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Cytotoxicity of the Aqueous Extract of *Clerodendrum splendens* on HaCaT Keratinocytes

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Authors' contributions

This work was carried out in collaboration between all authors. Author EOB designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Author CA wrote the protocol, managed the analyses of the study and the literature searches. Author SB developed the HPLC chromatogram of the extract. All authors read and approved the final manuscript.

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ABSTRACT

Clerodendrum splendens G. Don (Family Verbenaceae) has been used for centuries to treat various medicinal problems in Africa, including the treatment of wounds and other skin conditions. Though several studies have been conducted on the biological activities of this plant, none so far determines the effect of the leaves extract of *C. splendens* on skin cells and hence predicting its toxicity. This research aims at investigating the *in vitro* physiological effect of the aqueous extract of *C. splendens* on human adult low calcium high temperature keratinocytes (HaCaT). Keratinocytes are the main cells of the skin epidermis and any agent used for the treatment of skin conditions will

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first come in contact with these cells. The functional activities of the aqueous extract of *C. splendens* were investigated by determining effect on the cellular metabolic activity, proliferation and necrotic cytotoxicity by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bromodeoxyuridine (BrdU) and lactate dehydrogenase (LDH) assays respectively. The HPLC finger print chromatogram was also developed for quality control. There was no significant effect on the metabolic activity, rate of proliferation as well as LDH leakage from the HaCaT cells. According to these results, the aqueous extract of *C. splendens* did not show toxicity over the tested concentrations of the extract. The extract showed a tendency towards increased proliferation and viability at low concentrations of 0.1 and 1 µg/mL.
The positive effect at 0.1 and 1 µg/mL seems to suggest the absence of toxicity at those concentrations but a decrease in metabolic activity and proliferation at higher concentrations could be a dose dependent cytotoxic effect.

Keywords: Clerodendrum splendens; in vitro; toxicity; HaCaT keratinocytes.

ABBREVIATIONS

5-bromo-2'-deoxyuridine (BrdU); 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT); LDH (lactate dehydrogenase); rhEGF (Epidermal Growth Factor, Human, Recombinant); MTP (microtre plate reader); FCS (fetal calf serum).

1. INTRODUCTION

C. splendens G. Don (Family Verbenaceae) is a tropical shrub that can grow to become a climber [1]. It produces attractive red flowers during the dry seasons from December – April. The flowers are small complete pedicellate, pentamerous, zygomorphic, hermaphrodite and hypogynous [2,3]. Extracts of the roots, leaves and bark of *C. splendens* are used to treat malaria, coughs, buboes, venereal infections, gonorrhea, syphilis, skin diseases, ulcers, rheumatism, asthma, uterine fibroid and also used as a vermifuge and febrifuge [4,5,6,7]. Leaves of this plant have also been used topically as a medicinal wound-healing agent for bruises, blisters, sores and burns [8]. In addition to the above, this plant is also used traditionally in Ghana for the treatment of vaginal thrush and various skin infections [8]. Many biological activities of the extract from this plant have also been investigated. The methanolic extract of the aerial parts of this plant has been shown to have significant antimicrobial activity against Gram positive bacteria, Gram negative bacteria and fungi [9].

On its phytochemistry, carbohydrates, glycosides, unsaturated sterols, triterpenoids and flavonoids are reported to be present in the leaves [10] and volatile oil in the flowers [11]. Clerodane diterpenes and a phenylpropanoid have been isolated from the aerial parts of this plant [12]. High-molecular weight polysaccharide obtained from *C. splendens* has been shown to be a potent natural innate immunomodulator with a broad spectrum of agonist activity *in vitro* and has immunosupressive properties after chronic administration *in vivo* [7].

In wound healing, the extract is known to have beneficial effect on reducing the time required for epithelialization and decreasing scar area while increasing the tensile strength of the wound [9]. Though this plant is widely administered topically to treat various skin conditions, its cytotoxic effect has however not been determined on skin cells. Since the aqueous extract is the most common preparation for local application [7], we investigated its effect on skin keratinocytes. The keratinocytes form majority of the cells in the epidermis and their role in the skin and recovery after skin injury cannot be overstated. Epidermal injuries are caused by several conditions including physical, chemical or thermal destruction of the skin and even by infection or inflammation. Re-epithelialization is an important process during the early stages of epidermal wound healing and involves the migration, proliferation, as well as differentiation of keratinocytes from the wound margins [13]. Appropriate re-epithelialization requires not only the development of a continuous epidermal layer but also full epidermal differentiation and the formation of junctions between the epidermis and the dermis [13]. Epidermal keratinocyte differentiation to corneocytes therefore leads to the formation of the squamous epithelium that forms the protective covering of the skin [14]. Based on the state of differentiation of keratinocytes, the epidermis forms the Stratum

corneum, Stratum granulosum, Stratum spinosum and Stratum basale.

2. MATERIALS AND METHODS

2.1 Harvesting of Plant Material

The whole plant material was collected from Ayigya, Kumasi in the Ashanti Region of Ghana in July 2014 and was authenticated by Mr. Asare at the Department of Pharmacognosy, Kwame Nkrumah University of Science and Technology, Ghana. A voucher specimen (FP/07/0032) has been kept in the herbarium of the Department of Pharmacognosy, KNUST.

2.2 Extraction of Plant Material

The leaves of *Clerodendrum splendens* were separated from the rest of the plant and air dried at room temperature at approximately 30ºC for three weeks and pulverised to a coarse powder. One gram of material was extracted with 10 mL of water, by ultra-sonication for 15 min followed by centrifugation at 6000 × g for 10 min. The clear supernatant was collected and the residue extracted again with another 10 mL of the same solvent. The combined extracts were concentrated under vacuum at 40ºC and lyophilized to remove residual water from the crude extract. An extract yield of 12% w/w relative to the dried plant material was obtained.

2.3 Ethical Approval

Approval of the cytotoxicity studies was made by the local Ethical Committee of University of Muesnter, Muenster, Germany (2006-177-f-S).

2.4 Methods of Cell Biology

2.4.1 Passaging of HaCaT keratinocytes

HACAT keratinocytes were obtained from the Germany Cancer Research Center (DKFZ), Heidelberg, Germany by the assistance of Prof. Dr. Norbert Fusenig. The HaCaT cells were seeded in 96 well plates at a density of 5000 cells per well in 100 µL of HaCaT medium. The cells were passaged at 37ºC, in 5% CO_2 for 48 h and then incubated with 100 µL of freshly prepared test solutions of the extracts and controls which were prepared with MCDB 153 basal medium (Biochrom AG, Berlin, Germany) supplemented with bovine insulin, rhEGF, ethanolamine, hydrocortisone and L-glutamin. Untreated controls were incubated in MCDB complete medium only, while the positive controls were incubated in MCDB complete medium supplemented with 5% FCS. The aqueous extract was dissolved in the medium above and made sterile, by filtering through a syringe filter (Corning Incorporated, NY, U.S.A.) to obtain concentrations of 0.1, 1, 10 and 100 µg/mL. The cells were incubated with the test substances for a further 48 h at 37ºC, in 5% CO_2.

2.5 *In vitro* Tests to Determine Functional Activity

2.5.1 Metabolic activity (MTT assay)

The MTT colorimetric assay is a widely used tool in cell biology for measuring the viability of cells [15]. It is designed to determine the viability of cells as a function of mitochondrial succinate dehydrogenase activity. This assay makes use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which is reduced to a blue coloured formazan by mitochondrial dehydrogenase [16]. The coloured formazan is characterized by high absorptivity at λ 570 nm. After incubation with the test substances, 50 µL of MTT reagent (5 mg/ mL in PBS) was added to the cell and incubated for 6 h. The reagent was flicked off and a 50 µL quantity of DMSO was added to enable dissolution of the formazan crystals. The absorbance was measured with the MTP (microtitre plate) -reader at λ 595 nm to a reference wavelength of λ 690 nm. FCS 1% was used as the positive control. FCS is a substance which is highly nutritious and enriched with growth factors, vitamins etc, know to increase both the cellular viability and rate of proliferation of cells. Thus it was used as a positive control respectively in these assays.

2.5.2 BrDU proliferation assay

This assay was used to measure the effect of the test extracts on the rate of proliferation of the epidermal cells. This technique is based on the incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. After its incorporation into the DNA, BrdU is detected by immunoassay with monoclonal antibodies highly specific for BrdU [17]. In this assay, the BrdU proliferation ELISA kit (Roche, Mannheim, Germany) was employed and the assay was performed according to the manufacturer's instructions.

2.5.3 LDH cytotoxicity assay

One parameter for the measurement of cell death is the integrity of the cell membrane. Cell membrane integrity can be assessed by cytoplasmic enzyme activity, such as lactate dehydrogenase (LDH) that is released into the surrounding medium by damaged cells. This enzyme is a stable cytoplasmic enzyme present in all cells which is rapidly released into the cell culture medium upon damage of the cytoplasmic membrane [18]. The LDH leakage was determined with the LDH Cytotoxicity Detection Kit (Roche Diagnostics, Milan, Italy). In this assay, the first step is the reduction of NAD^+ to $NADH/H^+$ by the LDH catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan with a broad λ max at 500 nm [19,20]. Similar to the procedure as in section 2.3.1, cells were passaged and incubated with test substances. After the incubation period, 30 µL of the medium in each well was transferred into a new well. Thirty microlitre (30 µL) of the 10% Triton-X 100 was added to the remaining cells for 30 min (as positive control). Thirty microclitres (30 µL) of the reaction mixture (INT-Catalyst solution 44:1) was added to the supernatant and the plate incubated in darkness, at room temperature for 30 min. The reaction was stopped by adding 10 µL of 1 M HCl acid followed by 5 µL of ethanol (96%) to prevent the formation of bubbles. The cytotoxicity of the test solution, which is the percentage LDH released relative to the maximum lysis, was performed as per the manufacturer's instruction and calculated with the formula below. Triton-X 100 is a nonionic detergent capable of disrupting cellular membrane by solubilizing membrane proteins leading to cellular necrosis, hence was used as the positive control.

$$\text{Cytotoxicity}[\%] = \frac{A_{\text{test-solution (supernatant)}} - A_{\text{untreated control}}}{A_{\text{lysed-cells-solutions}} - A_{\text{untreated control}}}$$

2.6 HPLC Finger-printing

The HPLC finger-printing of *C. splendens* was determined to serve as a reference for future identification of this plant extract, as well as for the purpose of quality control. The HPLC fingerprinting was determined with Chromatographic conditions: mobile phase: water: acetonitrile (98:2) v/v (isocratic condition), Flow rate: 1 mL/min, Column: Phenomenex; Luna 5micron C18; 150 x 4.60mm, temperature: 22℃, Detector: Perkin Elmer 785A UV-Visible detector, Pump: Spectra series P100, Integrator software: Powerchrom, injection volume: 10 μL, detection wavelength: λ254 nm, and run time: 10 min.

2.7 Statistical Analysis

GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Data are presented as mean ± SEM (n = 10) and analyzed by the Kruskal-Wallis test.

3. RESULTS

The aqueous extracts from *C. splendens* did not affect mitochondrial activity significantly at concentrations of 0.1 to 100 µg/mL (Fig. 1). A non-significant increase in cell energy status was accompanied by a non-significant increase in cellular proliferation at 0.1 and 1 µg/mL (Fig. 2). In the necrotic cytotoxicity assay, the extract showed no significant cytotoxicity or cytoprotective effect over the concentrations tested (Fig. 3).

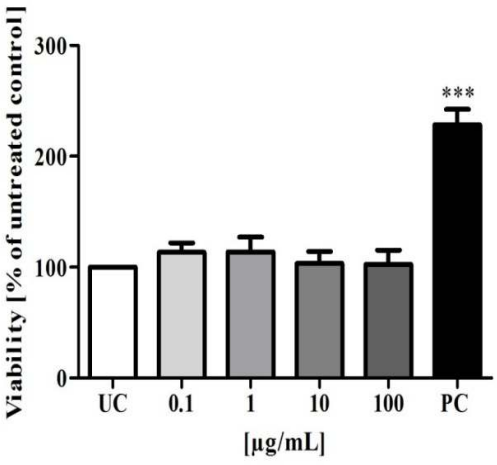

Fig. 1. Effect of the aqueous extract of *C. splendens* on *in vitro* cell viability of HaCaT keratinocytes treated with 0.1, 1, 10 and 100 µg/mL. UC: Untreated control, PC: Positive control (5 % FCS)

Bars represent mean ± SEM (n = 10), the results being replicates of 3 independent experiments, compared to the untreated control

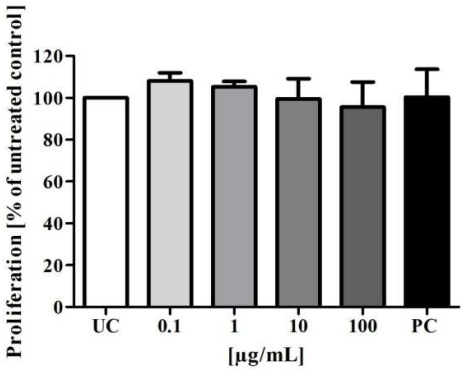

Fig. 2. Effect of the aqueous extract of *C. splendens* on *in vitro* cellular proliferation of HaCaT keratinocytes treated with 0.1, 1, 10 and 100 µg/mL. UC: Untreated control, PC: Positive control (1 % FCS)

Bars represent mean ± SEM (n = 10), the results being replicates of 3 independent experiments, compared to the untreated control

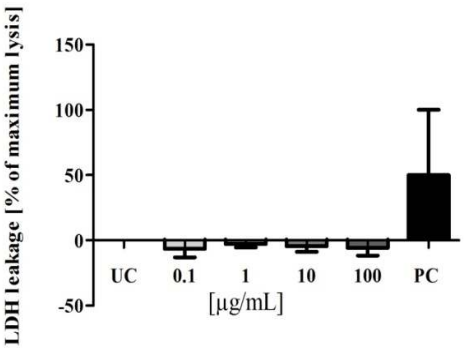

Fig. 3. Effect of the aqueous extract of *C. splendens* on *in vitro* necrotic activity on HaCaT keratinocytes treated with 0.1, 1, 10 and 100 µg/mL. UC: Untreated control, PC: Positive control (10 % Triton-X 100)

Bars represent mean ± SEM (n = 10), the results being replicates of 3 independent experiments, compared to the untreated control. UC: untreated control, PC: Positive control, 10 % Triton X-100 for LDH assay

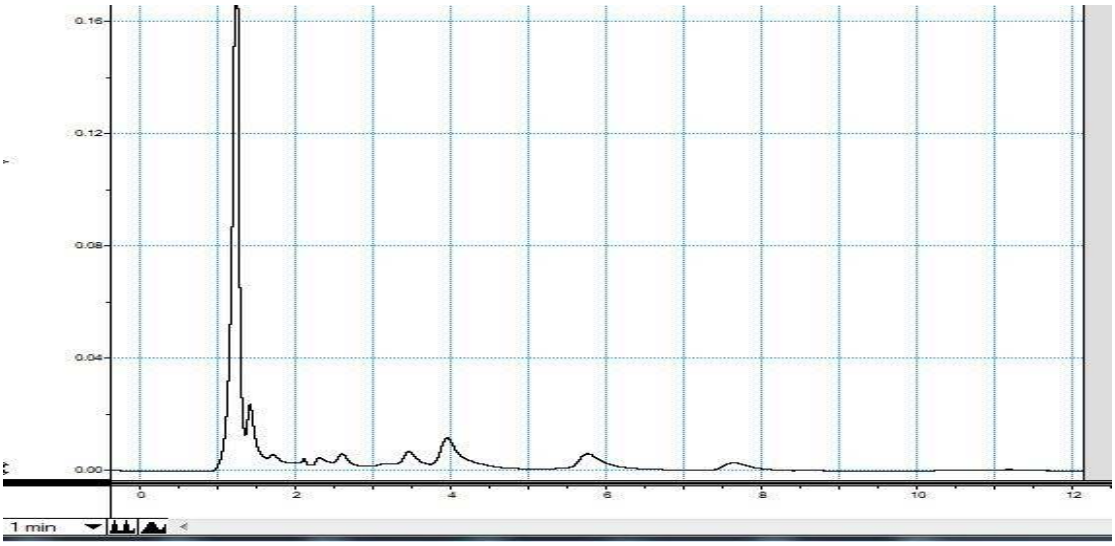

Fig. 4. HPLC fingerprint chromatogram of the aqueous extract of *Clerodendrum splendens*, λ 254 nm

4. DISCUSSION

Cytotoxicity assays is highly recommended before any investigation of mechanisms as well as further *in vivo* experiments are carried out, because it enables toxic concentrations of drug substance to be identified before further in-depth research [21]. *In vitro* cytotoxicity testing provides a crucial means of ranking compounds for consideration in drug discovery [22], hence the importance of this study. The treatment of HaCaT keratinocytes with the aqueous extract of *C. splendens* at concentrations of 0.1, 1, 10 and 100 µg/mL resulted in no significant effect on viability and proliferation of HaCaT keratinocytes (Figs. 1 and 2) when compared to the untreated control. The insignificant effect on energy status and proliferation will be beneficial in the long run for the skin cells since it indicates the absence of toxicity. The insignificant dose dependent reduction in LDH leakage could possibly have been a result of a cytoprotective activity of the extract or due to decrease in the number of cells as the concentrations increases, as shown by the

reduction in rate of proliferation, as seen in the BrdU proliferation assay. For keratinocytes, maintenance of viability with a corresponding non-significant effect on proliferation indicates that the cells could be undergoing cellular differentiation [23]. Differentiation of basal keratinocytes are known to result in a loss of growth potential [24]. This is beneficial for skin regeneration after injury or infection since differentiation of keratinocytes results in the formation of the epidermal skin layers.

It is also well known that the therapeutic effects of a herbal extract is based on the synergistic effect of its mass constituents [25]. Hence, constituents of *C. splendens* such as carbohydrates, glycosides, unsaturated sterols, triterpenoids, flavonoids and polysaccharides [10,7] are likely to be responsible for the biological effect seen on the HaCaT cells. Polysaccharides for example are well known phytoconstituents that positively affect the wound healing process by increasing the rate of proliferation and differentiation of epidermal keratinocytes [26-28]. Flavonoids are also known to positively affect epidermal wounds by inducing the proliferation of fibroblasts, production of matured collagen fibres which result in better angiogenesis [29,30].

5. CONCLUSION

In addition to its already known antimicrobial activities, anti-oxidant activities and *in vivo* wound healing activities, the aqueous extract of *C. splendens* has been shown to have no significant effect on the rate of *in vitro* cellular proliferation, viability and LDH leakage of HaCaT keratinocytes. The absence of toxicity is a good effect for wound healing activity. These results indicate that the extract from *C. splendens* could be a non-toxic topical agent and this may justify its extensive use in the treatment of the various skin conditions. From the above results, there is also a possible indication that at higher concentrations than a 100 μg/mL, there could be an increase in adverse effect of the extract on the viability and rate of proliferation of the keratinocytes of the skin. Hence doses higher than these may not be used during therapy. However further investigations will have to be made on the effect of the extract on other skin cells such as fibroblast and melanocytes and *in vivo* studies conducted on the extracts to ascertain the pharmacological activities of specific compounds that are present in the extract.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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