the deposition of collagen to form a cross link with other collagen for the contraction of wound during healing. The high accumulation of fibroblasts may be due to the tannins, flavonoids or antioxidants properties present in the leaf extract of *C. anisata* or their synergistic effect [10,25]. There is also orthohyperkeratosis in the dermo-epidermal junction which is further supported by an increase in the proliferation of fibroblasts, which are responsible for collagen synthesis and therefore better contraction of the wound. The untreated wound tissues specimen showed a thin layer of fibrosis of the epidermis indicating a slow wound healing process (Fig. 2). Agyepong et al. [10] reported that *C. anisata* leaf extract possess antimicrobial activity which may contribute to the wound healing activity of the plant and also sufficient oxygen supply in wounds may inhibit microbial infection in wounds.

The effect of the extract on various growth factors such as beta transforming growth factor-1 (TGF-ß1), fibroblast growth factor (FGF), keratinocytes growth factor (KGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) as well as the biosynthesis of collagen and other skin tissues should be evaluated. There is a need to isolate the bioactive agents or principles in *C. anisata* which are responsible for the above biological activities.

5. CONCLUSION

Ethanol leaf extract of *C. anisata* exhibited antioxidant property with IC_{50} of 32.9 µg/mL. The ethanol leaf extract of *C. anisata* enhanced the rate of wound closure and also exhibited high influence on proliferation of fibroblasts and levels of fibrous connective tissues in the wound bed.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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