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PHARMACOGNOSTIC, MYCOBACTERICIDAL, AND MUTAGENIC INVESTIGATIONS OF THE LEAVES OF CLERODENDRUM SPLENDENS G. DON

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ABSTRACT: *C. splendens* is a medicinal plant used for various ailments within the West-African Community. This study sought to investigate the pharmacognostic characteristics, mycobactericidal, and mutagenic activities of the hydro-ethanolic leaves extract *C. splendens*. The organoleptic, microscopic, macroscopic and physicochemical characteristics were investigated according to WHO, 2011 quality control methods for herbal materials. The mycobactericidal and mutagenic activities were investigated by the agar well diffusion assay and the Ames test (Muta-ChromoPlateTM) respectively. Present in *C. splendens* are tannins, flavonoids, glycosides, saponins, steroids and alkaloids which are comparable to published literature. The leaves of had anomocytic stomata, and starch granules, while calcium oxalate crystals were visibly absent. The 50% ethanol extract of *C. splendens* was not active against *M. smegmatis* at the highest concentration of 100 mg/mL. *C. splendens* was also non-mutagenic. Though the extract exhibited no mycobactericidal activity at the highest tested concentration, the documented pharmacognostic characteristics can be used for the purposes of authentication. Also, the non-mutagenic potential indicates a lack of toxicity in that respect.

INTRODUCTION: *Clerodendrum splendens*, belongs to the family Verbenaceae, andis commonly referred to as bleeding heart vine, flaming glory bower and pagoda flower [1]. It is also referred to as 'ekenyieya' among the Nzemas of Ghana, 'nruchu among the Igbo of Nigeria and 'trupatru' among the Kweni of Ivory Coast [2]. It is a twining shrub found in the tropics that grows to a climber and bears red flowers during the months of December through April.

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It grows to about 5 m in height. It is predominantly found in Western Africa, however *C. splendens* can also be found in Central Africa. Traditionally, extracts from the roots and leaves have used for centuries to treat a number of diseases[3] including cough, malaria and venereal diseases such as gonorrhoea and syphilis. It is also used to manage asthma [1].

Topically, the leaves and lotion have been used as a wound healing remedy for bruises, blisters, sores and burns and skin diseases such as shingles and ulcers [1, 3, 4]. It is also used to treat spleen disorders in children, asthma, rheumatism, and malaria. On the basis of doctrine of signatures, the plant is believed to have antihaemostatic properties in parts of Ivory Coast due to the redness of its flowers [2].

Mystically, the Yuroba's of Nigeria believe in its ability provide protection for widows who dream about their dead husbands [5]. *C. splendens* has been shown to possess anti-inflammatory, antioxidant, and antimicrobial properties [4]. The extract is antimicrobially active against *Bacillus subtilis, Staphylococcus aureus, Streptococcus faecalis, Micrococcus flavus*, as well as resistant strains of *Staphylococcus aureus* (SA1199B, RN4220 and XU212), Gram negative bacteria which includes *Escherichia c*oli, *Pseudomonas aeruginosa, Proteus mirabilis*, and *Klebsiella pneumoniae* as well as the fungi *Candida albicans*. Its phytochemical constituents include carbohydrates, unsaturated sterols, flavonoids, glycosides and triterpenoids [6]. This study thus further investigated possible activity of the leaves extract of this plant against *Mycobacterium smegmatis*, and also basic pharmacognostic parameters for identification. The possible effect on mutagenesis was also investigated on the T29 strain in the Ames test.

MATERIALS AND METHODS:

Plant Collection: *C. splendens* was collected from the Campus of the Kwame Nkrumah University of Science and Technology in January 2017 and authenticated at the Ghana Herbarium, Department of Plant and Environmental Studies, University of Ghana with Voucher specimen of (PSM65/1/17). The leaves were washed, air dried for three weeks and milled into a coarse powder.

Macroscopic and Microscopic Analysis: The leaves of *C. splendens* were examined macroscopically for characteristics such as colour, shape, texture, leaf venations and leaf margin. The microscopic characteristics of these samples were studied using standard procedures according to WHO guidelines on Quality control methods for herbal materials, 2011 [7]. Four-millimeter square (4 mm^2) sizes of the mature lamina of the leaves were cut with a sharp edge. They were then boiled for about two hours in chloral hydrate solution to clear the chlorophyll content. The sections were mounted on a clean glass slide with the help of glycerin and covered with a clean glass cover slip. The slides were observed under light microscope at an objective magnification of x10. Qualitative features such as the presence of calcium oxalate crystals, stomata and trichomes were observed. Quantitative parameters such as vain islet number, veinlet termination numbers, stomata number and stomatal index were determined according to standard protocols as described by WHO, 2011. This was done by counting each determination under ten different fields of view and calculating the number per millimeter square of each leaf surface.

Physico-chemical Analysis: Air-dried samples of the plant materials were analyzed to determine their physicochemical parameters. The moisture content was estimated by the loss on drying method. Total ash, acid insoluble ash and water-soluble ash were also determined using the WHO, 2011 guidelines.

Fluorescence Studies: Fluorescence analysis of the crude powdered drugs were also carried out to determine the characteristic fluorescence of the pulverized leaf samples and when the samples were dissolved in specific solvents according to methods described by Ranjith, 2018 [8]. Observations were made under visible day light and UV light of short wavelength (254 nm) and UV light of long wavelength (365 nm) for their characteristic colour [8].

Extractive Values Determination: Twenty grams (20 g) of coarsely powdered air-dried leaves of plant material was weighed and 200 mL of solvent (either water, 50% ethanol or petroleum ether) was added. The plant material was sonicated for 45 minutes and allowed to cool down in between. The extractives were filtered and concentrated *in-vacuo* at 40°C. The concentrates were then freeze-dried to obtain dry crude extracts and stored at -20 °C prior to use. The percentage yield of petroleum ether, water and 50% ethanol soluble extractive values were calculated with reference to the air-dried material**.**

Extraction and Phytochemical Screening: The crude plant material in were screened phytochemically for the presence of tannins, glycosides, saponins, alkaloids and flavonoids using standard methods [9, 10].

Determination of Mycobactericidal Activity:

***M. smegmatis*:** The test organism, *Mycobacterium smegmatis* (MC2 155) was obtained from the Noguchi Memorial Institute for Medical Research, Legon, Ghana. Middlebrook 7H9 powder, nutrient agar, and all reagents used for experiments were purchased from VWR, U.S.A. An inoculating

loopful of isolated *M. smegmatis* culture was introduced into 20 mL of 5H9 Middlebrook broth and incubated for 24 hours at 37 °C. The bacterial culture was then standardized to contain 1 x 10^6 cell/mL by the help of previously calibrated bacterial suspension curve at 680 nm using a calorimeter.

Preparation of Test Extract: A concentration of 100 mg/mL of each of the water, 50% ethanol and petroleum ether extracts were prepared in sterile distilled water and sterile filtered through a 0.22 µM membrane filter to obtain sterile extracts.

Agar well Diffusion Method: Hundred microliters (0.1 mL) of 1x 10^6 cell/mL culture of *Mycobacterium* were added to 20 mL of stabilized nutrient agar at 45°C, mixed, poured into a sterile petri dish and the agar allowed to set. Five holes were bored equidistant from each other using a sterile bore of diameter 13 mm (cork borer number 6). A volume of 0.15 mL of each extract was introduced into a separate well appropriately labelled as such. Sterile water of 0.15 mL used for dissolution was introduced into the fourth well as the negative control and rifampicin 10 µg/mL was introduced into the fifth well as the positive control. The extracts were allowed to diffuse for 15 minutes at room temperature and then incubated at 37 °C for 24 hours. The diameter of the zones of inhibition were measured. The experiment was performed in triplicates.

Mutagenic Test:

Sample Preparation: *C. splendens* leaves were pulverized and extracted by decoction. Fifty grams (50.0 g) of pulverized material was boiled in 500 mL of distilled water for 20 min. The decoction was centrifuged at 6000 RPM for 10 min. The supernatant was collected and concentrated under vacuum at 40^{o}C and lyophilized to obtain dried crude extracts. The extracts were stored at -20 ^{o}C for use. The samples were prepared and diluted with sterile water on the day of the assay to a concentration of 10 µg/mL and sterile filtered using a 0.22 µM membrane filter.

Induction of Mutation: The Muta-ChromoPlateTM two strain kit (manufactured by Environmental Bio-Detection Products Inc, Ontario, Canada) which works on the principle of the Ames test was employed to determine the mutagenicity potential of the extracts [11]. The experiment was carried out in accordance with protocol provided by the manufacturer. Salmonella typhimurium TA98 was grown overnight for 14 h at 37^{o}C in 10 mL nutrient medium. The metabolic reaction mixture consisting of 4% S9 fraction, 1% 0.4 M MgCl2, 1% 1.65 M KCl, 0.5% 1 M D-glucose-6-phosphate disodium, 4% 0.1 M NADP, 50% 0.2 M phosphate buffer and 39.5% sterile distilled water were prepared into sterile 50 mL Falcon tubes. A 2.5 mL aliquot of the reaction mixture was added to each tube. Sterile water or sample material to be tested or 100 µL of the positive control (nitrofluorene) was added to the respective tubes. Five microliters of the bacteria suspension were added to each tube, except for the blank tube. The mixture was vortexed for 15 minutes and contents of each tube poured into sterile reagents boats and 200 µL dispensed into each well of a sterile 96-well plate and incubated for 5 days at 37^{o}C. Mutagenicity was detected either as an increase in the number of histidine revertants with reference to scores provided in the test kit to determine whether the mutation was significant or not. If a reverse mutation occurs, the bacteria in the colony have the ability to synthesize histidine and will continue to grow, turning the colour in the well from purple to yellow. The number of positive wells scored in the 96 well plates determined the significance of mutation in the fluctuation test [12].

The statistical table provided in the kit was used to compare the natural background rate of reverse mutation to the rate of reverse mutation within a sample assay. Based on these samples, p values of 0.001 were classified as highly mutagenic, p values of 0.01 moderately mutagenic and p values of 0.05 weakly mutagenic.

RESULTS AND DISCUSSION:

Pharmacognostic Parameters: The fresh leaves of*C. splendens*was observed to bedeep green in colour and had non-specific odour and tastes. A summary of these features is given in **Table 1**. The leaves exhibit entire margins and smooth-glabrous surfaces. This examination showed the leaves of *C. splendens* **Fig. 1** to be elliptical in shape, with a unicosate and reticulate leaf venation.

FIG. 1: LEAVES OF *CLERODENDRON SPLENDENS*

TABLE 1: MACROSCOPIC CHARACTERISTICS THE LEAVES OF *C. SPLENDENS*

Morphology	*C. splendens*
Shape	Elliptical
Margin	Entire
Venation	Unicosate and reticulate
Texture	Smooth

The vein islet number for *C. splendens* was 2 per mm^2 respectively with a veinlet termination of 2. Details of the results are shown in **Table 2.**

TABLE 2: LEAF CONSTANTS OF *C. SPLENDENS*

Parameter	*C. splendens*
Stomatal Number	5
Epidermal Cell Number	20
Vein islet Number	2
Veinlet termination number	2
The stomatal index [stomata per square mm of epidermis]	20

n=3, data given is mean±SEM

The leaves of *C. splendens* had anomocytic stomata and starch granules **Fig. 2A**, calcium oxalate crystals were visibly absent. **Fig. 2B** also displays the epidermal cells and vein terminations of *C. splendens*.

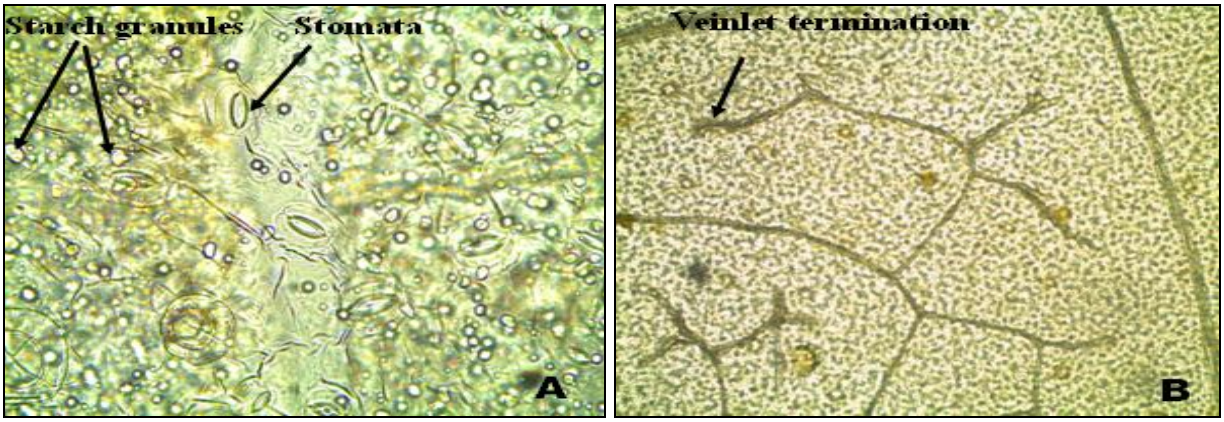

FIG. 2: MICROSCOPIC CHARACTERISTICS OF THE LEAF EPIDERMIS OF *C. SPLENDENS*

The yield of *C. splendens* in water, 50% ethanol and petroleum ether were 1.9, 5.7 and 1.4 %w/v respectively **Table 3** and **4** displays details of the phytochemical components of the extract of *C. splendens.*

TABLE 3: EXTRACTIVE VALUES

Solvent	*C. splendens* (% W/W)
Water	1.9
Ethanol 50%	5.7
Petroleum Ether	1.4

n=3, data given is mean±SEM

TABLE 4: PHYTOCONSTITUENTS OF *C. SPLENDENS*

Constituent	Results
Saponins	+
Alkaloids	+
Flavonoids	+
Tannins	+
Steroids	+
Glycosides	+

+: Present; - : Absent

TABLE 5: ASH VALUE PARAMETERS OF THE LEAVES OF *C. SPLENDENS*

Parameters	*C. splendens*
Total ash	6.17
Acid insoluble ash	7.00
Water soluble ash	11.50
Moisture content	11
Foreign organic matter	-
Foaming index	<100
Swelling index [mL]	2.33

n=3, data given is mean±SEM

Fluorescence Analysis: Analysis for characteristic fluorescence of the samples at short and long wavelengths showed varying colours for all the samples in specific solvents. These results are useful for both identity and quality determination of the samples and are presented in **Table 6.**

TABLE 6: FLUORESCENT STUDIES OF POWDERED *C. SPLENDENS* LEAVES IN VARIOUS SOLVENTS

	Day light	254 nm	365 nm
Powdered Sample	Greenish brown	Dark brown	Dark brown
Distilled water	Light brown	Blue	Pale blue
1N HCl	Light brown	Pale blue	Pale blue
1N NaOH	Black	Deep blue	Pale blue
50 % H_2SO_4	Deep green	Deep blue	Blue
Methanol	Light green	Bright red	Light red
Glacial acetic acid	Green	Red	Light red
Nitric acid	Orange	Blue	Pale blue
Chloroform	Dark green	Bright red	Light red
50 % $FeCl_3$	Dark brown	Deep blue	Light blue
95 % Ethanol	Light green	Bright red	Light red

Mycobactericidal Activity: Tests to evaluate the antimycobacterial activity of the extract of *C. splendens* showed no significant inhibitory effect against *M. smegmatis*

Mutagenic Activity: Mutagenic tests conducted on the extract of *C. splendens* showed that it was non-mutagenic for TA98. It is estimated that about 25% of drugs prescribed worldwide are derived from plants [13]. Even at the beginning of this century, 11% of the 252 drugs considered as basic and essential for healthcare by the WHO were exclusively of plant origins [14]. And in the current advent of antimicrobial resistance, it has become more imperative to actively search for more antimicrobial agents. The treatment of infections from both tuberculous and nontuberculous mycobacteria are currently daunting challenges to healthcare [15]. In that respect, the mycobactericial activity of the 50% ethanol extract of *C. splendens* was investigated, however no activity was detected in the agar well diffusion method at the concentration of 100 µg/mL against, *M. smegmatis*, a soil dwelling saprophyte related to *M. tuberculosis* [16]. The documented pharmacognostic characteristics provide preliminary data for identification of crude plant samples[17] of *C. splendens*. Ash analyses of the powdered sample yielded total ash of 6.17%, water soluble ash of 11.50% and acid insoluble ash of 7.00%. The ash values give an idea of inorganic constituents and other impurities that could be present in the sample. Acid insoluble ash gives a measure of the amount of silica present, especially in the form of sand and siliceous earth [18].

Hence, when this figure is excessively exceeded, it could be an indication of the presence of impurities. Phytochemical analysis revealed the presence of saponins, tannins, alkaloids, glycosides, flavonoids, tannins, and steroids which is in agreement with already published literature. The presence of Carbohydrates, steroids, terpenoids and flavonoids is reported in literature [4, 19]. The extractive values indicated weights of the extractable chemical constituents of the crude drug when different solvents are used for extraction [18]. The leaves of *C. splendens* contain more mid polar constituents as compared to the polar and non-polar ones.

In the evaluation of the test extracts of *C. splendens* against *M. smegmatis* for possible anti-mycobacterial activity using the agar diffusion method, the extracts were shown to have no significant mycobactericidal properties at the highest concentration of 10 µg/mL used for the assays even though the plant, from the same region, had been shown to have antimicrobial activity against some Gram-positive and Gram-negative organisms [19].

In the studies of the 100 µg/mL extract of *C. splendens,* it was determined to be non-mutagenic againt TA 98 strain. Mutation in *Salmonella typhimurium* TA 98 is indicative of a frame shift mutation (FSM), this could therefore suggest that these plant samples could contain molecules that can insert (intercalate) between the normal bases to create mistakes during DNA synthesis. The determined pharmacognostic indices can be employed for identification, purity and quality assessment of the respective *C. splendens*.

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