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PHYTOCHEMICAL ANALYSIS AND MYCOBACTERICIDAL STUDIES OF THE LEAVES OF *C. MUCRONATUM* SCHUMACH. & THONN.

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ABSTRACT: *Combretum mucronatum* Schumach & Thonn is a scandent shrub whose leaves are widely used by Ghanaians and other West-African herbalists for the treatment of various infectious and non-infectious diseases. It is used to treat wounds, coughs, dysentery, worm infestation, neurological disorders and bacterial infections. Some coughs are as a result of mycobacterial infection. Despite the success gained in minimizing antibiotic resistance over the past decades, there still remains a need for new antibiotics. Due to the constant development of multi-drug-resistant bacteria to existing antibiotics, particularly those directed against multidrug-resistant Gram-negative bacteria such as the tuberculous bacteria, new antibiotics are needed. Folklorically used antimicrobial herbs are good sources of new antibiotics. In that regard, the aim of this study was to assess *C. mucronatum* leaves for its possible mycobactericidal activity and investigate its extracts phytochemically. The macroscopic, microscopic, and physicochemical characteristics were determined as preliminary means of quality assessment of the plant materials. Phytochemically, *C. mucronatum* is rich in a wide variety of tannins and flavonoids, and their corresponding TLC chromatograms can be used to confirm the identity and purity of the crude plant material. The 50% ethanol extracts of *C. mucronatum* had activity against *M. smegmatis* with a minimum inhibitory concentration of 50 mg/ml. The mycobactericidal activity can be further exploited for development into an anti-infective.

INTRODUCTION: *Combretum mucronatum* Schumach. and Thonn. is a scrambling shrub belonging to the family Combretaceae. It is known locally in the Twi Ghanaian language as 'Hwiremoo [1]. It is a scrambling shrub that can grow to become a forest liane with pubescent branchlets, oblong leaves, pubescent on nerves beneath, glabrous above, inflorescence variable, numerous flowers, petals and filaments that are white with pale pink winged young fruits [1].

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This plant is widely distributed in the tropical climates of West Africa and especially in the Savanna forests of the region [2, 3]. The leaves of *C. mucronatum* are used in the form of a poultice, decoction and tincture extensively by Ghanaian and Nigerian herbalists for the treatment of both new and old wounds, boils, burns, fever, guinea worm, malaria, septicemia, thrush, rheumatism, gonorrhea, cough, dysentery, worm and bacterial infestation [1, 3-5].

The roots, which are cut into small pieces, are boiled with capsicum peppers or wood ash, and the concoction is drunk for chest pains, gonorrhea, and nervous disorders [2]. The aqueous extract from the leaves of *C. mucronatum* is reported influence mitochondrial activity and proliferation of dermal fibroblasts and epidermal keratinocytes significantly

as a means of enhancing wound healing [6]. The methanol root extracts of *C. mucronatum* has also been shown to have anti-inflammatory effects *in-vivo* by interaction with the opioid pathway [2].

The methanol extract has also been shown to have activity against some pathogenic bacteria, namely: *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus,* and *Bacillus subtilis* [3]. Recent investigations have also shown that *C. mucronatum* prevents scopolamine-induced memory deficit in mice due to its significant anti-cholinesterase, antioxidant and anti-dementic properties, and may be useful in the management of Alzheimer's disease [2].

Other known pharmacological activities of this plant are anti-pyretic, choleretic, diuretic, vulnerary, and cholagogue. The main compounds identified in *C. mucronatum* include proantho-cyanidins, flavonoids, and fatty acids [7, 8].

Despite the success gained in minimizing antibiotic resistance over the past decade, there consequently still remains a need for new antibiotics. New antibiotics and particularly those directed against multi-resistant Gram-negative bacteria and especially against agents such as the tuberculous bacteria, are needed.

Unless antibacterial development is re-energized, there is a serious risk that a growing proportion of infections will become effectively untreatable [9]. Due to the broad spectrum of anti-microbial activity demonstrated by *C. mucronatum* and also in accordance with its folkloric use in the treatment of coughs, the *mycobacterial* activity needed to be investigated.

MATERIALS AND METHODS:

Plant Collection and Identification: *C. mucronatum* leaves were harvested from the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, in April 2011 and authenticated by Mr. Amponsah of the Ghana Herbarium, currently the Department of Plant and Environmental Biology, University of Ghana. Herbarium voucher (number PSM002/11) has been kept at the Herbarium of the Department of Pharmacognosy and Herbal Medicine, University of Ghana, Legon. The leaves were air-dried for two weeks and pulverized.

Macroscopic and Microscopic Analyses: Macroscopic and microscopic characteristics of samples were studied using standard procedures according to WHO guidelines on quality control methods for herbal materials, 2011 [10]. Four-millimeter square (4 mm^2) sizes of the mature lamina of the leaves were cleared with chloral hydrate solution and mounted in glycerin at a magnification of x^{10}. The presence of calcium oxalate crystals, stomata, and trichomes was observed, and quantitative parameters such as vein islet number, veinlet termination number, stomata number, and stomatal index were estimated.

Physico-chemical Analysis: The plant materials were analyzed for moisture content and ash values. The moisture content was estimated by the loss on drying method. Total ash, water-soluble ash, and acid insoluble ash were also determined using the WHO, 2011 guidelines [10].

Determination of Extractive Values: One gram (1 g) of pulverized plant material was extracted with 10 ml of water, ethanol 90% v/v, ethyl acetate, dichloromethane, hexane, and petroleum ether, respectively. The extraction of the plant materials was performed by ultrasonication for 15 min, followed by centrifugation at 5000 rpm for 10 min. The clear supernatant was collected and the residue extracted again with another 10 ml of the respective solvent. The combined extracts were concentrated under a vacuum at 45 °C and freeze-dried.

Phytochemical Screening of Ethylacetate Extract by Thin-Layer Chromatography (TLC): The ethyl acetate extract obtained was screened phytochemically for the presence of flavonoids and proanthocyanidins using standard methods described by Wagner and Bladt, 1996 [11]. The extract was dissolved in methanol to obtain a concentration of 2 mg/ml, and 7.2 µl of the resulting solution was loaded onto silica gel 60 F_{254} plates.

The plates were then developed in the solvent system, H_2O:HCOOH: EtOAc (5:5:90). The underivatized and derivatized plates were documented at daylight, λ 254 and λ 366 nm before and after spraying with detecting reagents. Detection spray reagents used in this respect were Natural product reagent (1% w/v of diphenyl-

boryloxyethylamine) for flavonoids and vanillin-HCl acid reagent (1% w/v of vanillin prepared in MeOH and subsequently with concentrated HCl) for proanthocyanidins.

Determination of Tannin Content: The tannin content of the aqueous extract of *C. mucronatum* was determined according to the European Pharmacopoeia 7.0, Monograph "Bestimmung des Gerbstoffgehaltes P flanzlicher Drogen", 2011. The tannin content was determined with 750 mg (m1) of pulverized dried plant material. The plant material was extracted with 250 ml of water, filtered, and transferred into a 250 ml volumetric flask, and the volume made up to the graduated mark. A 30 ml aliquot of the extract was centrifuged at 6000 rpm for 10 min.

The clear solution was labeled as the stock solution. Total phenol solution (TPS), was prepared by pipetting 5 ml of the stock solution into a 25 ml volumetric flask and diluting with distilled water to the graduated mark. The remaining Phenol Solution (RPS) was prepared by adding 10 ml of the stock solution to 100 mg of slightly chromated hide powder. The mixture was shaken for 60 min and then filtered. The filtrate (5 ml) was diluted to 25 ml. Reference Solution (RS) of pyrogallol was prepared by dissolving 0.05 g in 100 ml of milli pore water and 5 ml of the resultant solution diluted to 100 ml.

Folin-Ciocalteu's phenol reagent of 1.0 ml was added to 2 ml of TPS and diluted with 10 ml of distilled water. The resultant solution was diluted to 25 ml with 14.06 % w/v of sodium carbonate solution. The above procedure was repeated for RPS and RS. After 30 min of incubation in darkness, the absorbances (A) of the solutions were taken at λ 760 nm using water as the blank.

The percentage of tannin content was calculated using the average of three separate measurements with the following formula.

$$(\%) = (62.5 \times (A_{\text{Total phenol solution}} - A_{\text{Total phenol solution}}) \times M_2 / A_{\text{Refrences}} \times M_1$$

Fluorescence Studies: Fluorescence analysis of the powdered crude drug was also carried out to determine the characteristic fluorescence when dissolved in specific solvents. This was performed according to published methods [12-14]. The samples were observed under daylight and UV light of short and long wavelengths (254 and 365 nm respectively) for their characteristic color [15].

Mycobactericidal Activity:

Microorganism: The test organism, *Myco-bacterium smegmatis* (MC2 155) was obtained from the Noguchi Memorial Institute for Medical Research, Legon, Ghana. Middle brook 7H9 powder, nutrient agar, and all reagents used for experiments were purchased from VWR, U.S.A. *M. smegmatis* was culture din 20 ml of Middle brook 7H9 broth for 24 h at 37 °C. The bacterial culture was then standardized to 1×10^6 cell/ml with the aid of a previously calibrated bacterial suspension curve at 680 nm.

Micro Broth Dilution Method: Due to the richness of the ethyl acetate extract with flavonoids and procyanidins, this extract was chosen for the mycobactericidal activity investigations. A sterile stock concentration of 400 mg/ml of the ethyl acetate extract of *C. mucronatum* was prepared. An extract volume of 250 µl, 500 µl of double strength nutrient broth, 50 µl of sterile water, and 200 µl of bacterial culture was added to a 24 well plate and mixed to get an in-well concentration of 100 mg/ml.

Another 500 µl of double strength (D/S) nutrient broth, 125 µl of the stock plant extract, 175 µl of sterile water, and 200 µl of culture was added to the next well to get a concentration of 50 mg/ml.

Similar procedures were used to obtain concentrations of 12.5 and 6.25 mg/ml. A growth control was set up with 500 µl of D/S nutrient broth, 300 µl sterile water, and 200 µl of bacteria culture. The sterile control contained 500 µl D/S nutrient broth, 250 µl plant extract, and 250 µl sterile water. The plates were incubated at 37 °C for 24 h. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION:

Macroscopic Characteristics: *C. mucronatum* leaves are deep green with a non-specific odor and astringent taste. A summary of its features is given in **Table 1. Fig. 1** is a picture of *C. mucronatum* displaying the leaves and flowers.

FIG. 1: ***COMBRETUM MUCRONATUM***

TABLE 1: MACROSCOPIC CHARACTERISTICS OF *C. MUCRONATUM* LEAVES

Morphology	*C. mucronatum*
Shape	Obtuse
Margin	Entire
Venation	Pinnate
Texture	Smooth

Microscopic Characteristics: Microscopic analysis gave the vein islet number for *C. mucronatum* as 14, and the veinlet termination as 6. Details of the results are shown in **Table 2**.

The upper surface of *C. mucronatum* leaves had anomocytic stomata, wavy epidermal cells, and rosette calcium oxalate crystals, which were abundant within the lamina **Fig 2**.

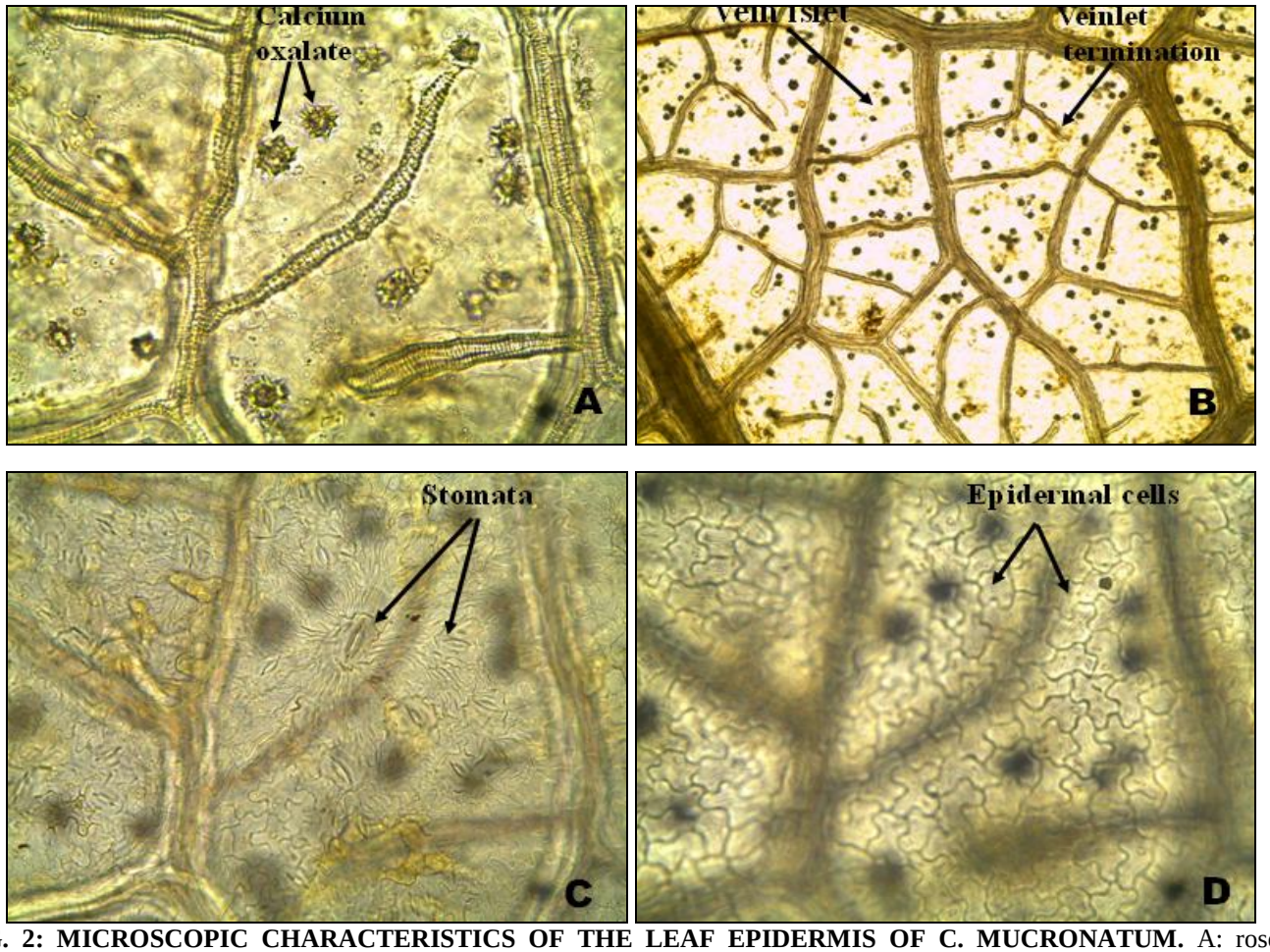

FIG. 2: MICROSCOPIC CHARACTERISTICS OF THE LEAF EPIDERMIS OF C. MUCRONATUM. A: rosette calcium oxalate crystals, B: vein islet and veinlet terminations, C: anomocytic stomata, D: wavy epidermal cells

TABLE 2: MICROSCOPIC CHARACTERISTICS

Parameter	*C. mucronatum*
Stomatal Number [mm^2]	16
Epidermal Cell Number [mm^2]	65
Vein islet Number [mm^2]	14
Veinlet termination number [mm^2]	6
The stomatal index [%]	8.5

Physicochemical Characteristics: The pharmacognostic characteristics are a prerequisite for their standardization. These characteristics can be used as a good indication of identity, purity, and quality and provide a simple means of detecting adulteration and substitution of these plant materials [16]. These parameters will, in the long run, also assure efficacy and safety. The physicochemical parameters are provided in **Table 3**. Moisture content values, for example, are useful in reducing errors in the estimation of the actual weight of drug material and also indicate the stability of the plant material against degradation by moisture and microbes [17]. The higher the moisture content, the higher the possibility of degradation. For *C. mucronatum* leaves, the average moisture content was estimated at 20.5 %w/w of the dried plant material. Extractive values indicate weights of the extractable chemical constituents of the crude drug under different solvent environments [18]. The results show that leaves of *C. mucronatum* contain more polar constituents, as water had the highest extractive value of 16% w/w **Table 4**.

TABLE 3: PHYSICOCHEMICAL CHARACTERISTICS

Parameters	*C. mucronatum*
Total ash [%w/w]	7.50
Acid insoluble ash [%w/w]	6.00
Water-soluble ash [%w/w]	10.17
Moisture content [%w/w]	20.50
Foreign organic matter [%w/w]	-
Foaming index	<100
Swelling index [ml]	4.67

TABLE 4: EXTRACT YIELDS *C. MUCRONATUM* LEAVES

Solvent	Water	Decoction	Ethanol 90%	Ethyl acetate	Dichloromethane	Hexane	Petroleum ether
Extract yield (%w/w)	16.0	7.0	7.0	3.0	2.0	1.0	1.0

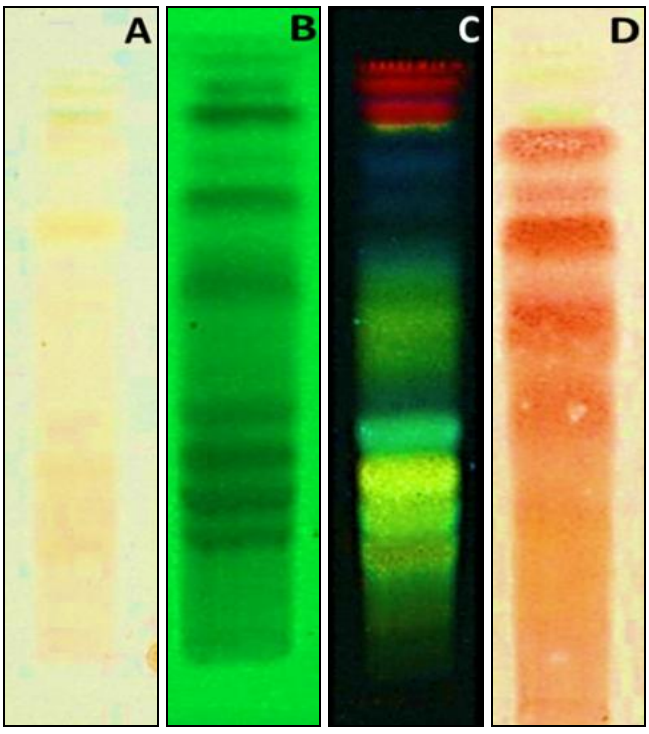

FIG. 3: TLC CHROMATOGRAM OF THE ETHYL ACEATED EXTRACTS OF *C. MUCRONATUM* LEAVES. A: daylight; B: λ 254 nm; C: λ 366 nm, detection with natural product reagent, D: daylight, detection with vanillin-HCl

Phytochemical Constituents: Fig. 3 displays the TLC fingerprint chromatograms of the ethylacetate extract of the leaves of *C. mucronatum*. Flavonoids were detected in the chromatograms because when underivatized, they quench fluorescence at λ 254 nm **Fig. 3B** but give characteristic fluorescence at λ 366 nm after spraying with Natural product reagent. Phenol carboxylic acids typically turn blue while flavonols and flavones turn orange, yellow, or yellow-green (as seen in **Fig. 3C**). Proanthocyanidins, when sprayed with vanillin-HCl, gave typical red bands under daylight, as seen in **Fig. 3D**. The presence of flavonoids and proanthocyanidins in the leaves of *C. mucronatum* is in agreement with published data [1, 2, 7, 8]. The mean tannin content was estimated to be 12 ± 5.7 % w/w of dried plant material.

Fluorescence Studies: Analysis for characteristic fluorescence of *C. mucronatum* leaves in various solvents **Table 5** at short and long wavelengths showed varying colors. These results are useful for confirming both the identity and quality of the crude plant materials. Compounds in plant

materials may fluoresce under UV light but may not show such activity when observed in daylight. This phenomenon may be due to the compounds already present in the plant material or fluorescent derivatives formed after treatment with the specific reagents [13]. In methanol, for example, *C. mucronatum* fluoresces red, while in nitric acid, this same sample fluoresces blue at 366 nm.

TABLE 5: FLUORESCENT STUDIES OF *C. MUCRONATUM* LEAVES IN VARIOUS SOLVENTS

	Daylight	254 nm	366 nm
Distilled water	Green	Deep blue	Pale blue
1N HCl	Brown	Deep blue	Pale blue
1N NaOH	Deep red	Deep blue	Pale blue
50% H_2SO_4	Black	Deep blue	Pale blue
Methanol	Brown	Bright red	Deep red
Glacial acetic acid	Brown	Bright red	Pale red
Nitric acid	Red	Blue	Blue
Chloroform	Deep green	Bright red	Pale red
50% $FeCl_3$	Deep green	Deep blue	Pale blue
95% Ethanol	Light green	Bright red	Pale red

Mycobactericidal Activity: The ethylacetate extract of *C. mucronatum* exhibited myco-bactericidal activity at a minimum inhibitory concentration of 50 mg/ml, while that of rifampin, the positive control, was 0.1 µg/ml.

A number of works have demonstrated the anti-infective effects of flavonoids. Recent studies by Cao *et al.,* have demonstrated that a mixture of plant flavonoids exhibit anti-infective activity against the mycobacteria specifically, *Myco-bacterium tuberculosis* [19]. Thus the presence of flavonoids in the leaves of *C. mucronatum* could account for this effect, making it a potential anti-mycobacterium agent.

CONCLUSION: This study provides basic phytochemical data that can be used in identifying and assessing the quality of crude materials of *C. mucronatum* in areas such as West-Africa, where it is widely used. It has also established the mycobactericidal activity of the ethyl acetate extract and has provided the basis for further investigations into its *myco-bactericidal* activity.

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CONFLICTS OF INTEREST: Nil

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