Journal of Applied Biological Sciences
E-ISSN: 2146-0108 16(3): 503-526, 2022
DOI: 10.5281/zenodo.7114250
Research Article

ANTI-INFLAMMATORY EFFECTS AND KERATINOCYTE REGENERATIVE POTENTIAL OF *CASSIA ALATA* (LINN) LEAF EXTRACTS AND THEIR IMPLICATIONS FOR WOUND HEALING

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(Received 23th November 2021; accepted 01th June 2022)

ABSTRACT. In this study, dried and ground plant leaves of *Cassia alata* were extracted with methanol and water, which were then subjected to various analyses. Morphological changes of human keratinocytes in response to plant extracts were observed with a differential interference contrast (DIC) microscope imaging system. The cell viability and proliferation effects of the plant extracts were also evaluated via colorimetric cytotoxicity assays and the CyQUANT® assay. Anti-inflammatory effects of the plant extracts were evaluated by flow cytometry with a cytometric bead array (CBA) kit and also cyclooxygenase (COX)-1, COX-2 and 5-lipoxygenase (5-LOX) enzyme inhibition assays. Results of the DIC microscopy assay indicated that Human keratinocytes treated with either the *C. alata* methanol (CAM) or aqueous (CAA) extracts (0.1-0.2 mg/mL) achieved 100% confluency. Cytotoxicity testing confirmed that the plant extracts were not toxic to keratinocyte cells at the concentrations used in the study. Only two cytokines *i.e.* interleukin (IL)-6 and IL-8 were detected by the CBA method, with the results confirming that none of the plant extracts induce a pro-inflammatory effect. Moreover, CAM and CAA extracts showed strong anti-inflammatory effects in suppressing IL-8. Both plant extracts (6.25-100 µg/mL) demonstrated strong inhibitory effects on COX-1 and 5-LOX enzymes compared to the standards *i.e.* indomethacin and nordihydroguaiaretic acid, respectively. However, inhibition of the COX-2 enzyme was less compared with indomethacin. The CAM extract at a concentration of 6.25 µg/mL, produced strong inhibition of COX-1 and 5-LOX enzymes, which was greater than that of the respective control treatments at the same concentration. Hence, our results indicate that the *C. alata* leaf extracts have a strong anti-inflammatory potential, which could be used to treat wounds and inflammation associated with the skin.

Keywords: *Cassia alata, anti-inflammatory, cytotoxicity, cell viability, proliferation, keratinocyte.*

INTRODUCTION

Cassia alata, also known as *Senna alata* [1] and more commonly "candle bush", belongs to the Family Fabaceae [2, 3]. *Cassia alata* is a beautiful flowering shrub that grows approximately 1-2 m in height and produces dazzling bright yellow flowers in a column that resemble candlesticks [2, 4]. Dark green leaves consist of 10-12 pairs of leaflets that are oblong in shape with an approximate length of 7-11 cm and a width of approximately 3-8 cm [5]. Bilateral leaves are alternately arranged and fold upon itself at night [2]. The floral structure consists of sepals, petals, and stamens fuse together [5], whilst the fruit manifests as a pod containing 50-60 flattened seeds and measures 15-30 cm [2]. This plant grows amply in sunny areas where the soil is damp [5]. Native to the

Amazon rainforest, the plant can also be found elsewhere in South America, tropical regions of Asia and Africa, Central America, Polynesian Islands, Australia and India [2, 4].

Plants in the *Cassia* genus generally produce a variety of secondary metabolites, which include anthraquinones, alkaloids, flavonoids, and pyrrolizidine alkaloids, triterpenes, steroids and tannins, thus, they have attracted attention as potential sources of medicinal treatments [5, 6]. Chatterjee and colleagues [2] reported that across the globe, *C. alata* is used for a wide variety of treatments. The plant species is also widely used as a traditional medicine in India and Southeast Asia for various ailments [7]. Moreover, the efficacy of the plant is not limited to infection and biological illness as the leaves are an ingredient in various dermatology products [5]. Recently, biotechnological applications of *C. alata* extracts have been proposed for use within the cosmetic industry [8]. Chatterjee and colleagues [2] reported multiple accounts from different cultures that relates to the use of *C. alata* for the treatment of skin disorders and wounds. In Northern Nigeria, the root, stem and leaves of *C. alata* are used to treat burns, wounds and skin infections [9]. Preparations of *C. alata* leaves are also used topically to treat wounds by local healers in the Sangli district, Maharashtra, India [10].

Wound closure is a dynamic mechanism that involves a combination of proliferation and migration of skin cells at the wound site [11]. The human epidermis is composed of multiple layers of keratinocytes that are constantly renewed to replace worn out or damaged cells [11]. Fibroblasts and keratinocytes are the main component of the wound closure mechanism [11]. Proliferation and migration of keratinocytes are essential for the re-epithelialisation of skin wounds [12], while fibroblasts at the wound site, secrete extracellular proteins facilitating the underlying keratinocytes to proliferate rapidly [13]. Cytokines interleukin-α (IL-α) and transforming growth factor-β (TGF-β) perform a critical role in fibroblast-stimulated keratinocyte proliferation [12]. HaCaT cells are a spontaneously immortalised, human keratinocyte cell line, which has been widely studied in skin biology [14]. Moreover, HaCaT cells not only have minimal variability in their growth characteristics *in vitro*, but they also show miniscule changes in proliferation and differentiation characteristics as passage number increases. Hence, under carefully optimised *in vitro* conditions, HaCaT cells provide a reliable model to screen anti-inflammatory compounds [15].

Inflammation, an immune response to infection and injury, is an essential process that aids in the removal of pathogenic factors, thereby restoring normal physiological function [16]. Cytokines are also intrinsically involved and are principal mediators in inflammation [17]. Keratinocytes, vascular endothelial cells and leukocytes such as neutrophils, monocytes and macrophages, secrete pro-inflammatory cytokines during the wound healing process [18, 19]. Interleukins (IL) are a subset of cytokines [20] and their over expression, including tumour necrosis factors (TNF), have been linked to activation of pathogenic inflammation [17]. TNF-α and IL-6 are important pro-inflammatory cytokines with multiple vital functions in pathogenesis [21, 22, 23]. IL-6 induces neutrophil and macrophage infiltration and mediates collagen deposition, angiogenesis and epidermal cell proliferation, however, elevated levels of IL-6 is a sign of a prolonged inflammatory phase within the wound healing cycle [24]. IL-8 has numerous functions, including activation and chemotaxis of white blood cells, production of endothelial cell adhesion proteins and the promotion of keratinocyte maturation [25]. However, the production of IL-8 is also an indicator of infection [26, 27].

Inflammation can be suppressed by the inhibition of cyclooxygenase (COX) enzymes [28]. The chemical entities which can block the active site of cyclooxygenase-2 are more commonly known as COX-2 inhibitors [28]. COX enzymes catalyse the biosynthesis of prostaglandins and thromboxanes from arachidonic acid [29]. Arachidonic acid released from phospholipids in the cell membrane is the precursor for many inflammatory mediators, depending on different pathways involved in the inflammatory response [16, 30, 31]. COX and lipoxygenase (LOX) are two essential enzymes involved in generating arachidonic acid derivatives [31, 32, 33]. While COX-1-derived prostaglandins are responsible for the initial phase of acute inflammation, COX-2 regulation occurs within several hours from the initial onset of the process [16]. COX-2 expression is induced by pro-inflammatory stimuli and results in the generation of prostaglandins [31], which play a significant function as mediators of acute inflammation [30]. LOX enzymes catalyse the oxygenation of polyunsaturated fatty acids such as arachidonic and linoleic acids to produce biologically active hydroperoxides mediators such as leukotrienes that are involved in important inflammatory cell signaling mechanisms [31, 34]. Both prostaglandins and leukotrienes are potent signaling molecules that prolong inflammation [32, 33]. Hence, COX and LOX are potential targets of anti-inflammatory drug development [35]. Potential anti-inflammatory agents can be used to suppress the over secretion of pro-inflammatory mediators as an intense inflammatory response may ensue [36].

Formulations such as decoctions and topical applications of *C. alata* are claimed to be effective against wounds and relieve inflammation, however, the pharmacological properties of the plant have not yet been scientifically validated. Moreover, its wound healing properties to date, remain unknown. As such, this project investigated the functional responses of human skin cells to bioactive compounds present in *C. alata* and assessed anti-inflammatory effects, with regards to potentiating wound healing.

MATERIALS AND METHODS

Plant Material

C. alata plants were commercially obtained and grown in native potting mix. Mature, healthy leaves collected from one-year old plants, were dried at 40 °C in an oven for 48 hours and ground into a fine powder.

Extraction

Ground plant material (20 mL of methanol per 5 g of plant material) was incubated in methanol (99.8%) for 48 hours at room temperature (RT) followed by vacuum filtration. Extracts were concentrated and dried at 40 °C/2,000 rpm (Scan Speed 40, LabGear, Australia) to obtain a dry residue. For the aqueous extraction, ground plant material (100 mL of water per 5 g of plant material) was added to a beaker containing boiling milli-Q water (arium™ pro, Sartorius, Germany). The plant solution was boiled continuously until the volume reduced to approximately 20 % v/v and then allowed to cool to RT. Next, the contents were transferred to a separate clean mortar, the liquid was squeezed out using a clean pestle, collected and centrifuged (Microfuge™18 Centrifuge, Labtronics, Australia) at 12000 rpm for 5 minutes at RT. The supernatants were evaporated in a rotary vacuum concentrator (Scan Speed 40, LabGear, Australia) at 40 °C/2,000 rpm until a dry residue was obtained.

Human Keratinocyte Cell Culture

Human immortalized keratinocytes (HaCaT, Addexbio Technologies, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v 50 mL foetal bovine serum (FBS), 1% v/v (5mL) 200 mM L-glutamine, 1 % v/v (5 mL) of 50 Units/mL of penicillin and 5 mg/mL streptomycin. Complete DMEM was denoted as full growth media (FGM). Cells were maintained in a humidified incubator at 37 °C, 5% CO_2 and passaged at approximately 80 % confluence using 1x trypsin-ethylenediaminetetraacetic acid (EDTA). An additional media solution was made with DMEM supplemented with 2% v/v (10 mL) FBS, 0.2% v/v (1 mL) of 200 mM L-glutamine, 0.2% v/v (1 mL) of 50 Units/mL of penicillin and 5 mg/mL streptomycin, which was referred to as reduced serum media (RSM). Pure DMEM, without any additions, was designated as serum free media (SFM).

Morphological Assay

HaCaT cells were observed using a Leica AF 6000LX bright field differential interference contrast confocal microscope system (Leica Microsystems, Germany), encased in a special humidified incubator at 37 °C, 5% CO_2. Cells were plated at a density of 1 x 10^5 cells/mL in a 96-well plate in FGM and allowed to attach for 24 hours at 37 °C in 5% CO_2. The media was then aspirated, and cells were separately treated with a 100 µL solution of *C. alata* extracts in SFM, at concentrations ranging from 0.05-0.8 mg/mL. Positive and negative controls were provided by untreated cell suspensions prepared in FGM and SFM, respectively. Plates were placed in a humidified incubator at 37 °C, 5% CO_2 for 72 hours. Images at 1000x magnification were captured every 15 minutes throughout a 72-hour time course. All images were analysed with LAS-X software platform (Leica Microsystems, Germany).

Human Keratinocyte Cell Viability Assay

Metabolic viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell metabolic assay (Sigma-Aldrich, Australia). Absorbance of the converted dye in cell suspensions were analysed by a microplate reader (POLARstar Omega, BMG Labtech, Germany). Optical density (OD) was measured at a wavelength of 570 nm and was proportional to its concentration. The OD was expressed as the mean value of the relative percentage viability of the treated samples, which was calculated using the following equation: (P-T)/P×100 where, P is the OD of the positive control at 570 nm and T is the OD of the test samples at 570 nm. Keratinocytes were plated in FGM at a density of 1x10^5 cells/mL in a 96-well plate. After overnight growth, the media was aspirated, and each sample well was treated separately with 100 µL of *C. alata* extracts at concentrations ranging from 0.05 - 1.6 mg/mL in RSM. Plates were incubated for 24 hours at 37 °C, 5% CO_2, after which 10 µL of 5 mg/mL MTT in phosphate buffered saline (PBS) [37] was added and further incubated for 4 hours at 37 °C, 5% CO_2. At the end of the incubation period, 110 µL of acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol) was added and the plates were gently shaken in the incubator at 37 °C, 5% CO_2 for 1 hour. The positive control was supplied by untreated cells in 100 µL of RSM, while the blank control consisted of 100 µL of RSM, which provided the background reading.

Direct Cell Proliferation Assay

The CyQUANT® direct cell proliferation assay kit (Thermo Fisher Scientific, Australia) was used to evaluate the potential effects of *C. alata* extracts on HaCaT cell viability. As per manufacturer's instructions, HaCaT cells cultured in FGM were seeded at a density of $1x10^5$ cells/mL in a 96-well plate (ThermoFisher, Australia) and incubated at 37 °C, 5% CO_2 for a 24 hour period to allow attachment. The media was aspirated and 100 µL of *C. alata* extracts at concentrations ranging from 0.05-1.6 mg/mL in RSM, were added separately to the cells. Untreated cells in 100 µL of RSM acted as the positive control while 100 µL of RSM free of cells served as the negative control. The microplate was incubated at 37 °C, 5% CO_2 for 24 hours. 100 µL of 2X detection reagent in RSM was added to the cells and incubated for 60 minutes at 37 °C. A reference standard curve was also performed using nine suspensions of known cell concentrations (0 = blank control, $1.25x10^4$, $2.5x10^4$, $5.0x10^4$, $1.0x10^5$, $2.0x10^5$, $4.0x10^5$, $8.0x10^5$, $1.6x10^6$ cells/mL) in RSM. Fluorescence was read at an excitation wavelength of 480 nm and emission wavelength of 520 nm in a microplate reader (POLARSTAR Omega, BMG Labtech, Germany). Since the fluorescence intensity is linearly dependent on cell number, the fluorescent reader quantifies the number of healthy cells present, using the prepared standard curve. Data were processed using Optima data analysis software version 2 (BMG Labtech, Germany).

Cytometric Bead Array (CBA) for Analysing Cytokine Suppression

A CBA human inflammatory cytokine kit (BD Biosciences, USA) was used to measure the level of cytokine expression of interleukin (IL)-12p70, tumour necrosis factor (TNF)-α, IL-10, IL-6, IL-1β and IL-8 in the supernatants of post lipopolysaccharide (LPS) stimulated HaCaT cell suspensions subsequent to treatment with the plant-derived extracts. The protocol by Hong *et al* [38] was followed in the preparation of the mammalian cells, while the implementation of the assay was executed as per manufacturer's instructions [39]. 500 µL of HaCaT cells in FGM were seeded at $5×10^5$ cells/mL in 24-well plates (ThermoFisher, Australia) and incubated at 37 °C, 5% CO_2 for 24 hours. After incubation, the media was aspirated, and cells were submitted to four separate treatments. Plant extracts (400, 200 and 100 µg/mL) were prepared in 500 µL of RSM and separately added to 500 µL of cell suspension in RSM previously treated with a 1 µg/mL solution of LPS *E. coli* serotype 055:B5 488 conjugate (Alexa Fluor™, Thermo Fisher Scientific, Australia). The resulting suspensions with plant extracts (200, 100 and 50 µg/mL) constituted the test samples. The positive controls were provided by a 1 mL cell suspension in RSM treated with 1 µg/mL solution of LPS. The negative control was provided by a 1 mL cell suspension in RSM without LPS and zero extracts. The 1 mL suspensions treated with the extracts (200, 100 and 50 µg/mL) in RSM represented the baseline controls (BC). The plates were then incubated at 37 °C, 5% CO_2 for 48 hours. Next, the cells were removed with a disposable cell scraper and their suspensions collected and centrifuged at 300 g for 10 minutes at RT. Supernatants were then collected and stored at -80 °C until required. Samples were analysed on the flow cytometer CytoFLEX™ S (Beckman Coulter Life Sciences, Australia), along with 10 standards made from a vial of mixed lyophilized human cytokines included in the kit. A standard curve of cytokines was established at the following concentrations: 0, 20, 40, 80, 156, 312.5, 625, 1250, 2500, 5000 pg/mL. Each cytokine had a standard curve which was made by using their mean fluorescence intensities and concentrations. Each mean was fitted by

linear regression and justified by their own coefficient of determination. The interpolation of each cytokine concentration within unknown test samples, BCs and positive control were normalised against the negative control and averaged. Results were analysed and processed with BD FCAP Array 3.0 (Soft Flow Limited, Hungary) software.

Enzyme Inhibition Assays

Cyclooxygenase (COX) Inhibition Assay

The COX-1 and COX-2 peroxidase end-point assays were carried out according to the method described by Gierse and Koboldt [40] with slight modifications. 20 µL of plant extracts dissolved in dimethyl sulfoxide (DMSO) were plated in triplicate at different concentrations (6.25-100 µg/mL) in a 96-well microtiter plate (ThermoFisher, Australia), followed by 20 µL of 10 U/mL of COX-1 or COX-2 (Cayman Chemical Company, USA) enzymatic solution. 160 µL of the endpoint assay mix consisting of 100 µM bovine hemin chloride, 10 mM of arachidonic acid, 17 mM of TMPD and 1 M of tris-chloride buffer at pH 8.0 was added and incubated for 10 min at 25 °C. Absorbance was recorded at 590 nm using a microplate reader (CLARIOSTAR^{TM} BMG Labtech, Germany). Concentrations of indomethacin ranging from 3.125-100 µg/mL were used as the standard. A sample with 20 µL of buffer instead of inhibitors was used as the positive control (assumed as 100% activity), while the negative control was provided by a sample without inhibitors and enzyme. Absorbance readings of the samples, standards and positive control were normalised against the negative control. Percentage inhibition of the COX enzymes were determined with absorbance of samples relative to positive control using the formula: $(E- S)/E\times100$, where E is the absorbance without any inhibitor and S is the absorbance of test sample.

Lipoxygenase (LOX) Inhibition Assay

The LOX assay was conducted according to the method described by Baylac and Racine [41] and Kamatou *et al* [42] with slight modifications. Ice-cold tris-chloride buffer, pH 7.4 at 4 °C was mixed with 100 U of the thawed 5-LOX enzyme (Cayman Chemical Company, USA). 20 µL of plant extracts dissolved in DMSO were plated in triplicate in a 96-well microtiter plate (ThermoFisher, Australia) at different concentrations (6.25-100 µg/mL), followed by 160 µL of 0.1 M tris-chloride buffer pH 7.4 and maintained at 25 °C. A 20 µL of the 5-LOX enzyme was added to each well, mixed and agitated with 20 µL of arachidonic acid and incubated for 10 min at 25 °C. Concentrations ranging from 3.125-100 µg/mL nordihydroguaiaretic acid (NDGA) were used as the standard. A sample with 20 µL of buffer instead of inhibitors was used as the positive control (assumed as 100% activity) while the negative control was provided by a sample without inhibitors and enzyme. Samples were measured at 243 nm using a microplate reader (CLARIOSTAR^{TM}, BMG Labtech, Germany). All absorbance readings were normalised against the negative control. Percentage inhibition of the 5-LOX enzyme with absorbance of samples relative to positive control using the formula: $(E-S)/E\times100$, where E is the absorbance without any inhibitor and S is the absorbance of test sample.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, USA). Triplicate samples were assayed in each technical experiment and replicated three times (n=9). The comparison among multiple groups was performed by

one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Levels of statistical significance were determined based on the P values <0.05, <0.01, <0.001 and <0.0001.

RESULTS AND DISCUSSION

The results obtained from the morphological assays clearly indicate that keratinocytes grown in FGM (positive control) proliferated rapidly within the first 24 hours and achieved full confluence (Fig. 1 a-d). However, within the course of the next 48 hours, rapid apoptosis in the control cells was observed. In contrast, cells grown in SFM (negative control) did not reach 100% confluence even after the 72 hour time point (Fig. 1 e-h). At 24 hours, apoptotic cells (which were identified as the dark immobile entities) were evident in the negative control, which continued to increase in number for the duration of the experiment.

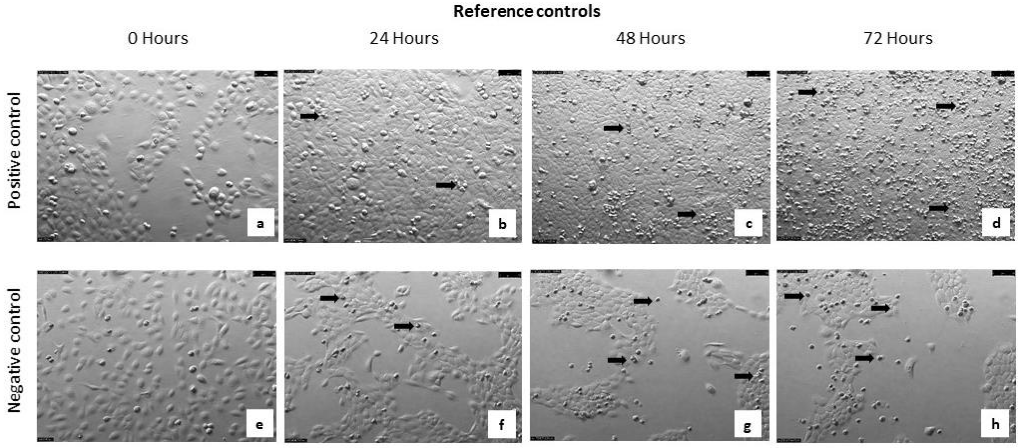

Fig. 1. *The proliferative effects of HaCaT cells treated with full growth media (FGM, a-d) which acted as the positive control and cells in serum free media (SFM, e-h) which served as the negative control. Cells were subjected to 72 hours microscopic examination and images were captured every 15 minutes. (→) indicates necrotic cells. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v (50 mL) fetal bovine serum (FBS), 1% v/v (5mL) 200 mM L-glutamine and 1% v/v (5 mL) of 50 Units/mL of penicillin and 5 mg/mL streptomycin, was denoted FGM. Pure DMEM, without any additions, was designated as SFM. Images were captured at 1000x magnification. Results indicate that keratinocytes grown in positive control proliferated rapidly within the first 24 hours and achieved full confluence, however, showed rapid apoptosis within the remaining 48 hours of the assay. In contrast, cells grown in negative control never reached 100 % confluence.*

In general, the *C. alata* methanol (CAM) and *C. alata* aqueous (CAA) extracts were found to induce cell proliferation (Fig.s 2 and 3). Further, an inverse relationship was observed, since as the concentration of plant extract decreased (0.8 to 0.05 mg/mL), keratinocyte proliferation increased (Fig. 2 a-t and 3 a-t). Specifically, irrespective of the concentration, the CAM extract improved proliferation by 70% within the first 24 hours (Fig. 2 b, f, j, n and r). Moreover, a reduction in extract concentration *i.e*. 0.2-0.1 mg/mL,

further enhanced cell growth to 100% within 48 hours of treatment (Fig. 2 k and o), which was maintained until cessation of the assay (72 hours) (Fig. 2 l and p). However, the CAA extract promoted keratinocyte proliferation by 60% at 24 hours (Fig. 3 b, f, j, n and r), although, only those concentrations ranging between 0.2-0.1 mg/mL augmented cellular confluency by 100%, at 72 hours (Fig. 3 l and p). The degree of apoptosis from treatment with *C. alata* extracts (Fig. 2 a-t and Fig. 3 a-t) was less compared to those cells treated with FGM (Fig. 1 a-d), although the level of apoptosis induced by CAA extracts was slightly higher (Fig. 3 d, h, l, p and t) compared to CAM extract treated cells (Fig. 2 d, h, l, p and t) at the end of the 72 hour time period.

Interestingly, a high degree of morphological changes was observed in those HaCaT cells treated with CAM and CAA extracts (0.8-0.05 mg/mL) by producing cytoplasmic projections. The morphological response observed in the cells representing the control treatments were comparatively very low. Similarly, keratinocytes treated with plant extracts (CAM and CAA at 0.8-0.4 mg/mL) showed a high degree of motility during the first six hours of the assay.

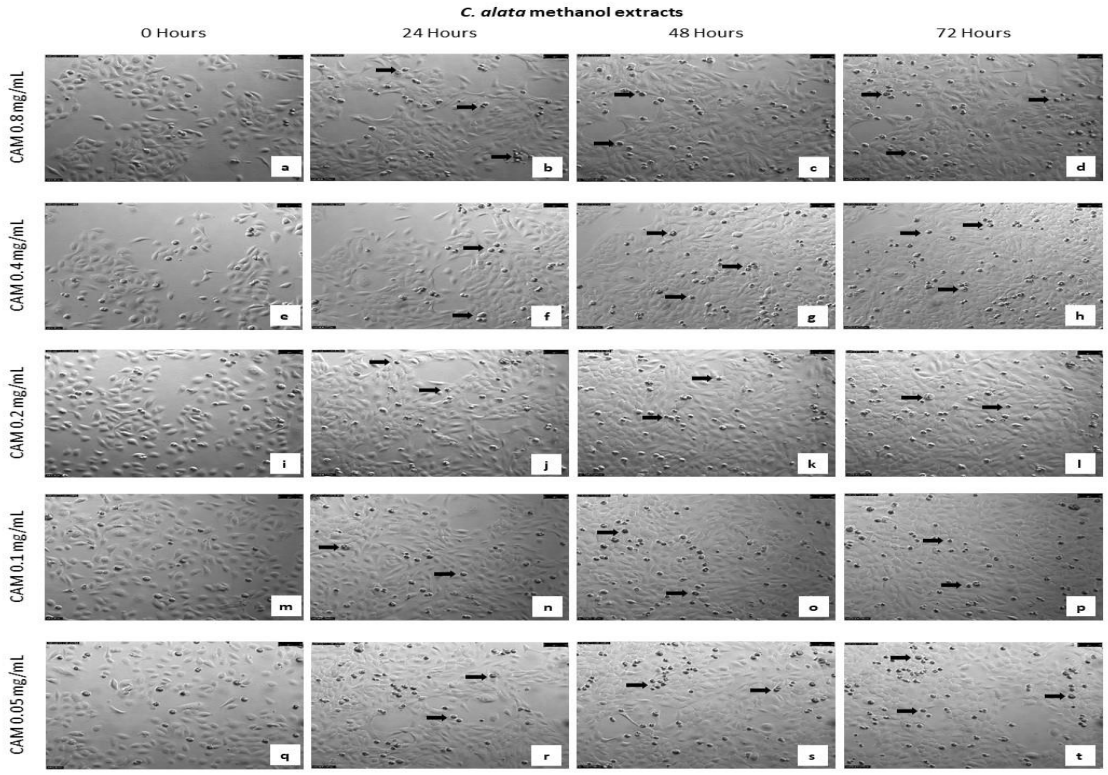

Fig. 2. *Proliferative effects of C. alata methanol extracts (at concentrations ranging from 0.8-0.05 mg/mL) on the HaCaT cells. Treated HaCaT cells were subjected to 72 hours microscopic examination and were compared with untreated cells grown in full growth media (FGM, Fig. 1 a-d) and untreated cells in serum free media (SFM, Fig. 1 e-h). CAM extract was able to improve proliferation by 70 % within the first 24 hours. 0.2-0.1 mg/mL CAM extracts enhanced HaCaT cell growth to 100 % within 48 - 72 hours. Less apoptotic effects were observed upon treatment with the CAM extract within 24-72 hours, compared to the positive control (Fig. 1 b-d). CAM: C. alata methanol. (→) indicates necrotic cells. Images were captured at 1000x magnification. CAM extract improved proliferation by 70% within the first 24 hours and a reduction in extract concentration (0.2-0.1 mg/mL), further enhanced cell growth to 100% within 48 hours of treatment.*

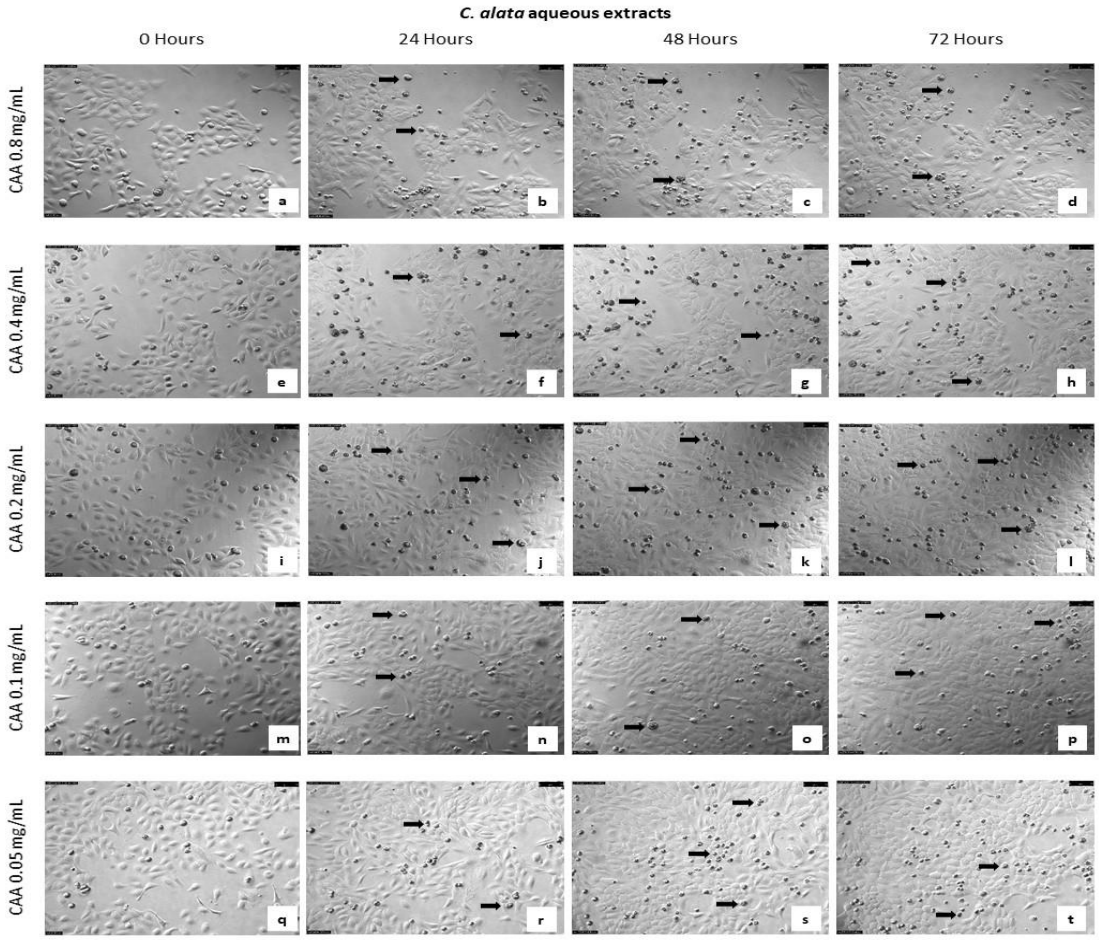

Fig. 3. *The proliferative effects of C. alata aqueous extracts (at concentrations ranging from 0.8-0.05 mg/mL) on keratinocytes. Treated HaCaT cells were subjected to 72 hours microscopic examination and were compared with untreated cells grown in full growth media (FGM, Fig. 1 a-d) and untreated cells in serum free media (SFM, Fig. 1 e-h). Less apoptotic effects were observed upon treatment with the CAA extract within 24-72 hours, compared to the positive control (Fig. 1 b-d). CAA extract promoted keratinocyte proliferation by 60% at 24 hours. Specifically, 0.2 - 0.1 mg/mL CAA extract augmented cellular confluency by 100% at 72 hours. CAA: C. alata aqueous. (→) indicates necrotic cells. Images were captured at 1000x magnification. CAA extract promoted keratinocyte proliferation by 60% at 24 hours, although only those concentrations ranging between 0.2 - 0.1 mg/mL augmented cellular confluency by 100%, at 72 hours. The degree of apoptosis from treatment with C. alata extracts was less compared to those cells treated with FGM (Fig. 1 a-d).*

Despite the concentration, HaCaT cells treated with *C. alata* extracts were less viable than that of the control (Fig. 4 a and b) as determined by the MTT assay. All *C. alata*-derived treatments, significantly ($P<0.0001$) decreased keratinocyte viability compared to the control. Although it was significantly less ($P<0.0075$) compared to the control, the CAM extract at 0.05 mg/mL showed slightly higher rate of cell viability compared to the other *C. alata*-derived treatments.

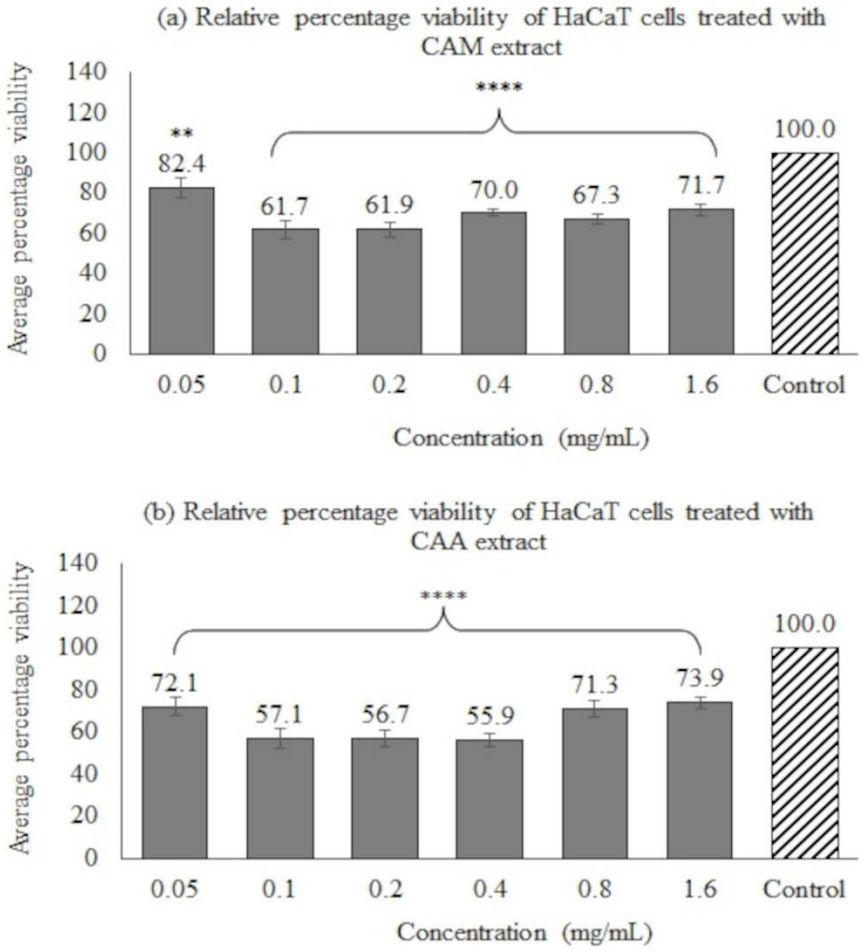

Fig. 4. *Relative percentage of keratinocyte viability treated with C. alata plant extracts; CAM (a) and CAA (b) at different concentrations (1.6 - 0.05 mg/mL) using the MTT assay. Relative viability of the cells treated with CAM or CAA did not improve up to the level of the positive control. However, keratinocyte viability was slightly higher when treated with CAM extracts at concentrations 0.05 - 0.4 mg/mL compared to CAA at the same concentration levels. Of all the treatments, it was evident that a concentration of 0.05 mg/mL CAM extract enhanced HaCaT cell survival the greatest compared to the other dosing levels. Each concentration represents the average percentage relative viability (±SEM) of three independent experiments performed in triplicate (n=9). The positive control represents the untreated cells cultured in reduced serum media (RSM).Significance levels **** P<0.0001 and ** P<0.01 compared to positive control. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, CAM: C. alata methanol, CAA: C. alata aqueous and RSM: reduced serum media.*

An overall trend showed that keratinocyte cell numbers increased in response to a decrease in plant extract concentration (Fig. 5 a-d). However, none of the plant extract treatments increased the number of cells beyond that of the control. In fact, the cells treated with the methanol extract (CAM) at 0.05 mg/mL (P=0.0031) and 1.60 mg/mL (P=0.0066), resulted in a significant reduction in cell number compared to the treatment

control (Fig. 5 a). The optimum concentration for maximum cell viability proved to be the most efficacious for CAM and CAA extracts were, 0.2 mg/mL and 0.05 mg/mL respectively.

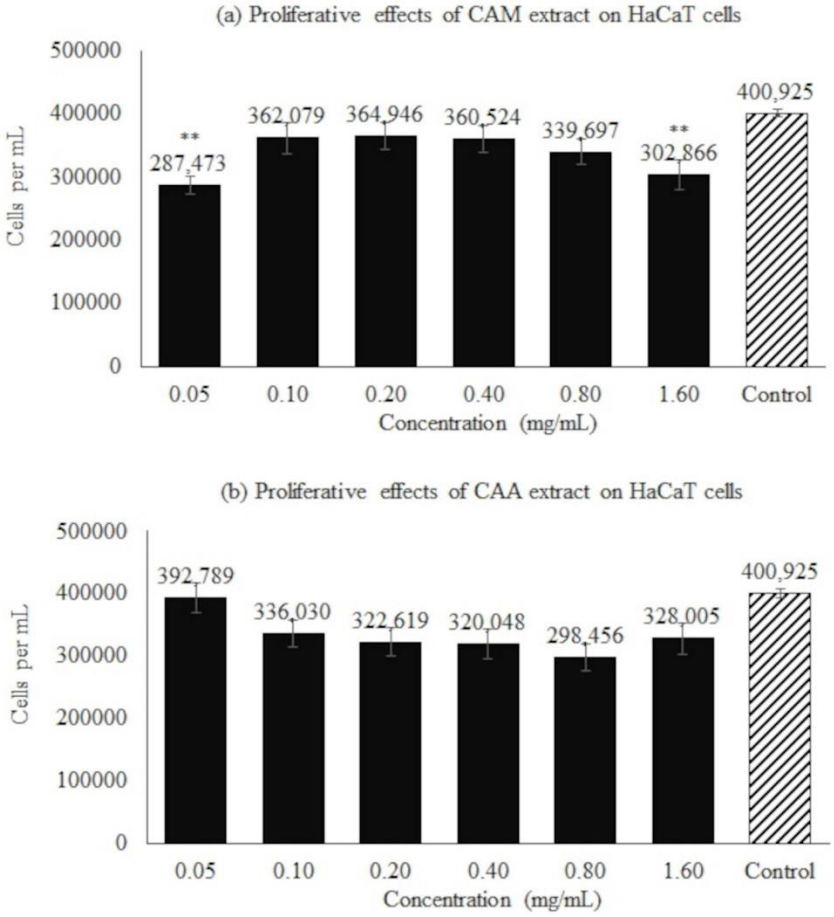

Fig. 5. *Viability of HaCaT cell treated with C. alata plant extracts; CAM (a) and CAA (b) measured by CyQUANT® assay. In general, both extracts (CAM and CAA) showed an increase in cell concentration when the extract concentration decrease. The proliferation effects of CAM extracts were contrary to CAA within the tested concentration range (0.05 - 1.60 mg/mL). Values were expressed as averaged final concentrations of healthy (cells/mL)±SEM, classified by extract and concentration. Each experiment performed in triplicate and repeated three times (n=9). The positive control represents the untreated cells cultured in reduced serum media (RSM). Significance levels **** (P<0.0001), ** (P<0.01) and * (P<0.05) compared to positive control. CAM: C. alata methanol and CAA: C. alata aqueous.*

Plant extracts were evaluated via use of the CBA assay for their capacity to stimulate or suppress cytokine (IL-12p70, TNF-α, IL-6, IL-8, IL-10 and IL-1β) expression in human keratinocytes (HaCaT), with or without LPS induction. Overall, the concentrations of cytokines IL-12p70, TNF-α, IL-10 and IL-1β detected in the test samples and baseline controls did not fall within the range of standards for each cytokine

as the amount was too small. Only IL-6 and IL-8 were in measurable levels. In general, IL-8 expression was comparatively higher than that of IL-6 (Fig. 6 a-f) for both extracts. Moreover, the cytokine levels of the test sample were found to be greater than the relevant BCs subsequent to individual treatment with the plant extracts at all concentrations. Hence, indicating they were not strong inducers of IL-6 and IL-8 (Fig. 6 a-f) secretion compared to the positive control, therefore, they were not pro-inflammatory. Further, the plant extracts were not able to suppress IL-6 production (Fig. 6 a-c) in those cells first stimulated with LPS (test samples) compared to the positive control, thereby indicating that they do not possess anti-inflammatory properties. Keratinocytes treated with *C. alata* aqueous (CAA) extracts showed an elevation in the production of IL-6 as extract concentrations increased. In general, all plant extracts, except CAA extract at 50 µg/mL, were able to suppress IL-8 production (Fig. 6 d-f) in LPS-stimulated HaCaT cells compared to the positive control, therefore, indicating an anti-inflammatory effect. Both CAM and CAA extracts caused a decrease in IL-8 secretion as the concentration of each extract increased, hence signifying an inverse dose response effect. Of the two extracts, CAA extracts resulted the highest production of IL-8 across all concentrations (Fig. 6 d-f).

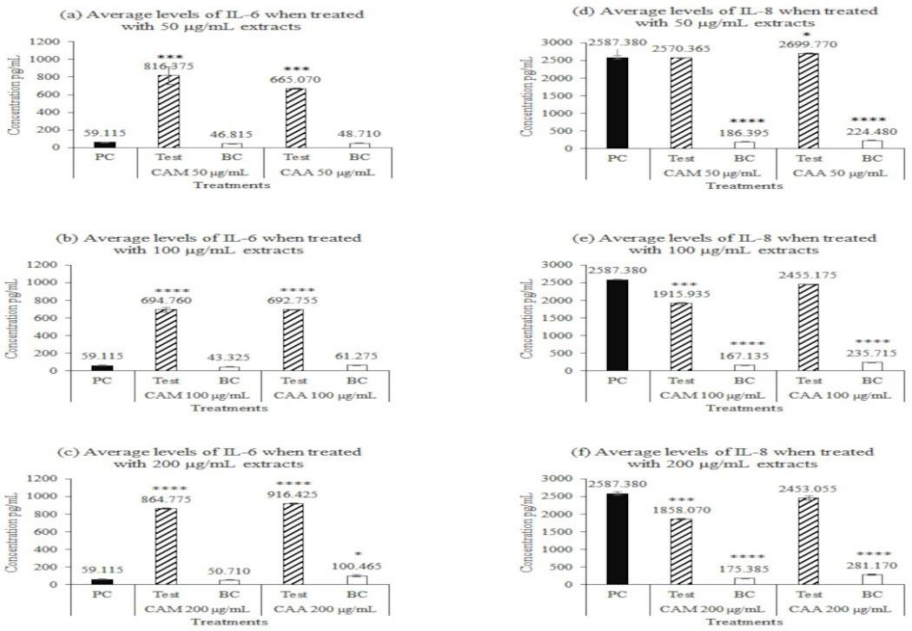

Fig. 6. *Average levels of interleukin (IL)-6 (a-c) and IL-8 (d-f) concentrations observed in the samples in pg/mL±SEM. Overall, IL-6 production was much less compared to IL-8. Both plant extracts (except 50 µg/mL CAA) exerted an anti-inflammatory effect against IL-8. None of the extracts irrelevant of the concentration imposed no anti-inflammatory effect against IL-6. However, both extracts were also not pro-inflammatory regarding IL-6 and IL-8. Cells pre-treated with lipopolysaccharides (LPS) followed by plant extracts (50, 100, 200 µg/mL) constituted the test samples (Test), while the cells that were only treated with extracts (i.e. minus LPS stimulation) represent the baseline controls (BC). Positive control (PC) was provided by suspensions with just LPS stimulated cells. Significance levels **** ($P<0.0001$), *** ($P<0.001$) and * ($P<0.05$) compared to positive control. CAM: C. alata methanol and CAA: C. alata aqueous.*

Percentage inhibition of COX-2 was slightly reduced compared to that of the COX-1 enzyme (Fig.s 7 and 8) by both extracts. At higher concentrations of the plant extracts (greater than 100 µg/mL), both COX-1 and COX-2 enzymes responded poorly. Therefore, the experiments were carried out at a concentration range of 6.25-100 µg/mL. Almost all extracts imposed 40 - 60 % inhibition of COX-1 compared to the indomethacin standards (Fig. 7 a-e). Treatment with aqueous extracts of the plant showed a decreasing trend in COX-1 inhibition upon an increase in concentration (Fig. 7 a-e). The CAM extract at 6.25-50 µg/mL elicited a higher percentage of COX-1 inhibition than that of indomethacin at comparative concentrations (Fig. 7 a-e). Moreover, indomethacin was superior compared to almost all plant extracts (excluding CAM at 12.5 µg/mL) in inhibiting the COX-2 enzyme regardless of concentration tested.

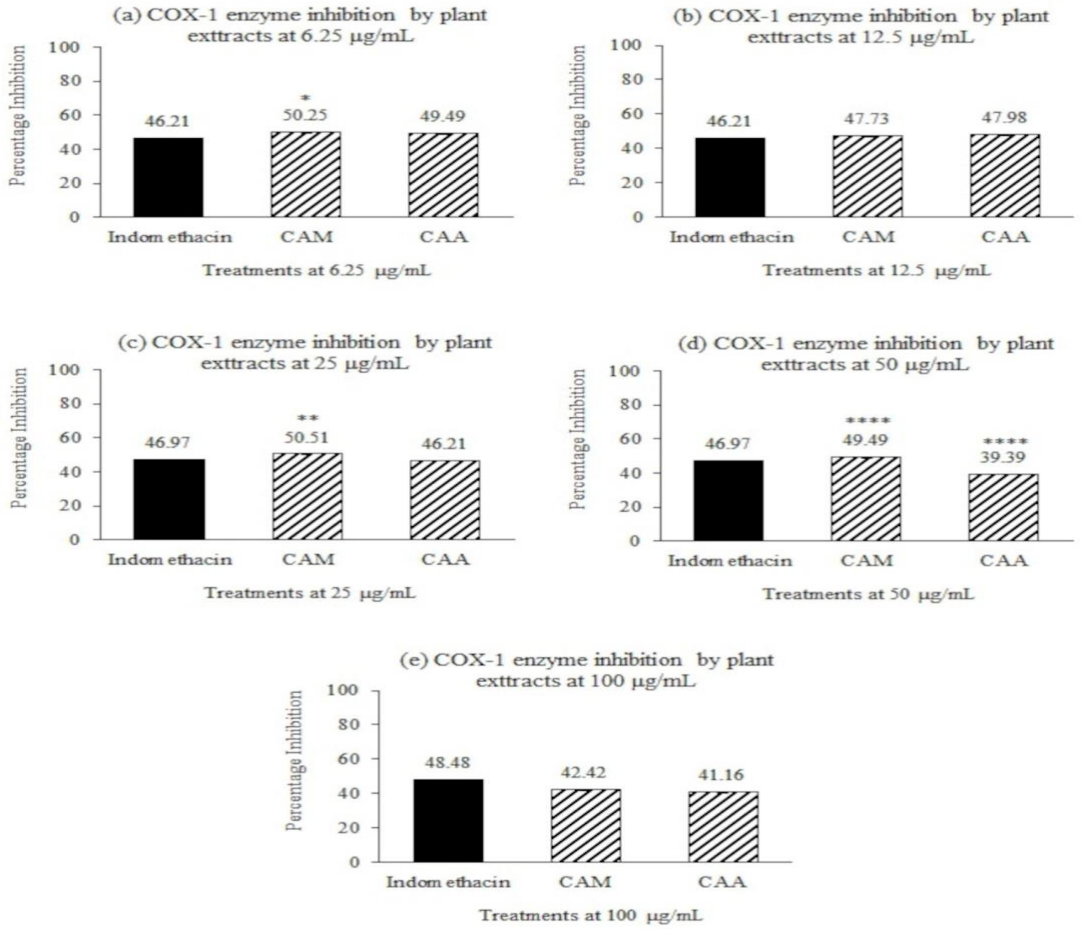

Fig. 7. *Cyclooxygenase (COX)-1 enzyme inhibition by plant extracts at five concentrations ranging from 6.25-100 µg/mL (a-e). Inhibition of COX-1 was higher with CAM extract compared to the control, Indomethacin at the concentrations 6.25-50 µg/mL. Inhibition imposed by CAM extract was almost always greater (except at 12.5 µg/mL concentration) compared to CAA treatment. Percentage inhibition (±SEM) of samples was calculated as the percentage reduction in absorbance compared to a positive control which had 100% activity without any inhibition. Indomethacin (INDO) ranging from 6.25-100 µg/mL was used as a standard inhibitor for comparison. Significance levels **** (P<0.0001), ** (P<0.01) and * (P<0.05) compared to standard inhibitor at its respective concentrations. CAM: C. alata methanol and CAA: C. alata aqueous.*

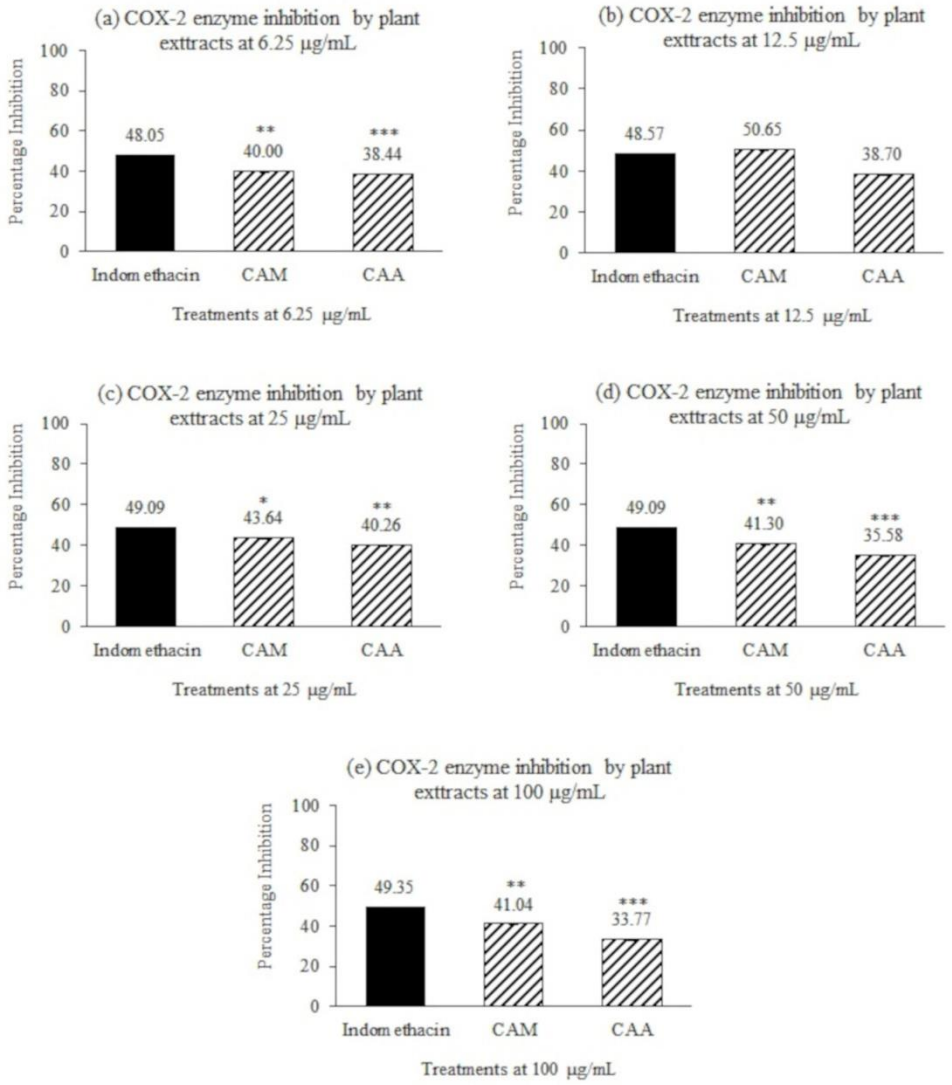

Fig. 8. *Cycloohygenase (COX)-2 enzyme inhibition by plant extracts at five concentrations ranging from 6.25-100 µg/mL (a-e). At all concentrations applied, COX-2 inhibition by CAM extracts were always greater compared to CAA treatment. Except CAM 12.5 µg/mL concentration, both plant extracts failed to inhibit COX-2 better than the control treatment (indomethacin) at their corresponding concentrations. Percentage inhibition (±SEM) of samples was calculated as the percentage reduction in absorbance compared to a positive control which had 100% activity without any inhibition. Indomethacin (INDO) ranging from 6.25-100 µg/mL was used as a standard inhibitor for comparison. Significance levels **** (P<0.0001), *** (P<0.001), ** (P<0.01) and * (P<0.05) compared to standard inhibitor at its respective concentrations. CAM: C. alata methanol and CAA: C. alata aqueous.*

The extracts were shown to inhibit the 5-LOX enzyme by 30 - 50 % compared to the NDGA standards (Fig. 9). Both plant extracts were superior to the positive control at inhibiting 5-LOX at all concentrations tested (Fig. 9 a-e). Moreover, moderate levels of 5-LOX inhibition were evident upon treatment with CAM extract at 6.25 µg/mL (41.51 %, P<0.0001) and 25 µg/mL (40.54%, P=0.0004) and CAA extract at 6.25 µg/mL (41.39

%, P<0.0001) and 12.5 µg/mL (40.78%, P=0.0017). CAM treatment almost always inhibited 5-LOX greater than CAA extracts, except at a concentration of 12.5 µg/mL.

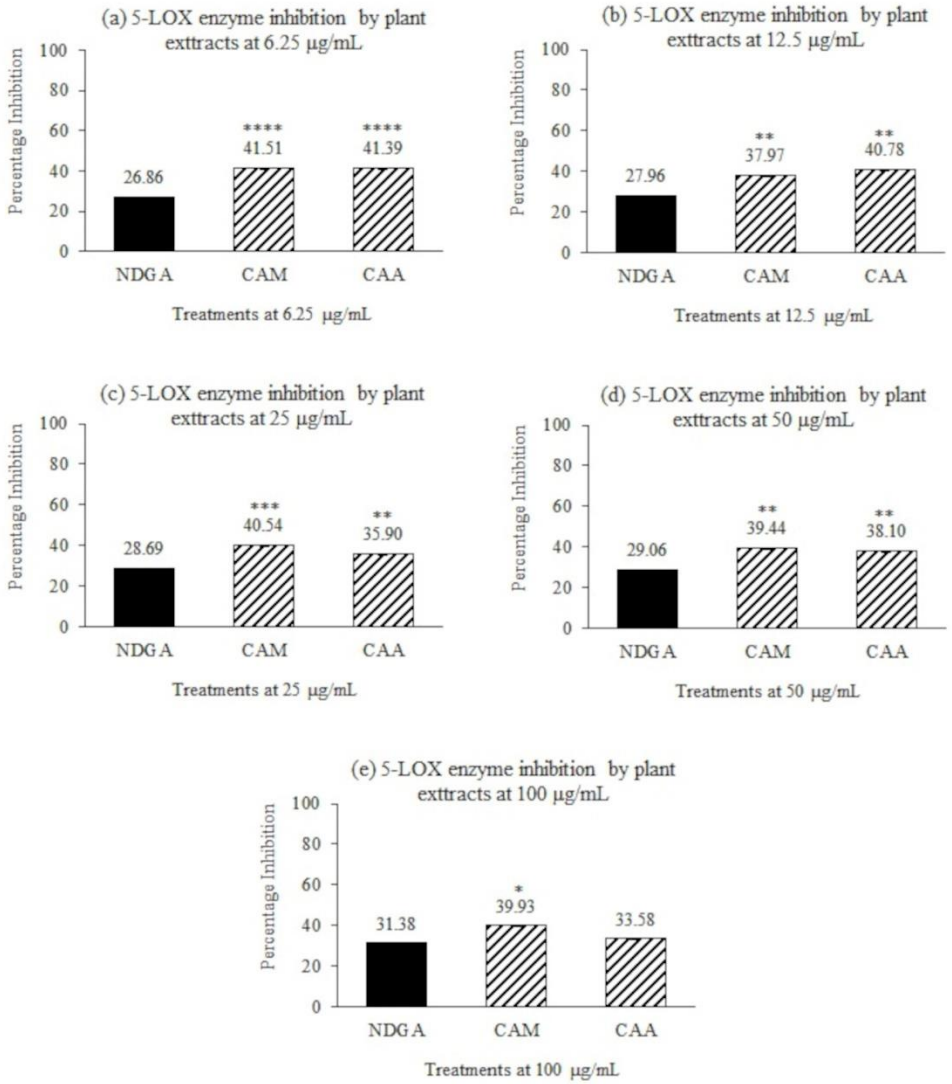

Fig. 9. *5-Lipoxygenase (5-LOX) enzyme inhibition by plant extracts at five concentrations ranging from 6.25-100 µg/mL (a-e). Both plant extracts inhibited 5-LOX superior to control treatment, Indomethacin at all concentrations applied. CAM extract inhibited the enzyme greater than CAA at all concentrations except at 12.5 µg/mL. Percentage inhibition (±SEM) of samples was calculated as the percentage reduction in absorbance compared to a positive control which had 100 % activity without any inhibition. Nordihydroguaiaretic acid (NDGA) ranging from 6.25-100 µg/mL was used as a standard inhibitor for comparison. Significance levels **** (P<0.0001), *** (P<0.001), ** (P<0.01) and * (P<0.05) compared to standard inhibitor at its respective concentrations. CAM: C. alata methanol and CAA: C. alata aqueous.*

In the current investigation, we utilized solvent extraction with methanol and an aqueous extraction method similar to that of a decoction, both resulting in water-soluble extracts. Decoction is prepared by boiling the plant material with water for a specified period of time or until the desired volume is attained [43]. Importantly, many compounds are heat stable *i.e.* high temperatures do not damage or destroy their innate medicinal properties. Moriyama *et al* [44, 45] reported that compounds isolated from *C. alata* remained stable during the process of isolation, which involved heat treatment (80°C for 1 hour). These results mirror our own as decoctions were prepared from the dried ground leaves of *C. alata* by boiling in an aqueous solution. Moreover, the fact that the extracts elicited a medicinal effect post-extraction clearly indicates that they remain stable and retain their bioactivity regardless of temperature used during the isolation process.

In this current study, morphological assays served multiple functions by determining the efficacy of plant extracts regarding keratinocyte viability and proliferation, potential cytotoxic effects, as well as identifying a suitable concentration range for subsequent experimentation. In fact, the results of the morphological and cell viability assays mirrored each other. In the morphological assay, it was observed that both methanolic and aqueous extracts of *C. alata* (CAM and CAA, respectively) could promote motility and morphological changes in keratinocytes compared to cells grown in both control treatments. Interestingly, in a study by Babitha and colleagues (2010), the authors showed that at a concentration of 25 μg/mL, the *C. alata* methanol extracts induced cell migration and dendrite formation in melanoblasts [46], similar to the morphological changes observed with HaCaT cells when treated with *C. alata* extracts in our current study. Such findings tend to indicate that CAM and CAA extracts potentially induce high metabolic activity in keratinocytes post- treatment.

The ability of a plant extract to alter cellular morphology or metabolism, interfere with cell adhesion, affect cell proliferation or cell differentiation or cause cell death is considered a cytotoxic effect [47]. Different varieties of cells may not respond universally when treated with a plant extract or specific compound [48]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, which is based on the capacity of functional respiring cells to metabolise tetrazolium salt into formazan, forming a purple colour complex by mitochondrial enzymes, can be applied to evaluate cell viability and proliferation [11, 49]. The amount of formazan produced is directly proportional to cell number in a homogeneous cell population, over a wide range [49]. Since active cells produce more formazan than those at rest [49], the measurement more accurately corresponds to cellular activity. Therefore, it is a reliable method of evaluating cell viability or potential cytotoxic effects. Alternatively, the advent of the CyQUANT® assay has made quantitating cell numbers more rapid and convenient. The average nucleic acid content per cell remains constant as the culture grows [50]. Thus, the total DNA content contained within the culture is accurately proportional to cell number [50]. Similarly, cell viability can be also evaluated with the CyQUANT® direct cell proliferation assay, which is a highly sensitive method for quantifying cells based on fluorescence produced when the dye binds to nucleic acids within viable cells [51].

In a study by Levy and Lewis [52], a hexane fraction from *C. alata* originally extracted with methanol, was shown to exert cytotoxic effects (143 μg/mL) against lung cancer cells (A549). Levy and Carley [53] reported significant cytotoxicity of the hexane fraction of a methanolic-derived *C. alata* extract on an ovarian cancer cell line (OV2008) at a concentration of 160 μg/mL. Moreover, the investigators suggested that *C. alata* hexane fractions could be capable of triggering a cascade of biochemical and morphological

changes involving caspases, which are implicated in the degradation of the cytoskeleton, hence causing apoptosis [54, 55]. In this current study, the CAM extract exhibited only low to moderate levels of cytotoxicity (Fig. 4a and 5a) within the concentration range (0.8-0.05 mg/mL) tested, but promoted the proliferation of keratinocytes up to 100% confluence (Fig. 2 k, l, o and p). The act of fractionating the CAM extract with hexane, may have increased the potential of confining and concentrating non-polar compounds, which were initially present within the methanol extract, thus supporting Levy and Carley's reported cytotoxic effects. In contrast, the treatments used in the current project were prepared only with methanol and aqueous extracts, which were then applied without fractionating in non-polar solvents. In a separate study [56], *C. alata* leaves extracted with petroleum ether, ethanol, acetone and chloroform elicited a 25 - 35% cytotoxic effect on colon cancer cells (HCT-15; concentration of 1.0 mg/mL). Moreover, cytotoxicity decreased non-linearly upon a reduction in concentration (1.0 to 0.01 mg/mL). A similar trend with regards to toxic effects was observed in this current study as cytotoxicity reduced as treatment concentration (from 0.8 to 0.05 mg/mL) decreased for both CAM and CAA extracts. Pieme *et al* [57] reported that an 80% v/v ethanol extract of *C. alata* leaves at 40-80 µg/mL inhibited the cellular proliferation of leukaemia (L1012) cells by 28-32%. However, in our current study, CAM extracts were able to enhance keratinocyte proliferation to a confluence of 100%. The leukaemic (L1012) cells used in Pieme's study may have altered cellular metabolic processes compared to the cell line (HaCaT) used in our study, thus resulting in a different cellular result.

Keratinocytes response in the pathogenesis of inflammatory skin diseases is vital [58] as they contribute significantly to the secretion of inflammatory mediators, specifically ILs and TNFs [36]. HaCaT cells, which have been widely used in various anti-inflammatory studies [59], are a non-tumorigenic monoclonal cell line, adapted to long term growth without feed-layer or supplemented growth factors [15] and are known to have the ability to secrete TNF-α, IL-6 and IL-8 [23, 60, 61]. LPS or endotoxins, found in the outer membrane of gram-negative bacteria, are predominant pathogenic factors for autoimmune diseases of the skin, therefore, they can be used to initiate an inflammatory response *in vitro* [23]. In our study, we attempted to measure the cytokine (IL-12p70, TNF-α, IL-6, IL-8, IL-10 and IL-1β) levels produced by human keratinocyte (HaCaT) cells, pre-induced with LPS or without, followed by treatment with plant extracts. Only the cytokines, IL-6 and IL-8 were detected out of the six cytokines present in the BD CBA human inflammatory cytokine kit as there is a theoretical limit of detection for the cytokines using this assay method [39] and thus, may explain why we did not detect IL-12p70, TNF-α, IL-10 or IL-1β. Moreover, the sensitivity for detecting the presence of IL-1β is less than the other cytokines available in the kit [39] , thereby making the measurement of small quantities of the secreted cytokine difficult. In our study, extracts from *C. alata* were able to suppress IL-8 levels compared to the positive control. However, all BCs (cells only treated with extract) did not induce IL-8 production compared to the positive control, thus, indicating that *C. alata* plant extracts had no pro-inflammatory effect. Various species of the genus *Cassia* have demonstrated significant anti-inflammatory activity [62]. Previous *in vivo* reports have indicated that *C. alata* possesses anti-inflammatory activity [63, 64]. However, this project was the first to investigate the anti-inflammatory activity of *C. alata* extracts in a broad context. Most of the previous reports of the anti-inflammatory action of the plant were based on animal models [65]. In one of the studies, the anti-inflammatory effect of *C. alata* leaf extract (500 mg/kg/day) in easing Freund's adjuvant arthritis in rats was demonstrated by a

reduction in swelling, leukocyte infiltration and cartilage degradation [64]. Moreover, the researchers reported that the extent of the anti-inflammatory effect by the *C. alata* leaf extract is comparable to that of diclofenac, a standard NSAID. Moreover, Sagnia *et al* suggested that the large amount of antioxidants present within the plant may be responsible for its anti-inflammatory action [66]. In our study, CAM leaf extracts were able to significantly (100 μg/mL, $P<0.0001$ and 200 μg/mL, $P=0.001$) reduce IL-8 expression in HaCaT cells induced with LPS compared to the positive control. However, none of the *C. alata* extracts (CAM and CAA) were able to inhibit the production of IL-6 irrespective of concentration tested.

CAM extracts were shown to impose strong inhibition against the COX-1 (25 μg/mL, 50.51%) and COX-2 (12.5 μg/mL, 50.65%) enzymes. In living cells, COX and 5-LOX enzymatic pathways mutually interact [67, 68]. The balance between the two is a critical parameter for the development of inflammatory reactions and many physiological processes [69]. Brooks [70] suggested that COX inhibitors could be associated with alternate processing of arachidonic acid into 5-LOX leukotrienes, while inhibitors of the 5-LOX pathway may lead to changes in the cellular handling of arachidonic acid into COX-mediated prostaglandin expression. Thus, dual inhibitors are extremely important. In this current study, CAM and CAA extracts demonstrated non-selective inhibition as they displayed higher affinity to COX-1 than the other two enzymes. Of the two isozymes, COX-1 activity is more physiologically important while, COX-2 activity is essential for its pathological significance [71, 72]. It has been reported that the COX-2 enzyme contains polymorphisms that might contribute to the variability in response to targeted inhibitors [73]. As the current results indicate, the non-selective inhibition by the compounds present in the plant extracts were not affected by the polymorphisms present in the enzyme. Compounds that inhibit 5-LOX have been shown to significantly decrease pro-inflammatory levels of IL-6 in human synovial fibroblasts [74]. The 5-LOX inhibition assays undertaken in our study indicated that the plant extracts successfully inhibited the enzyme. Hence, this effect may have resulted in the decreased level of IL-6 in the BCs we observed using the CBA method. Simeone and colleagues [75] reported that higher COX-2 expression was associated with increased levels of IL-8, which is a key factor in breast cancer invasion and metastasis. Hinson and colleagues [76] suggested that prostaglandins derived from COX-2 activity is responsible for the induction of IL-6, in macrophages. Maihofner and colleagues [77] reported that the COX-2 enzyme can be induced by pro-inflammatory cytokines, such as IL-6. Our results indicate that both CAM and CAA extracts were unable to inhibit the COX-2 enzyme to a greater extent compared with COX-1. As such, unregulated COX-2 activity could have resulted in increased levels of IL-6 in the test samples screened using the CBA method. Oxygenation reactions catalysed by COX and 5-LOX enzymes takes place at the endoplasmic reticulum and nuclear envelope [78, 79]. Thus, despite the anti-inflammatory effects they possess, a compound should be capable of crossing cellular membranes to exert its effect. This attribute could be a limitation to clinical success of selecting effective anti-inflammatory compounds, despite demonstrating efficacy during *in vitro* experimentation.

As the results indicate, *C. alata* plant extracts were not pro-inflammatory with regards to IL-6/IL-8 production, however, they were found to be strong suppressors of IL-8. In addition, they were able to impose strong inhibition on COX-1 and 5-LOX enzymatic activity *in vitro*. Thus, both plant extracts (CAM and CAA) and their active constituents have shown significant therapeutic importance in their ability to down-regulate pro-inflammatory cytokines and inhibit COX and 5-LOX enzymes. Such a good indication of

the strong anti-inflammatory potential of the two plant extracts and their active constituents may aid in the rapid healing of chronic wounds. However, future research should test their anti-inflammatory activity with other screening methods such as the human whole-blood assay [69], since it would be helpful to identify the pathological and clinical significance of the plant extracts and cognate bioactive compounds.

CONCLUSION

Current results indicate that *C. alata* extracts have significant potential to improve keratinocyte proliferation and viability, elicit significant anti-inflammatory activity by inhibiting IL-8 and pro-inflammatory enzymes and were also not cytotoxic to human-derived cells, which are important attributes of a potential therapeutic for the treatment of chronic wounds. Therefore, our results have scientifically validated the anti-inflammatory and regenerative effects of *C. alata* plant extracts potentiating wound healing.

Acknowledgement. We wish to extend our sincere gratitude to Institute of Research and Community Service at the University of Jember by "Hibah Penelitian Pemula" for support on funding in this research.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: V.A.A., T.C., Design: V.A.A., T.C., Data Collection or Processing: V.A.A., T.C., Analysis or Interpretation: V.A.A., T.C., Literature Search: V.A.A., T.C., Writing: V.A.A., T.C.

Financial Disclosure. This research received no grant from any funding agency/sector.

Abbreviations. ANOVA, analysis of variance; BC, baseline control; CAA, *Cassia alata* aqueous; CAM, *Cassia alata* methanol; CBA, cytometric bead array; COX, cyclooxygenase; DIC, direct interference contrast, DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FGM, full growth media; HaCaT, human keratinocyte cell; IL, interleukin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; OD, optical density; RSM, reduced serum media; TGF, transforming growth factor; TNF, tumor necrosis factor.

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