

Effects of Aqueous Leaf Extract of *Ageratum conyzoides* Linn on Keratinocytes and Fibroblasts Behavior

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ABSTRACT

Background and Purpose: Healing is a natural process that repairs injured tissue. Several medicinal plants, including *Ageratum conyzoides* Linn., have been tested for their wound-healing properties. Because the predominant cell populations in mammalian skin are fibroblasts and keratinocytes, our study aimed to evaluate the cell-activating and anti-inflammatory properties of the aqueous leaf extract of *A. conyzoides*. Specifically, we sought to show that the extract promoted fibroblast and keratinocyte proliferation.

Methods: Quantification of the major class of phytochemicals was done after aqueous extraction of powdered dried leaves. Keratinocytes and fibroblasts were extracted from the foreskin of newborn to two-year-old children, and cell viability was evaluated using the trypan blue test; proliferation tests, including MTT, CFSE, and PCNA measurement, were performed. Toxicity of the extract was studied using propidium iodide (PI) + annexin V assay.

Results: The extract contained 82.73 ± 0.76 mg GAE/g tannins, 164.13 ± 6.565 mg QE/g flavonoids, and 94.75 ± 1.13 mg GAE/g total phenols. In the presence of the extract, a higher percentage of dead cells (30.5% at 500 mg/mL) was observed; among them, more cells were apoptotic than necrotic. The percentage of dead cells decreased as the extract concentration decreased. Without the extract, cells were viable but did not proliferate; with the extract, cells proliferated as the extract concentration increased. Fibroblast proliferation increased slightly (15.4%), whereas keratinocyte proliferation increased markedly (64.7%).

Conclusion: This study shows that the aqueous leaf extract of *A. conyzoides* dose-dependently activated fibroblast and keratinocyte proliferation but induced moderate toxicity at the highest concentration.

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INTRODUCTION

Healing is a natural process that reconstructs an injured area and involves four phases: hemostasis, inflammation, proliferation, and maturation (Palmier and Garulo, 2016). The wound repair process begins with the activation of the coagulation cascade, inducing the production of growth factors such as platelet-derived growth factors (PDGF). Finally, platelets aggregate in the wounded area, leading to hemostasis (Molefe *et al.*, 2025). Simultaneously, inflammatory cytokines, such as interleukin-1, 6, and 8 (IL-1, IL-6, and IL-8), tumor necrosis factor-alpha (TNF- α), and different growth factors are also mobilized to the injured site (Molefe *et al.*, 2025). Neutrophils and macrophages are recruited for debridement and removal of bacteria by phagocytosis.

Bacteria are one of the main causes of wounds non healing, since an infected wound heals more slowly and wounds are highly susceptible to infections, especially when antibiotic-resistant biofilms develop. These biofilms can lead to the overproduction of inflammatory cytokines, resulting in a prolonged inflammatory phase that hinders cell proliferation, angiogenesis, and re-epithelialization (Molefe *et al.*, 2025). Moreover, antibiotic resistance is a public health issue. The World Health Organisation stated that antimicrobial resistance was one of the 10 greatest threats to public health facing humanity (WHO, 2020). Finding a solution to antibiotic resistance is therefore essential for faster wound healing. Various plants have demonstrated antibacterial effects (Karou *et al.*, 2007; Koudouvo *et al.*, 2011; Odeleye *et al.*, 2014). Akpalo *et al.* (2020) demonstrated the antibacterial properties of *Ageratum conyzoides* Linn., a plant belonging to the Asteraceae family.

Previous studies have associated the plant with the treatment of wounds, including burns, microbial infections, including those of the skin, pain, inflammation, and bleeding (Kotta *et al.*, 2020). An aqueous leaf extract of the plant showed anti-inflammatory activity by reducing the expression of pro-inflammatory cytokines IL-1 β , IL-18, TNF- α , and caspase-1 (Jamtsho *et al.*, 2024). The crude extract, organic fractions, and isolated compounds (5'-ethoxynobiletin, 1,2-benzopyrone, and eupalestin) from the Brazilian variety inhibited carrageenan-induced pleurisy in mice by inhibiting several pro-inflammatory mediators such as IL-17A, IL-6, TNF- α , and IFN- γ (Akhtar, 2022). As the predominant cell populations in mammalian skin are fibroblasts and keratinocytes, several *in vitro* studies utilize either or both of these cell types as effective tools to directly visualize cellular interactions (Talekar *et al.*, 2017). Our objective was to evaluate the use of *A. conyzoides* leaf extract as a cell activator and anti-inflammatory agent. Specifically, we sought to demonstrate that the extract promotes fibroblast and keratinocyte proliferation.

MATERIAL AND METHODS

Plant material

The plant material used in this study was the leaves of *Ageratum conyzoides* Linn. (Figure 1) and was collected in July and November, 2023. A specimen of the plant was identified by the Department of Plant Biology in the Faculty of Science at the University of Lomé; the herbarium specimen was preserved at the Laboratory of Botany and Plant Ecology as specimen "E 9°41'22.6"N 0°36'28.7" University of Lomé, based on data from the Togo National Forest Inventory (IFN 2015).



Figure 1: Leaves of *Ageratum conyzoides* Linn.

Bioethical approval and human tissue samples

This study received approval No.047/2022/CBRS from the Bioethics Committee for Health Research (CBRS) of the Togo Ministry of Health. Prepuce samples were obtained with parental consent at different medical centers in Lomé. The human tissue samples used in this study (Figure 2) were foreskins from newborn to two-year-old children.

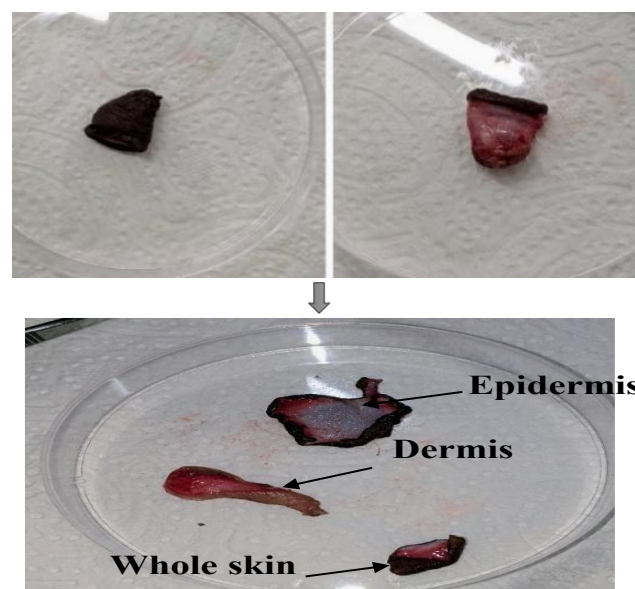


Figure 2: Pictures of pieces of dermis and epidermis before and after mechanical separation. Plate A shows newborns' foreskins and Plate B shows the dermis and epidermis of the foreskin of a newborn.

Aqueous Extraction of the Leaves

The extraction process followed Akpalo *et al.* (2020). Briefly, 50 g of the leaves were extracted with distilled water in a weight-to-volume ratio of 1:10 for 3 h at 50±2°C. The extraction was done three times, and filtrates were combined before drying. The extract was then centrifuged, filtered, and dried. The residue was reconstituted in physiological saline to the desired concentration. The extraction yield was calculated as follows:

$$Y = \left(\frac{\text{Weight of final extract}}{\text{Weight of leaves}} \right) \times 100$$

Thin Layer Chromatography (TLC)

Thin-layer chromatography was performed in normal phase on aluminum plates coated with silica gel (Kieselgel 60 F254). (Merck) 0.25 mm. The eluent (70% dichloromethane and 30% ethyl acetate) was poured to a height of about 0.5 cm into a beaker that was tightly sealed with aluminum foil. The samples were deposited on the TLC plate using a capillary glass pipette, and the spot was dried quickly between applications. The plate was introduced and placed vertically in the beaker, which was immediately closed. When the solvent front was about 1 cm from the top end of the plate, where a fine line was previously marked, the plate was removed from the beaker. The TLC was observed under UV light at 254 nm by fluorescence quenching, then at 366 nm, and finally revealed using vanillin–sulfuric acid, aluminum chloride, iron chloride, potassium hydroxide, anisaldehyde, or any developer, followed by heating and/or UV light exposure. After spraying, the TLC plate was heated at 110°C for about 5 min. Several colors appeared, depending on the compound type.

Quantitative Phytochemical Tests

Total Phenolic Content

The Folin and Ciocalteu (1927) colorimetric method was used. A 100 µL aliquot of each extract was mixed with 750 µL of Folin-Ciocalteu reagent, first diluted 1:10. After 5 min of incubation at 25°C, 750 µL of 20% sodium carbonate was added. The mixture was incubated in the dark for 90 min, and absorbance was monitored at 765 nm. For the blank, distilled water replaced the extract. Gallic acid (0–500 g/mL) was used as a standard, and the results were expressed in milligrams of gallic acid equivalent per gram (mg GAE/g) of dry extract by extrapolating the calibration line ($y=0.005018x + 0.055190$) obtained from the different concentrations of gallic acid.

Total Flavonoids Content

The method of Saloufou *et al.* (2018) was used with modifications. The total flavonoid content of the aqueous extract of *A. conyzoides* was estimated using colorimetric aluminum chloride (AlCl₃). Of the solution, 100 µL was added to 400 µL of distilled water, and 30 µL of 5% sodium nitrite was added to the mixture. After 5 min incubation at

room temperature, 20 µL of 10% aqueous AlCl₃ solution was added. A volume of 200 µL of aqueous Na₂CO₃ (1 M) was added to the mixture. After 5 min, 250 µL of distilled water was also added. The mixture was vortexed and the absorbance was measured at 510 nm using a spectrophotometer (DIV-UV5600, China). The total flavonoid concentration was determined using the quercetin calibration curve, and results were expressed as milligrams of quercetin equivalents per gram (mg QE/g) of dry extract.

Total Tannins Content

Casein (0.3 g) was introduced into a 53 mL flask, and 12.5 mL of the sample (1 mg/mL) was added. The mixture was kept in a water bath at 30°C for 1 h, then 2 mL of the solution was filtered and added to 10 mL of distilled water. The absorbance of the mixture was measured at 760 nm with a spectrophotometer (DIV-UV5600, China). The absorbance values were subtracted from the total phenol absorbances to determine the tannin absorbance values.

Extraction of Keratinocytes and Fibroblasts

Fibroblasts and keratinocytes were obtained from the foreskins of newborn to two-year-old children in various medical centers in Lomé. The dermis and epidermis were separated mechanically using forceps and scissors. The different parts were cut into small pieces and then decontaminated in Dulbecco's Modified Eagle's Medium supplemented with gentamicin, penicillin, streptomycin, and amphotericin B (DMEM+****) (Sigma-Aldrich, St. Louis, Missouri, USA). In a U-bottom culture dish, pieces of dermal tissue were soaked in collagenase (Roche Diagnostics, Mannheim, Germany) for 4 h on one hand. On the other hand, pieces of epidermal tissue were soaked in trypsin-EDTA (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 min. Collagenase and trypsin-EDTA are specific cell-digestion enzymes. After incubation, the skin pieces were removed, and the collagenase-extracted cells were stained with trypan blue (Life Technologies Corporation, Grand Island, USA) for microscopic observation at 10× and cell counting.

Trypan Blue Cell Viability Test

The extracted cells were cultured in a medium composed of 45 mL DMEM+**** mixed with 5 mL inactivated Fetal Calf Serum (FCS) at a concentration of 2×10^5 cells/mm³. They were stimulated with aqueous extract of *A. conyzoides* at three concentrations: 500 mg/mL, 250 mg/mL, and 125 mg/mL; the culture medium, 100% DMSO, and 10% DMSO served as controls. Briefly, stimulation was done by adding 100 µL of each stimulant (medium, extract, or DMSO) to 150 µL of cell culture. Those concentrations were chosen because they were used in a previous work (Akpalo *et al.*, 2021) for antibacterial properties, and the highest one (500 mg/mL) is the one used in a wound-healing hydrogel formulation (patent Number 21726 of OAPI,

delivered on December 2nd 2024, and published on December 12th, 2024). Incubations were carried out at 37°C in a humid atmosphere with 5% CO₂ for 18 h. Afterward, the cells were recovered, diluted by half in trypan blue, and mounted on a Neubauer cell chamber for counting under an optical microscope at 10× magnification. Live and dead cells were counted.

Proliferation Tests

Proliferation tests included MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Carboxyfluorescein succinimidyl ester (CFSE), and measurement of PCNA (proliferating cell nuclear antigen). For all proliferation tests, collagenase-digested cells were used.

MTT test

After 24 h of culture in a 96-well plate, cells were washed, then 10 µL of MTT was added, homogenized, and incubated at 37°C for 4 h. Next, 100 µL of 0.04 N HCl in isopropanol was added to each well; the plate was incubated for 1 h at room temperature, then read using a spectrophotometer (Human GmbH, Wiesbaden, Germany) at the wavelength of 630 nm (Figure 3). The culture medium served as a blank for this test.

CFSE test

To one vial of CFSE (Thermo Fisher Scientific, Eugene, Oregon, USA) staining solution, 18 µL of DMSO was added. The mixture was diluted in 20 mL of PBS (Life Technologies Limited, Paisley, UK) to a final concentration of 5 µM. The cells were suspended in 10 mL of diluted CFSE solution to a concentration of 2×10^5 cells/mm³; the aqueous extract of *A. conyzoides* at concentrations of 500, 250, and 125 mg/mL was added, and the cells were cultured in a 96-well microplate. After 20 min of incubation, the constituted Day 0 wells were read on the Cytoflex flow cytometer (Beckman Coulter, Brea, CA, USA); the other wells were incubated for reading after 24 h (Day 1), 48 h (Day 2), and 72 h (Day 3) (Figures 4 and 5). The culture medium, 10% DMSO, and 100% DMSO were used as controls for the test. Since it was not possible to observe successive peaks of fluorescence decline corresponding to the different cell generations using our flow cytometer, the mean fluorescence intensities were therefore used for data analysis.

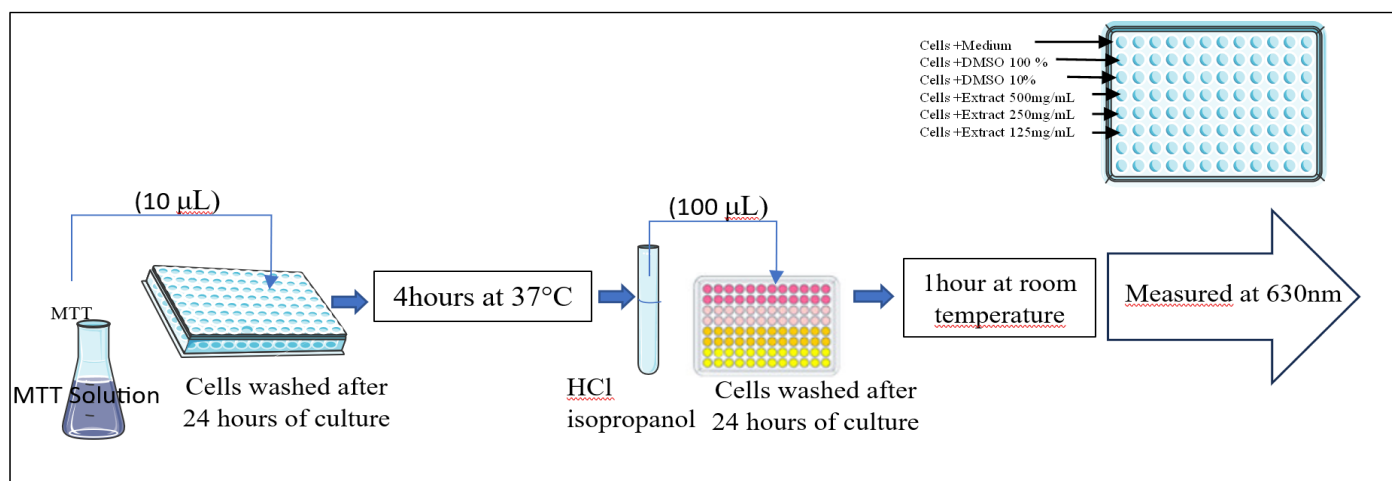


Figure 3: Schematic illustration of MTT test procedure used for cell proliferation.

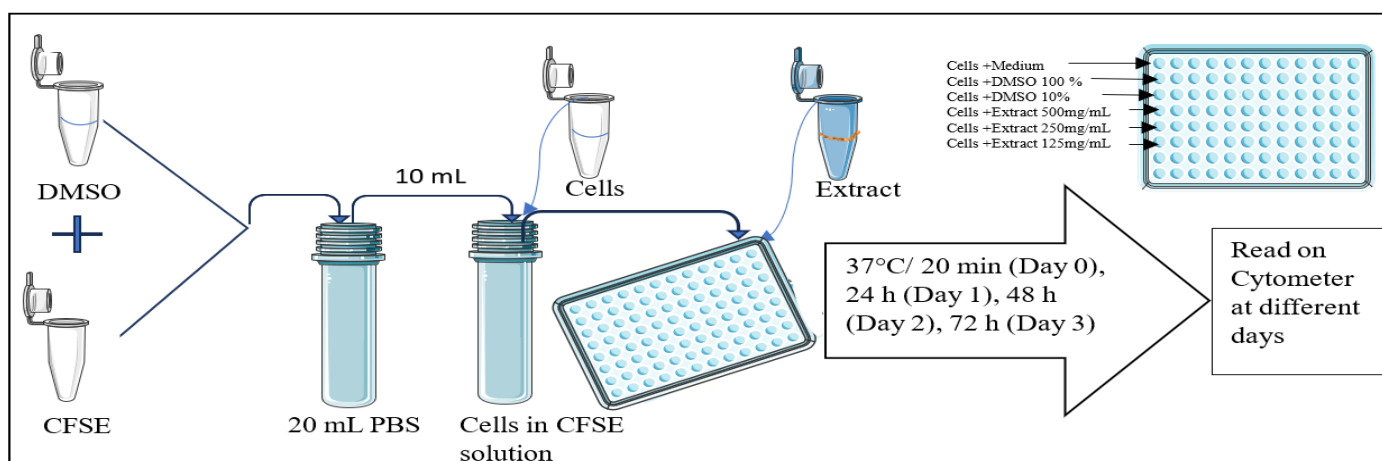


Figure 4: Schematic illustration of CFSE test procedure used for cell proliferation.

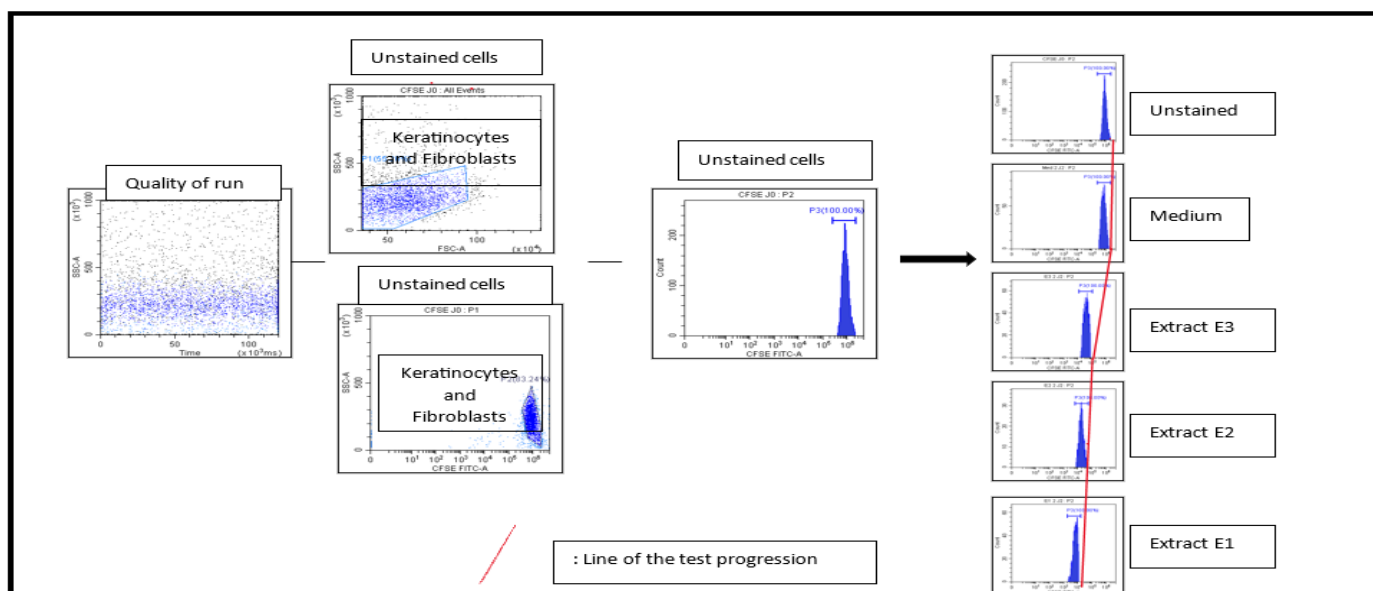


Figure 5: Gating strategy for CFSE-based cell proliferation analysis by flow cytometry. Cells were first gated according to acquisition stability (Time gate) and cell population (FSC-A vs SSC-A). Unstained cells were used as a negative control to establish baseline fluorescence. Keratinocytes and fibroblasts were then identified. CFSE fluorescence profiles were compared among the experimental groups [Unstained, Medium; Extract E3 (125 mg/mL), Extract E2 (250 mg/mL), Extract E1 (500 mg/mL)] to evaluate the effect of the plant extract on cell proliferation.

Measurement of PCNA (proliferating cell nuclear antigen) in the presence of specific markers CD140a and Involucrin

After 24 h of culture, the cells were washed with the washing solution (FACS Buffer: 49 mL PBS+1 mL FCS); 1 μ L of PCNA (Clone PC10, Life Technologies Corp, Carlsbad, USA), CD140a (Clone 16A1, Life Technologies, Czech Republic), and Involucrin were added to the cells and then incubated at 4°C for 30 min. The cells were then washed, and the pellets were resuspended in 100 μ L of FACS Buffer for cytometer reading (Figure 5). Figure 6 presents the gating strategy used.

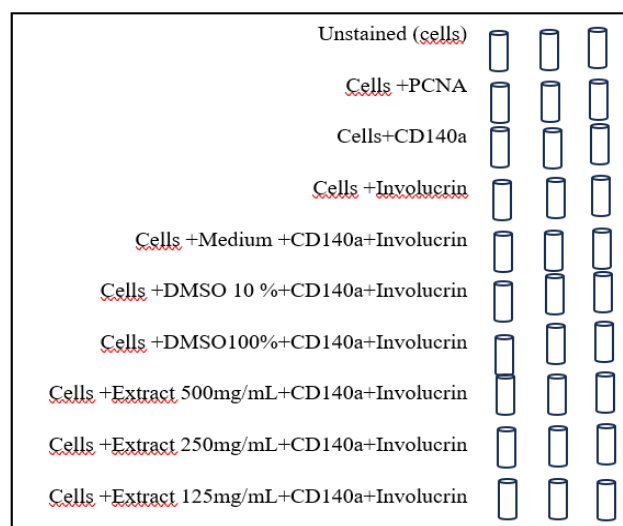


Figure 6: Staining panel for PCNA/CD140a/Involucrin analysis. Reading panel showing the staining setup for unstained cells and cells stained with PCNA, CD140a, Involucrin, or combined CD140a/Involucrin markers in the different treatment groups.

Toxicity Tests

PI (Propidium Iodide) + Annexin V Assay

After 24 h of culture in the presence of the extract, the cells were washed with the washing solution (FACS Buffer). In the dark, 100 μ L of PI (Avantor, Radnor, PA, USA) was added to the cells and incubated at 4°C for 15 min. The cells were then washed with FACS buffer; 1 μ L of Annexin V was added to the pellets and incubated at 4°C for 30 min. The cells were washed again, and the pellet was resuspended in 100 μ L of FACS buffer for reading on the cytometer (Figure 8); the gating strategy used is presented in Figure 7, and the gating strategy for the identification of apoptotic and necrotic cells using Annexin V and propidium iodide (PI) is presented in Figure 9.

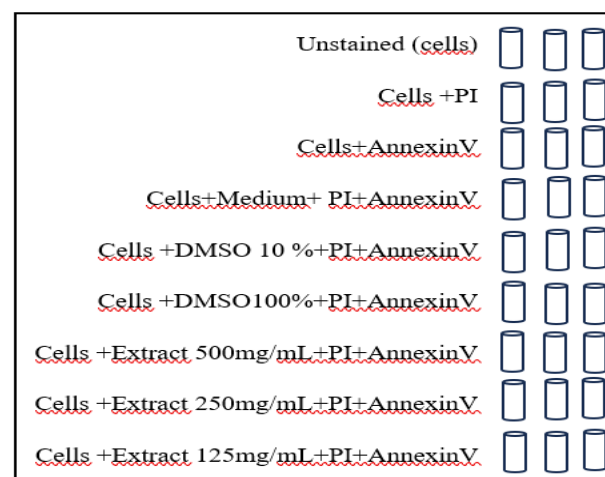


Figure 7: Staining panel for PI/Annexin V analysis. Reading panel showing the staining and flow cytometry analysis setup, including unstained cells, PI-stained cells, Annexin V-stained cells, and PI/Annexin V double-stained treated groups.

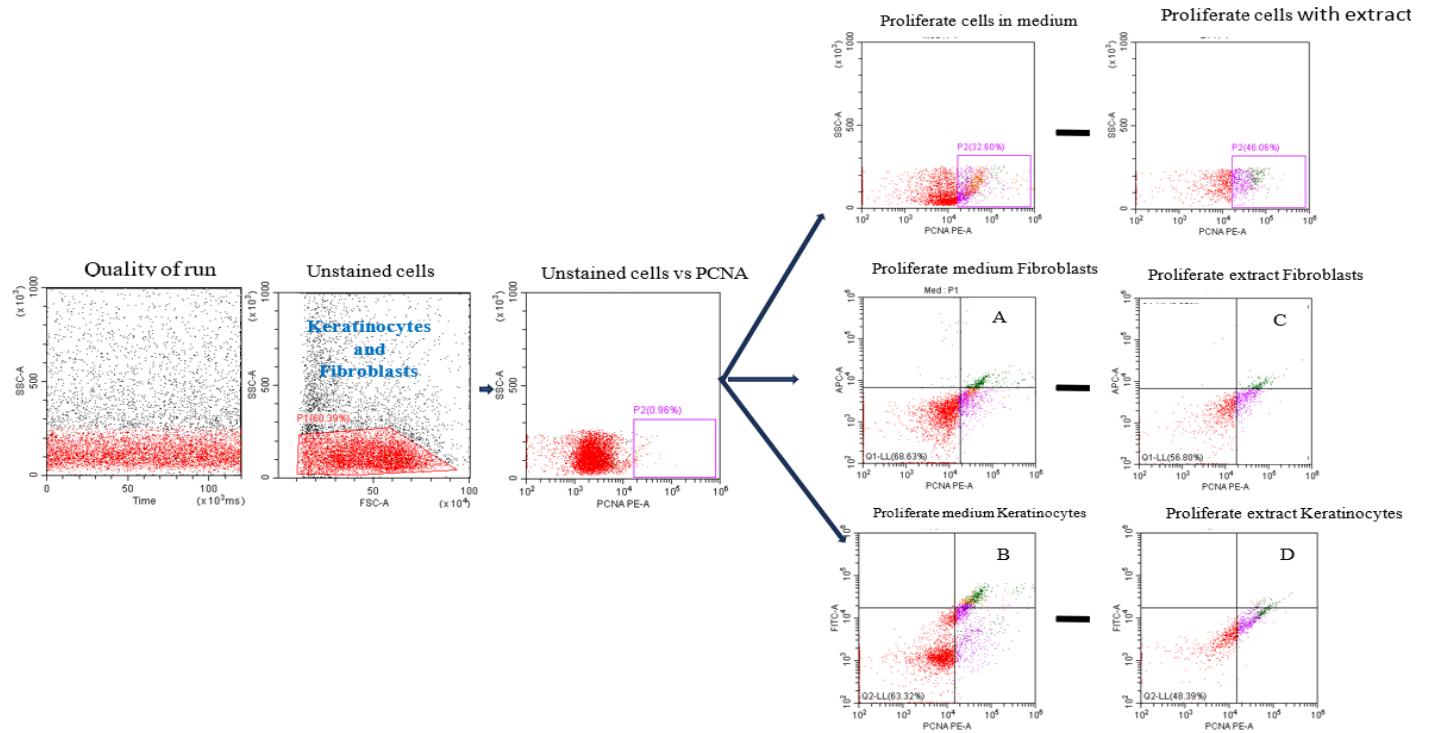


Figure 8: Gating strategy for identification of proliferating fibroblasts and keratinocytes based on PCNA expression by flow cytometry. Cells were first gated based on acquisition stability (Time gate) and cell population (FSC-A vs SSC-A). Unstained cells were used as a control to define baseline fluorescence. Identification of proliferating cells after treatment with plant extract; comparative analysis of fibroblast and keratinocyte proliferation based on PCNA fluorescence intensity was done. A = PCNA⁺-CD140a⁺; B = PCNA⁺-Involucrin⁺; C = PCNA⁺-CD140a⁺; D = PCNA⁺-Involucrin⁺.

Identification of Apoptotic and Necrotic Dead Cells

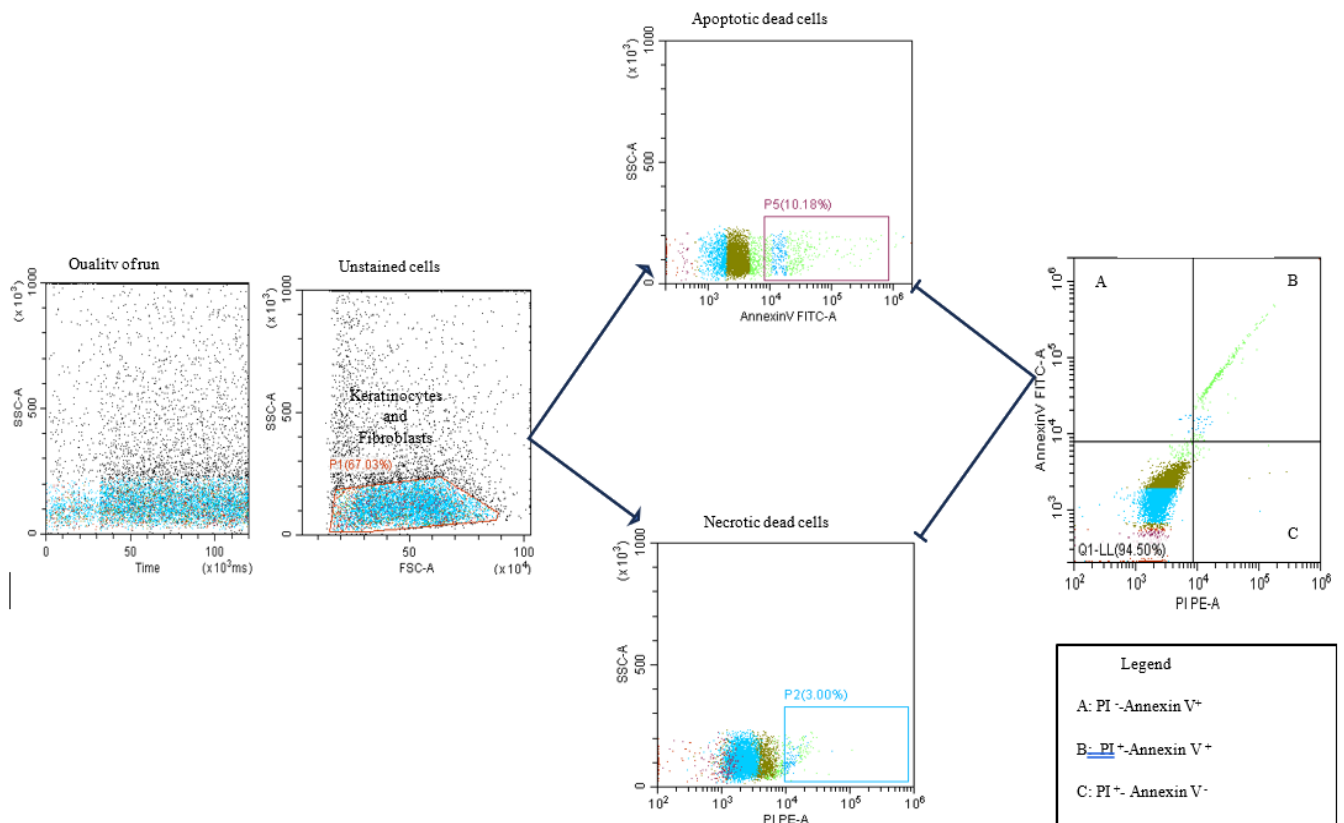


Figure 9: Gating strategy for the identification of apoptotic and necrotic cells using Annexin V and propidium iodide (PI). Cells were first gated based on acquisition stability (Time gate) and cell population (FSC-A vs SSC-A). Unstained cells were used as a control to define baseline fluorescence. Apoptotic cells were identified as Annexin-V⁺ and PI⁻, while necrotic cells were defined as the PI⁺ population

Inflammatory Activity of Keratinocytes and Fibroblasts

Primary antibodies against IL-6 and TNF- α , pro-inflammatory cytokines, were measured using the ELISA method in the supernatants of keratinocyte and fibroblast cultures to identify their pro-inflammatory activity in the presence of the aqueous extract of *Ageratum conyzoides* Linn.

Statistical Methods

Statistical analysis was performed using the GraphPad PRISM 8.0.2 software (GraphPad Software, Inc., La Jolla, CA, USA). Data normality was assessed using the Shapiro-Wilk test before comparing the groups. Data were compared using one-way ANOVA followed by Dunnett's post hoc test. $P < 0.05$ denotes statistical significance between compared data.

RESULTS

Aqueous Extraction Yield of *Ageratum conyzoides* Leaves

The aqueous extract of *A. conyzoides* had a very low yield.

From 50 g of leaves, 7.59 g of extract was obtained, corresponding to a 15% w/w yield.

Phytochemical Quantification

Table 1: Results of phytochemical quantitative tests.

	Levels in <i>A. conyzoides</i> Extract	Reference Compound
Tannins	82.73 $\pm$ 0.76 mg GAE/g	Gallic acid
Flavonoids	164.13 $\pm$ 6.57 mg QE/g	Quercetin
Total Polyphenols	94.75 $\pm$ 1.13 mg GAE/g	Gallic acid

Values are expressed as Means $\pm$ SEM, n=3. Total polyphenols and tannins are expressed in mg gallic acid equivalent/g extract (mg GAE/g), while flavonoids are expressed in mg quercetin equivalent/g extract (mg QE/g).

Purity of the Keratinocytes and Fibroblasts Extraction

STrypsin enabled the extraction of only keratinocytes (Figure 10A), while collagenase enabled the extraction of a mixed population of keratinocytes and some fibroblasts (Figure 10B). Immediately after extraction, 100% of the extracted cells were alive.

Table 1: Results of phytochemical quantitative tests.

Revelators	No Revelator	Sulfuric vanillin	AlCl ₃	FeCl ₃	KOH	Anisaldehyde
Inferences	Triterpenes Sterols	Triterpenes Sterols Terpenes Coumarins	Flavonoids	Flavonoids Tannins Coumarins	Flavonoids Coumarins Terpenes	Flavonoids

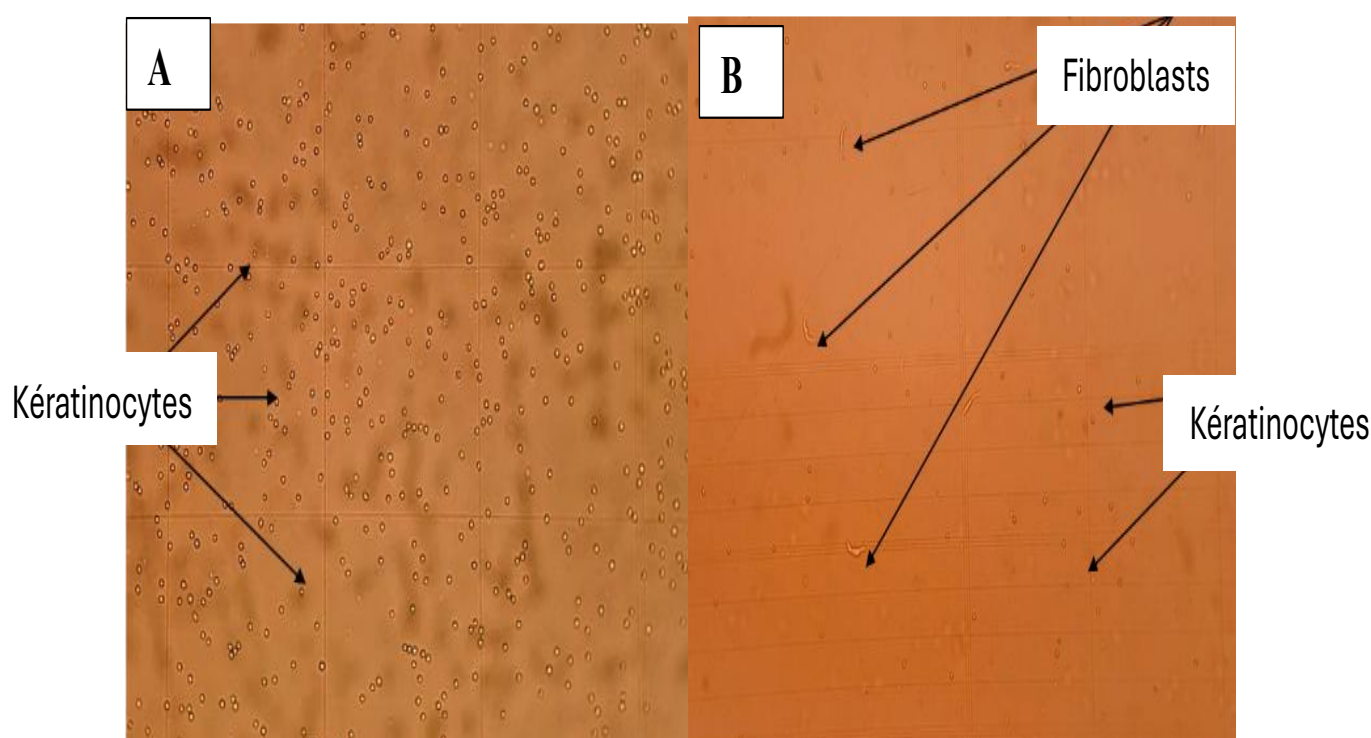


Figure 10: Microscopic observation ($\times 10$) of fibroblast and keratinocyte cells. (A) is trypsin-based extraction and (B) collagenase-based extraction.

Trypan Blue Cell Viability Test

The cell viability test results (Figure 11) show that, when exposed to Trypan Blue, more cells were alive than dead. In the culture medium negative control, up to 90.1% of cells were alive; similarly, in the wells containing the lowest extract concentration. In the wells containing DMSO 100, fewer cells were alive (78.5%). The highest percentage of dead cells (30.5%) was observed at the highest extract concentration (500 mg/mL). The percentage of dead cells decreased as the extract concentration decreased, to 26.1% and 12.4% for E2 (250 mg/mL) and E3 (125 mg/mL), respectively. The living-to-dead cell ratios were 2.3, 2.8, and 7.0 for E1, E2, and E3, respectively, compared with the medium, which had a ratio of 8.8.

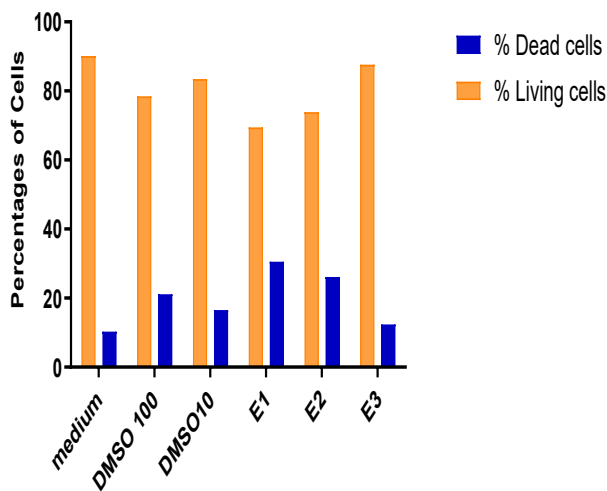


Figure 11: Comparative analysis of cell viability and cell death percentages in control and *A. conyzoides* extract-treated groups. E1: extract at 500 mg/mL; E2: extract at 250 mg/mL; E3: extract at 125 mg/mL. n = 3.

Proliferation tests

MTT test

Figure 12 presents the MTT test results. Optical density is proportional to the number of living cells; the higher the OD, the more living cells there are. In the absence of the extract, the cells were viable; in the presence of the extract, the cells proliferated as the extract concentration increased.

CFSE test

The CFSE results were analyzed using fluorescence intensity. The decrease in CFSE fluorescence intensity was considered indicative of increased proliferative activity. The lower the staining intensity, the more the cells have proliferated (Figure 13).

In this test, lower staining intensity indicates greater cell proliferation. In the absence of the extract, mean fluorescence intensities were high, indicating no or weak proliferation: (356 768.26 MFI), (1 447 328.00 MFI), and (439 524.8 MFI), respectively, for Medium, DMSO100, and DMSO10 at D3. DMSO 100 MFI was fourth, higher than

Medium MFI. In contrast, in the presence of the extract, the mean fluorescence intensities were reduced; there was slight staining, indicating cell proliferation: (10642,23 MFI), (52766,06 MFI), and (66202,57 MFI), respectively, for extract 500 mg/mL, 250 mg/mL, and 125 mg/mL, at D3. Proliferation was highest at the highest extract concentration.

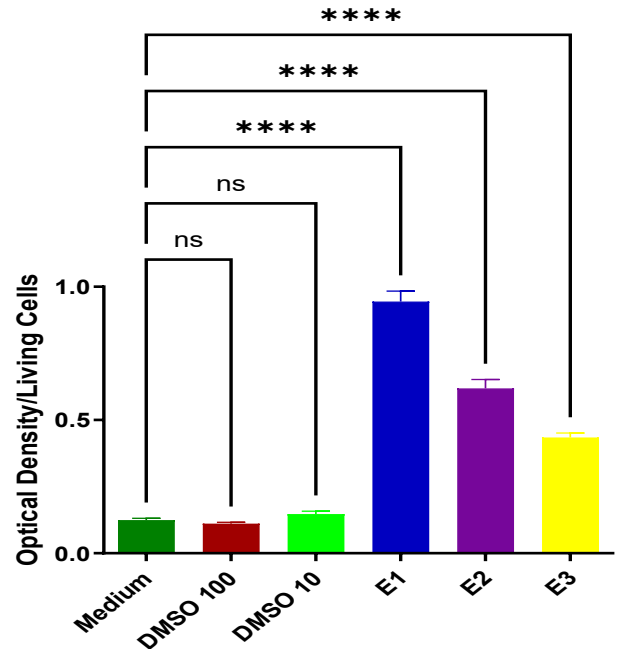


Figure 12: Effect of aqueous extract of *A. conyzoides* on cell proliferation assessed by MTT test. The negative control was medium, while other groups included DMSO (10% and 100%) and different extract concentrations (E1 = 500 mg/mL; E2 = 250 mg/mL; E3 = 125 mg/mL). **** $P < 0.0001$; ns, not significant; n = 9. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc. Each group was compared with the medium.

Measurement of PCNA (proliferating cell nuclear antigen) in the presence of specific markers CD140a and Involucrin

The results of the PCNA assay are presented in Figure 14. The higher the percentage of cells labeled by PCNA, the greater the cell proliferation. CD140a marker was used for fibroblasts, and Involucrin for keratinocytes. The results show that treatment with E1 (500 mg/mL), E2 (250 mg/mL), and E3 (125 mg/mL) affected cell proliferation differently. Fibroblast proliferation was slightly increased in the E1 (15.4%) and E2 (12.5%) groups compared with the controls (11.9%), whereas keratinocyte proliferation was markedly enhanced, with the highest proliferation rate observed in the E1 (64.7%) group. Keratinocyte proliferation rate decreased as the concentration decreased, same as MTT and CFSE test results

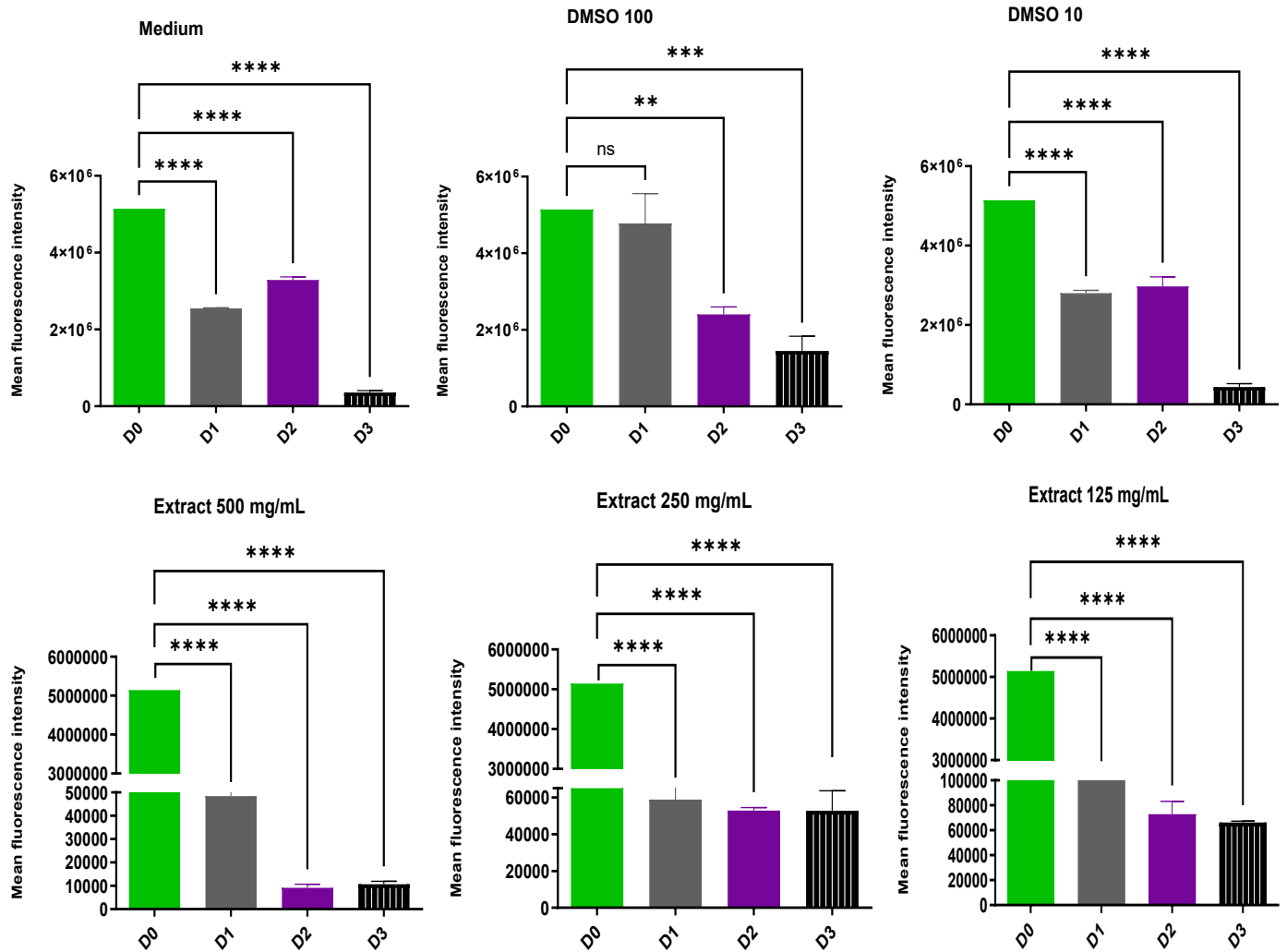


Figure 13: Evaluation of cell proliferation using CFSE staining after treatment with DMSO (100% and 10%) and aqueous extract of *A. conyzoides* (500 mg/mL, 250 mg/mL, and 125 mg/mL) on Days (D) 0, 1, 2, and 3. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data are expressed as mean $\pm$ SEM, $n = 3$. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc.

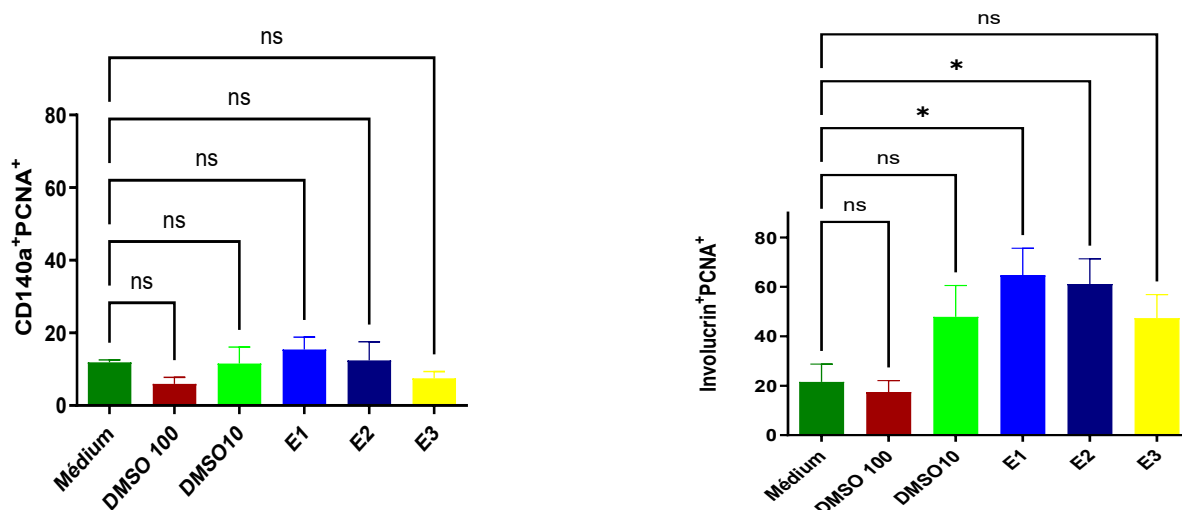


Figure 14: Effect of aqueous extract of *A. conyzoides* on fibroblast and keratinocyte proliferation assessed by PCNA assay. (A) Percentage of CD140a⁺/PCNA⁺ proliferating fibroblasts. (B) Percentage of involucrin⁺/PCNA⁺ proliferating keratinocytes. The negative control was medium, while other groups included DMSO (10% and 100%) and different extract concentrations (E1 = 500 mg/mL; E2 = 250 mg/mL; E3 = 125 mg/mL). * $P < 0.05$; ns, not significant, $n = 5$. Data are mean $\pm$ SEM, one-way ANOVA followed by Dunnett's post hoc.

Toxicity tests

PI (Propidium Iodide) + Annexin V Assay

The PI and Annexin V assay results are presented as follows: the percentage of dead cells reflects the extract's toxicity; the higher the percentage, the more toxic the extract (Figure 15). PI marks necrotic cell death, whereas Annexin V marks apoptotic cell death. Early apoptotic cells exclude PI, while late-stage apoptotic and necrotic cells stain positively because these dyes enter the nucleus and bind to DNA. The PI/Annexin V analysis revealed an increase in dead cell in extract-treated groups

compared with the control group (medium and DMSO10%). DMSO 100% showed the highest cytotoxic effect, while E1 appeared to induce the strongest apoptotic response among the tested extracts. The effect of the extract increased as the concentration of the extract increased.

Inflammatory Activity of Keratinocytes and Fibroblasts

The results of the TNF- α assay are shown in Figure 16. The amount of TNF- α in the wells containing the extract being lower than that observed in the culture medium shows that the extract did not produce a pro-inflammatory response.

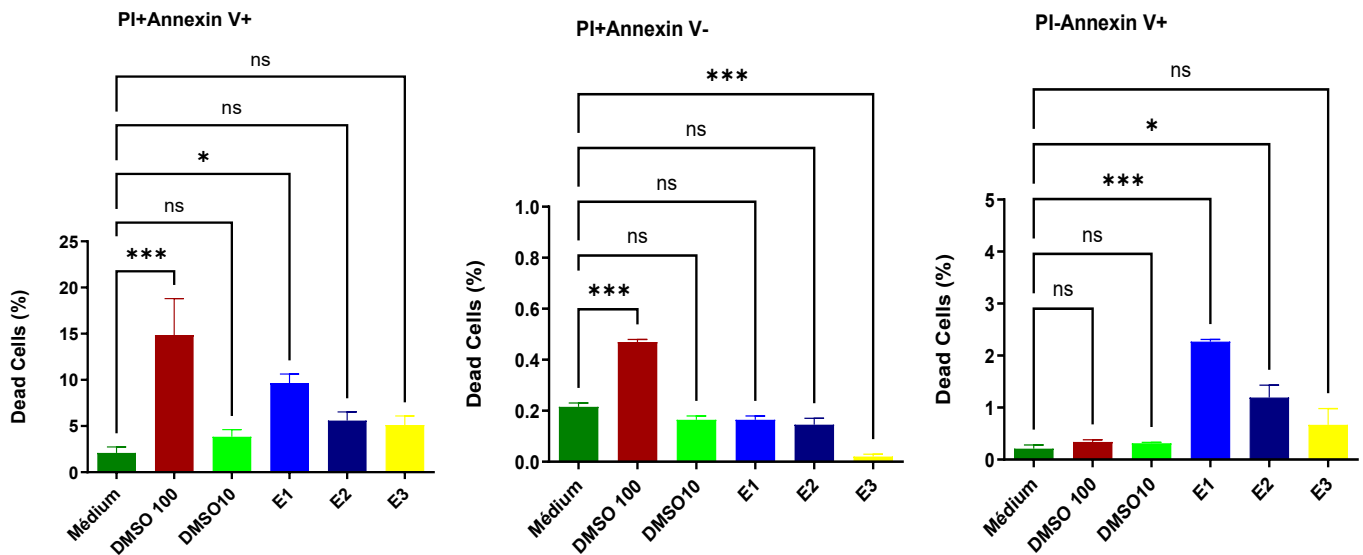


Figure 15: Analysis of cell death using PI and Annexin V staining in control (Medium), DMSO (100% 10%), and *A. conyzoides* extract-treated (500 mg/mL, 250 mg/mL, 125 mg/mL) cells. * $P < 0.05$, *** $P < 0.001$; ns = not significant; $n = 6$. Data are mean $\pm$ SEM, one-way ANOVA followed by Dunnett's post hoc.

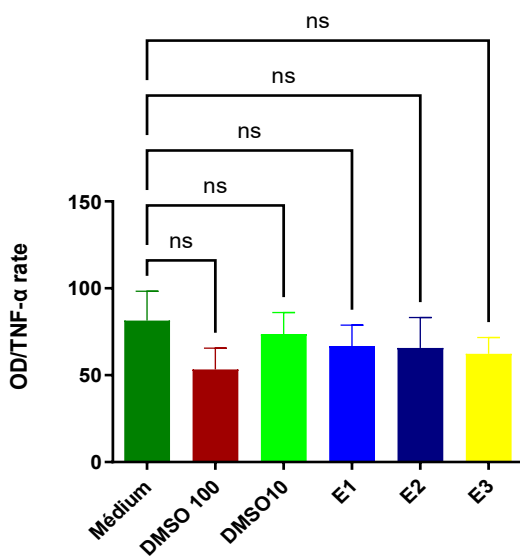


Figure 16: Effect of aqueous extract of *A. conyzoides* on TNF- α cytokine levels in control (Medium), DMSO (100% 10%), and extract-treated (500, 250, 125 mg/mL) cells. ns = not significant, $n = 8$. Data are mean $\pm$ SEM, one-way ANOVA followed by Dunnett's post hoc.

DISCUSSION

Proliferation and migration are two main events that occur during the proliferative stage of wound healing, involving many types of cells, including fibroblasts, keratinocytes, endothelial cells, and epithelial cells (Safwan Kamarazaman *et al.*, 2024). Fibroblasts and keratinocytes are key cells in re-epithelialization, so activating them would promote rapid healing. Several tests, including MTT, CFSE, and the PCNA assay (a recognized proliferation marker), confirmed this activation in the presence of the aqueous leaf extract of *Ageratum conyzoides*, a plant known for its healing properties (Odeleye *et al.*, 2014). Our results show that keratinocytes and fibroblasts proliferated as extract concentration increased. These results are consistent with previous studies reporting that topical application of extracts from this plant has been associated with a significant acceleration in wound contraction, an increase in the rate of re-epithelialization due to the proliferation of keratinocytes and fibroblasts, and repair of tissue (Dash and Narasimha, 2011; Arulprakash *et al.*, 2012). The greater

proliferation of keratinocytes than fibroblasts is thought to be because keratinocytes have a very high capacity for division (Alberts *et al.*, 2010).

Our results have also shown no inflammatory response. Indeed, a variety of plant-derived natural products have been shown to exhibit significant anti-inflammatory activity by inhibiting important pro-inflammatory mediators (Iswantini *et al.*, 2025). Flavonoids, phenolic compounds abundant in most plants, exhibit strong anti-inflammatory properties that are crucial for wound healing. The observed effects, namely increased proliferation and no inflammatory response, could be linked to the antioxidant and anti-inflammatory properties of the leaf components. This proliferation was obviously caused by the phytochemicals present in the extract, such as tannins and flavonoids, which possess antioxidant and wound-healing effects (Safwan Kamarazaman *et al.*, 2024). Phytochemical compounds found in plants, such as flavonoids, terpenoids, and alkaloids, exhibit potent bioactivities, including stimulating cell proliferation and differentiation (Molefe *et al.*, 2025).

Several studies have shown that *Ageratum conyzoides* is rich in flavonoids, phenolic compounds, and alkaloids, which can neutralize reactive oxygen species and modulate the inflammatory response (Okunade, 2002). In one of our previous studies, TLC analysis showed the presence of tannins, flavonoids, and terpenes (Akpalo *et al.*, 2020). Here we quantify flavonoids and tannins. Flavonoids, a prominent class of polyphenols found in plants, are most intensively studied for their antioxidant and biological activities (Wróblewski *et al.*, 2024).

In the post-inflammatory phase, anti-inflammatory agents suppress cytokines, reducing the inflammatory response and accelerating healing. Here, we quantify tannins, flavonoids, and total phenols. Furthermore, recent data suggest that *A. conyzoides* may inhibit NLRP3 inflammasome activation and reduce pro-inflammatory cytokine expression, such as IL-1 β , IL-18, and TNF- α , thereby limiting tissue damage and promoting the proliferative phase (Xu *et al.*, 2023). TNF- α plays a dual role, promoting inflammation and tissue repair in acute wounds but contributing to prolonged inflammation and tissue damage in chronic wounds when dysregulated (Molefe *et al.*, 2025). In the post-inflammatory phase, anti-inflammatory agents suppress these cytokines, reducing the inflammatory response and accelerating healing (Dubey and Dixit, 2023).

The cell viability test is often the first procedure performed in in vitro studies to determine the concentration of non-cytotoxic substances. This method measures cell viability in cell culture to assess the in vitro effects of pharmaceuticals on cell proliferation using cell-mediated cytotoxicity assays (Iswantini *et al.*, 2025). Cell viability assessment in our study using Annexin V/propidium iodide

(PI) staining revealed moderate toxicity of the extract at the highest concentration (500 mg/mL), with this toxicity being more apoptotic than necrotic. Annexin V+/PI- indicates early apoptosis, whereas Annexin V+/PI+ generally indicates late apoptosis/secondary necrosis. PI+/Annexin V- may represent primary membrane-compromised necrosis. In our case, these apoptotic cell deaths may relate to autophagy in keratinocytes, which promotes proliferation and migration, along with fibroblast activation (Jin *et al.*, 2025).

However, toxicity decreases as the extract concentration decreases, indicating a dose-dependent effect. This response is common with plant extracts, which contain complex mixtures of bioactive compounds that can be beneficial at low concentrations but harmful at high concentrations (Calabrese and Baldwin, 2003). Studies by Djeneb *et al.* (2021) also showed slight toxicity of the extract on different cells and a dose-dependent effect, which corroborates our results.

CONCLUSION

In this study, we showed that aqueous leaf extract of *Ageratum conyzoides* promotes fibroblast and keratinocyte proliferation but induces moderate toxicity at the highest concentration; both effects are dose-dependent. However, no pro-inflammatory effect was observed. Future studies would focus on improving cell culture conditions with specific culture media and identifying the range of concentrations that promote cell proliferation without inducing toxicity. Further studies would also evaluate the pro- and anti-inflammatory properties of the extract and the potential of keratinocyte autophagy.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

AUTHORS' CONTRIBUTION

OVK performed plant extraction, cell extractions, and all analyses. GK supervised cell technical analyses and made valuable comments on the manuscript. AEA proposed the study and gave valuable scientific advice.

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