

Antibacterial and anti-inflammatory activity of extracts and major constituents derived from *Stachytarpheta indica* Linn. leaves and their potential implications for wound healing

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Abstract

Wounds of various types continue to have a severe socioeconomic impact on the cost of health care. Globally, there has been increased interest surrounding the identification of bioactive compounds that promote or modulate the wound healing process. *Stachytarpheta indica* Linn. is traditionally used to heal wounds and relieve inflammation, however, the theorised pharmacological properties have not yet been scientifically validated. In this study, dried and ground plant leaves were extracted with water and methanol, which were then subjected to various analyses. Antimicrobial activity of the plant extracts and isolated compounds was determined using well diffusion assays, while the minimum inhibitory concentrations were determined with a colorimetric assay. Morphological changes of human keratinocytes in response to plant extracts were observed with differential interference contrast microscope imaging. Cell viability, proliferation and migratory effects post-treatment with the plant extracts were also evaluated via colorimetric cytotoxicity assays and a real-time cell analyser protocol. Anti-inflammatory effects of plant extracts and isolated compounds were evaluated by flow cytometry and cyclooxygenase and lipoxygenase enzyme inhibition assays. Three active compounds i.e. ipolamiide, verbascoside and iso-verbascoside, were isolated from *S. indica* leaves. Verbascoside demonstrated broad-range antibacterial activity and imposed strong inhibition at 9.77 µg/mL against *Staphylococci* spp. *S. indica* extracts (0.1-0.2 mg/mL) were shown to improve human keratinocyte proliferation up to 60 % and induce morphological changes by producing cytoplasmic projections at concentrations higher than 0.4 mg/mL. Plant extracts (6.25-100 µg/mL) and individual compounds (3.125-50 µg/mL) elicited strong anti-inflammatory effects by suppressing the expression of interleukin-8 and inhibiting cyclooxygenase-1 and 5-lipoxygenase enzymes. Collectively, these results indicate that plant extracts and isolated compounds derived from *S. indica* have the potential to inhibit bacterial growth, promote tissue regeneration and reduce inflammation, hence, potentially providing the basis for a novel therapeutic for the treatment of wounds.

Full Text

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Figures

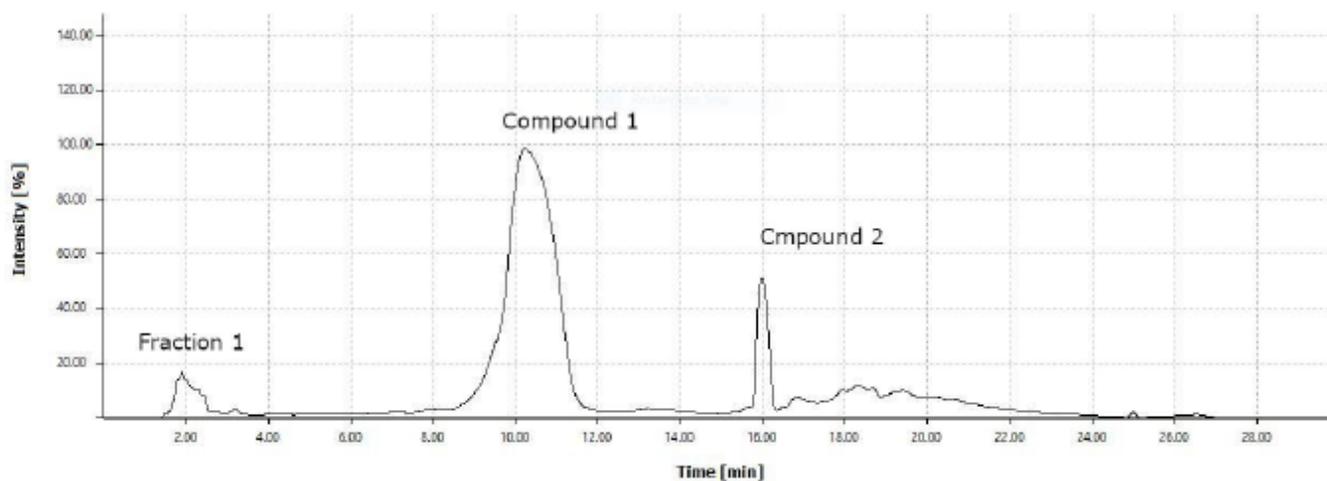


Figure 1

Chromatogram of eluted compounds from 30-80 % (v/v) methanol fractions of *S. indica* at 254 nm. Two compounds and a single fraction were isolated. (Chromatographic separation was performed using an Agilent ZORBAX™ preparative C18 column (150 mm x 21.2 mm, 5 μm); column temperature was maintained at room temperature; flow rate = 20 mL/min; injection volume = 900 μL).

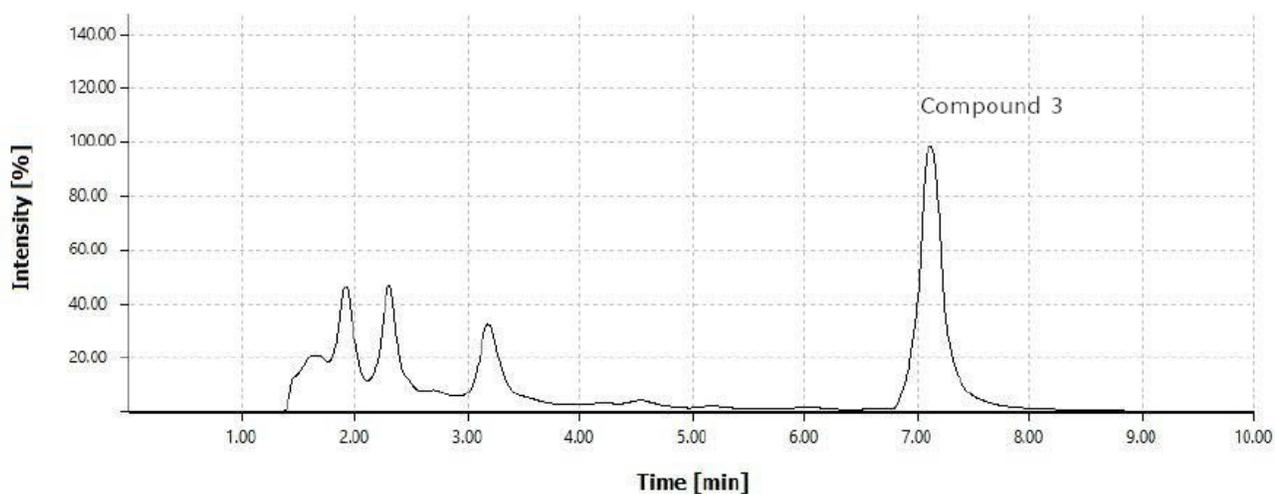


Figure 2

Chromatogram of *S. indica* HPLC fraction 1 at 254 nm. Compound 3 was isolated from this fraction. (Chromatographic separation was performed on Agilent ZORBAX™ preparative C18 column (150 mm x 21.2 mm, 5 μm); the mobile phase system comprised (A) 0.05 % v/v formic acid in Milli-Q water and (B) methanol; column temperature was maintained at room temperature; flow rate = 20 mL/min; injection volume = 800 μL).

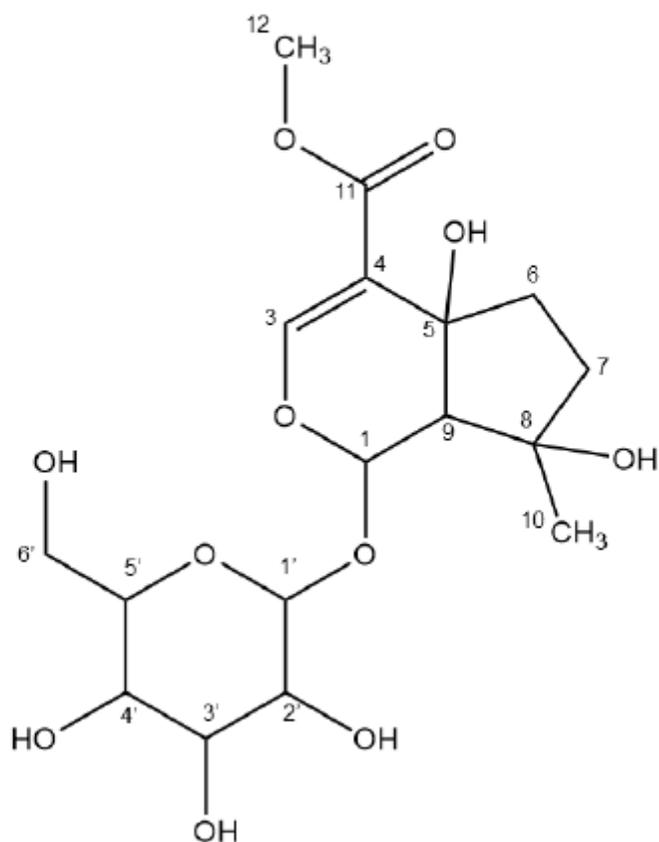


Figure 3

Chemical structure of compound 1 isolated from *S. indica* leaf extract. The compound was identified as ipolamiide (PubChem CID: 442425). The chemical structure illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

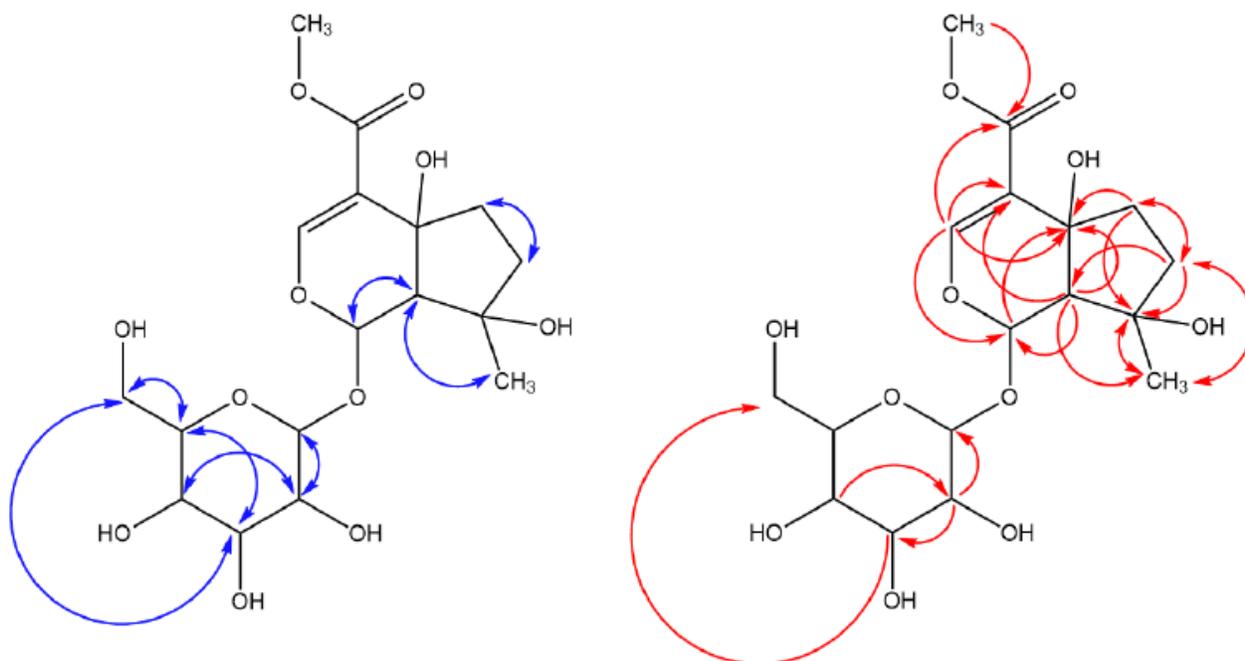


Figure 4

Correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) of compound 1 isolated from *S. indica* leaf extract. 2D nuclear magnetic resonance (NMR) results \boxtimes indicate COSY correlations, \boxdot indicate HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

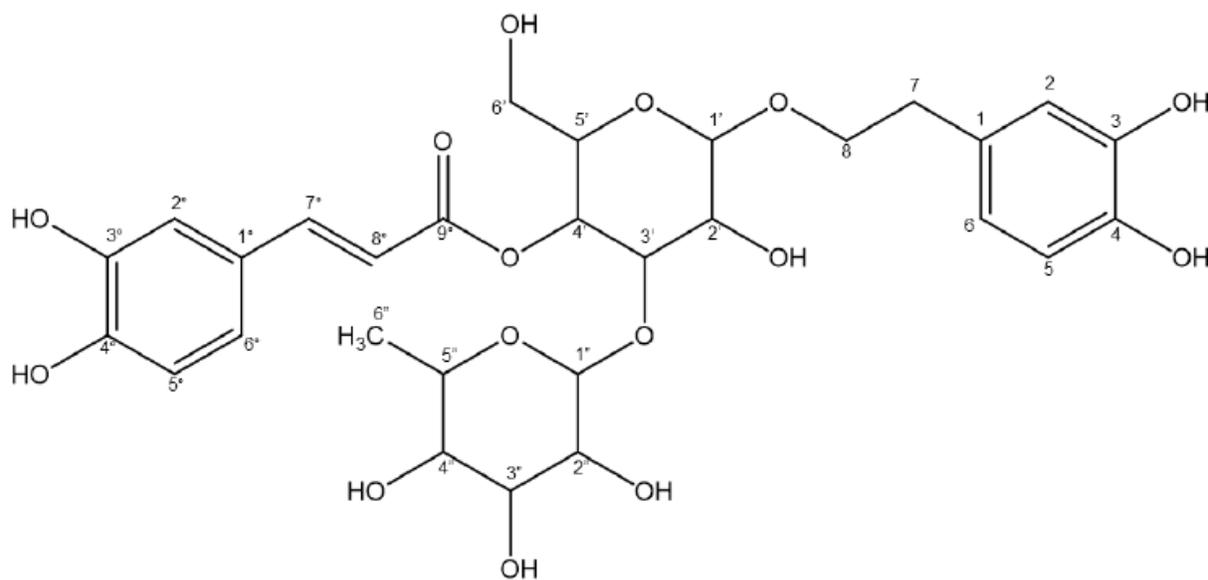


Figure 5

Chemical structure of compound 2 isolated from *S. indica* leaf extract. The compound was identified as verbascoside (also known as acetoside) (PubChem CID: 5281800). The chemical structure illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

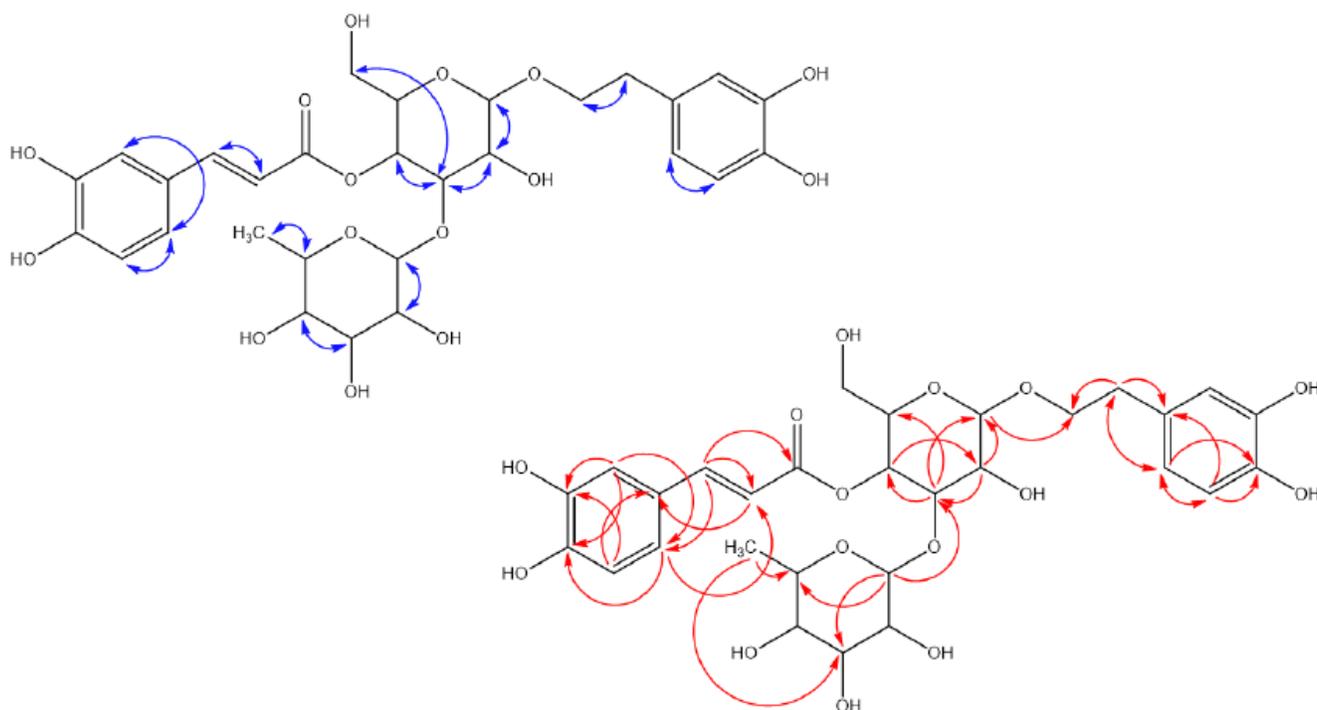


Figure 6

Correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) of compound 2 isolated from *S. indica* leaf extract. 2D nuclear magnetic resonance (NMR) results \curvearrowright indicate COSY correlations, \curvearrowleft indicate HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

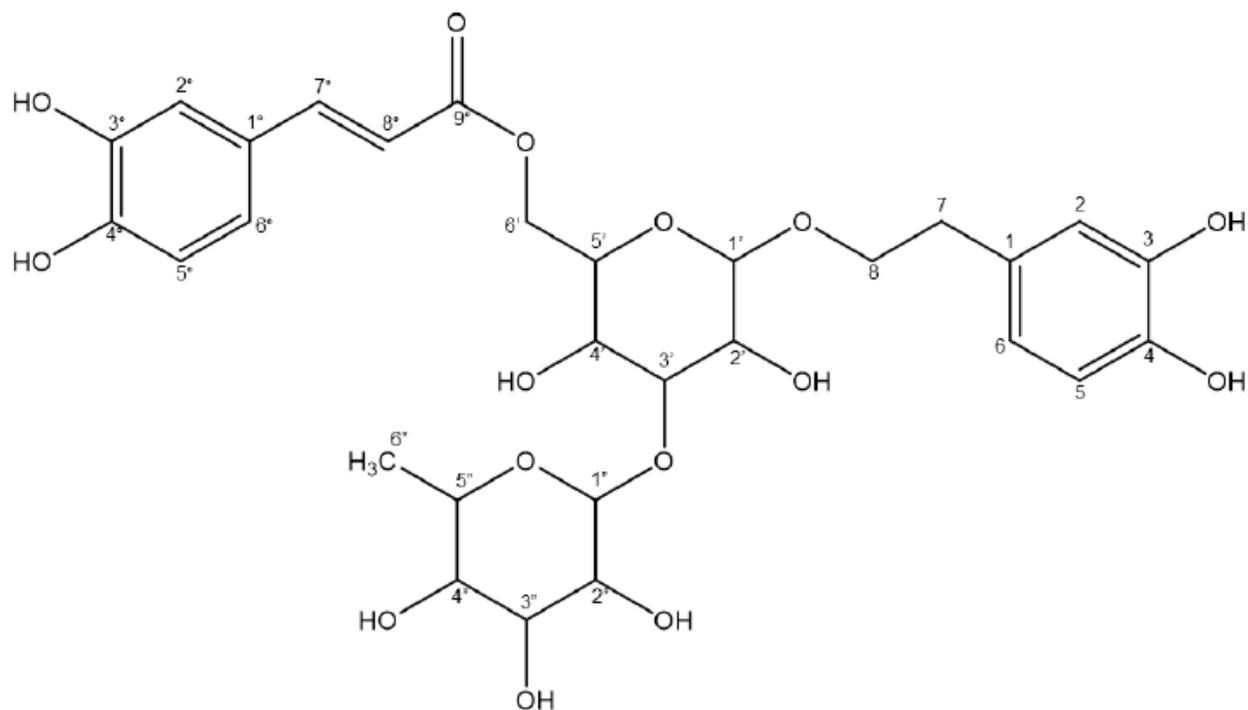


Figure 7

Chemical structure of compound 3 isolated from *S. indica* leaf extract. The compound was identified as isoverbascoside (also known as isoacetoside) (PubChem CID: 6476333). The chemical structure illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

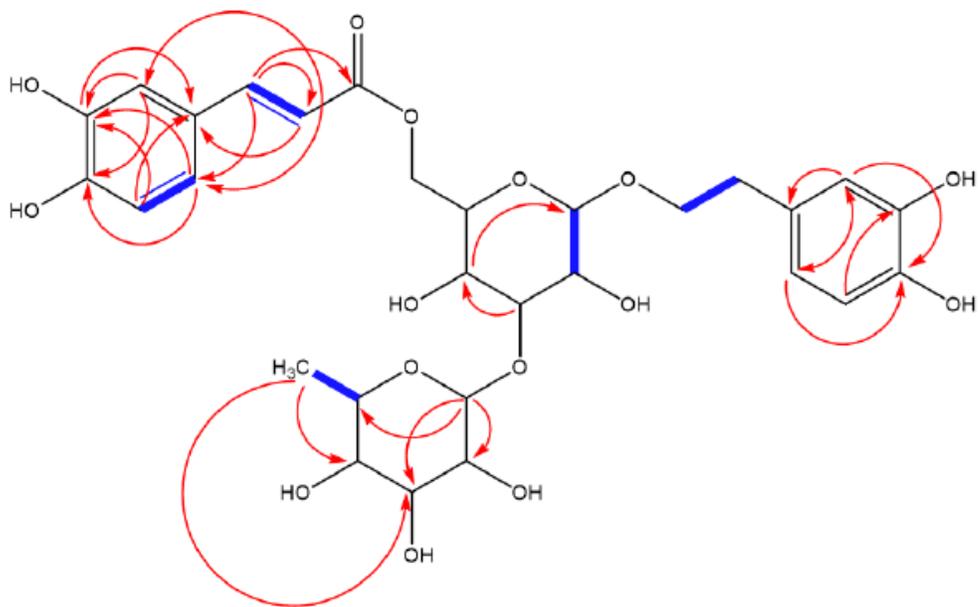


Figure 8

Correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) of compound 3 isolated from *S. indica* leaf extract. 2D nuclear magnetic resonance (NMR) results \curvearrowright indicate COSY correlations, \curvearrowleft indicate HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

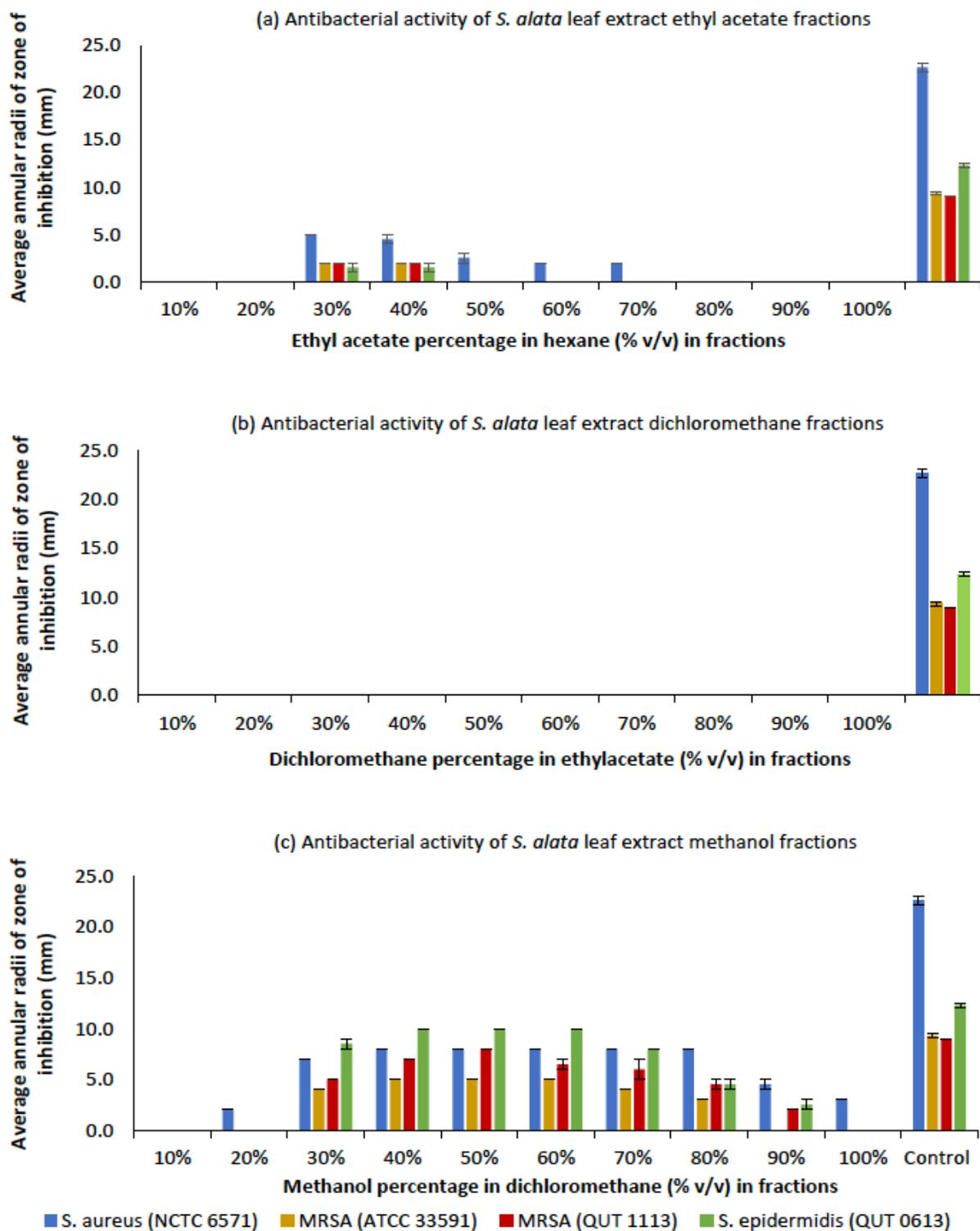


Figure 9

Antibacterial activity of *S. indica* leaf extract fractions at 10 mg/mL concentration. Averaged annular radii of clear zones \pm SEM. Thirty fractions were tested against four bacterial species which were susceptible to *S. indica* extracts in the preliminary antibacterial screening. (A) antibacterial activity of ethyl acetate fractions, (B) antibacterial activity of dichloromethane fractions and (C) antibacterial activity of methanol fractions. 30 – 80 % (v/v) methanol fractions showed antibacterial activity against

all four bacterial strains tested. Only 30 – 40 % (v/v) ethyl acetate fractions elicited antibacterial activity against all four bacteria. None of the dichloromethane fractions were bactericidal against any of the bacteria tested.

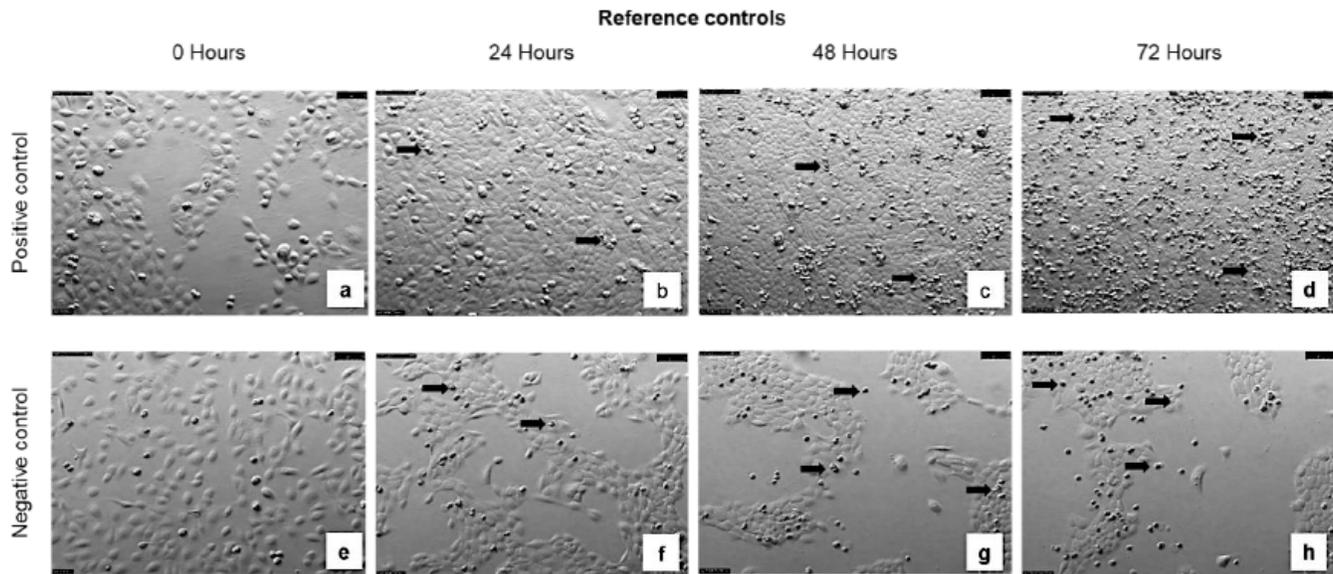


Figure 10

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) foetal 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.

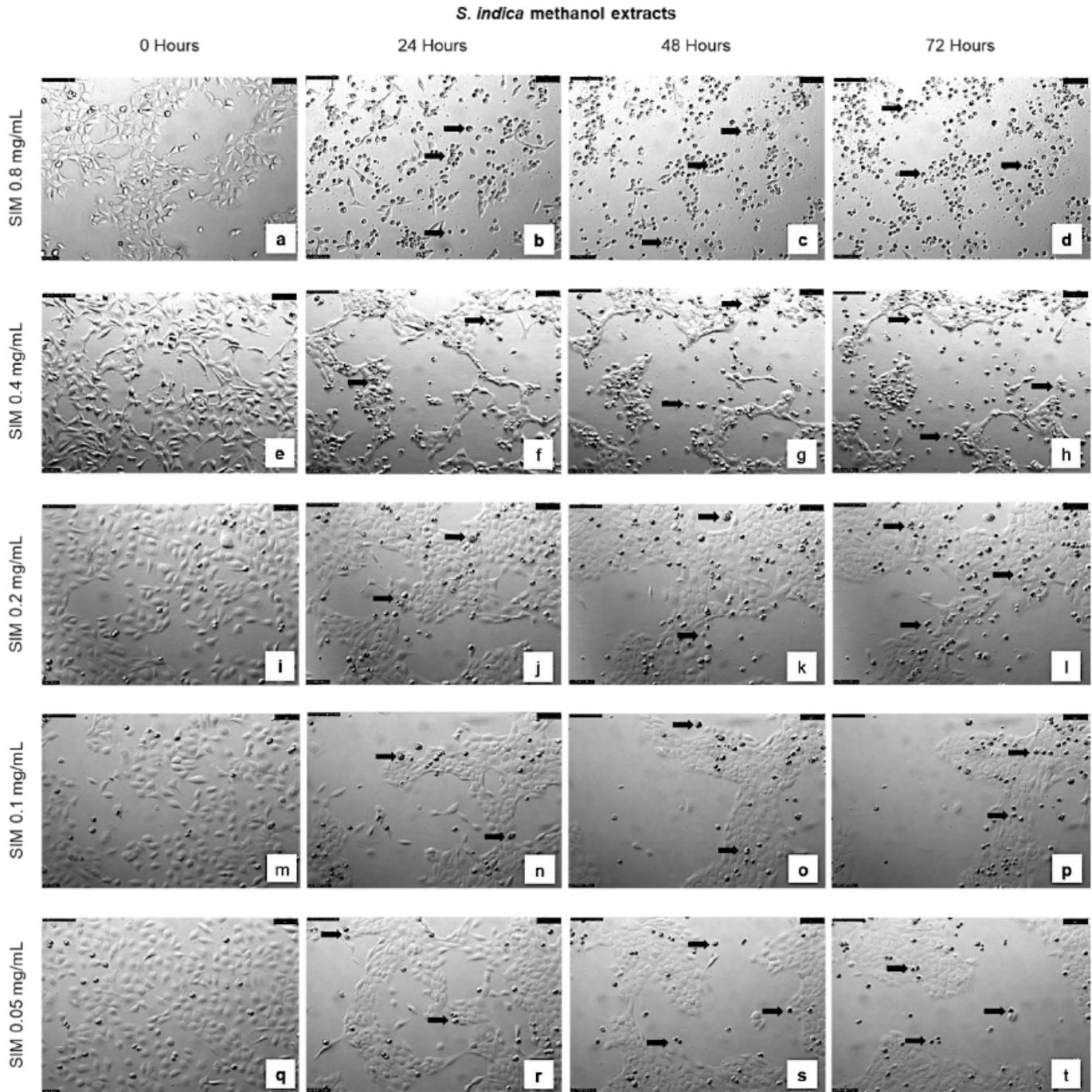


Figure 11

Proliferative effects of *S. indica* methanol extracts (at concentrations ranging from 0.8 - 0.05 mg/mL) post-treatment. Treated HaCaT cells were subjected to 72 hours microscopic examination and were compared with untreated cells grown in full growth media (FGM, Fig. 10 a-d) and untreated cells in serum free media (SFM, Fig. 10 e-h). Noticeable reduction in apoptosis was observed in HaCaT cells treated with SIM extracts within 24 - 72 hours, compared to positive control (Fig. 10 b-d). 0.05 mg/mL SIM extract maintained 50 % proliferation from 24 - 72 hours. 0.8 mg/mL SIM extract completely killed HaCaT cells within 24 hours. SIM: *S. indica* methanol. (→) indicates necrotic cells. Images were captured at 1000x magnification.

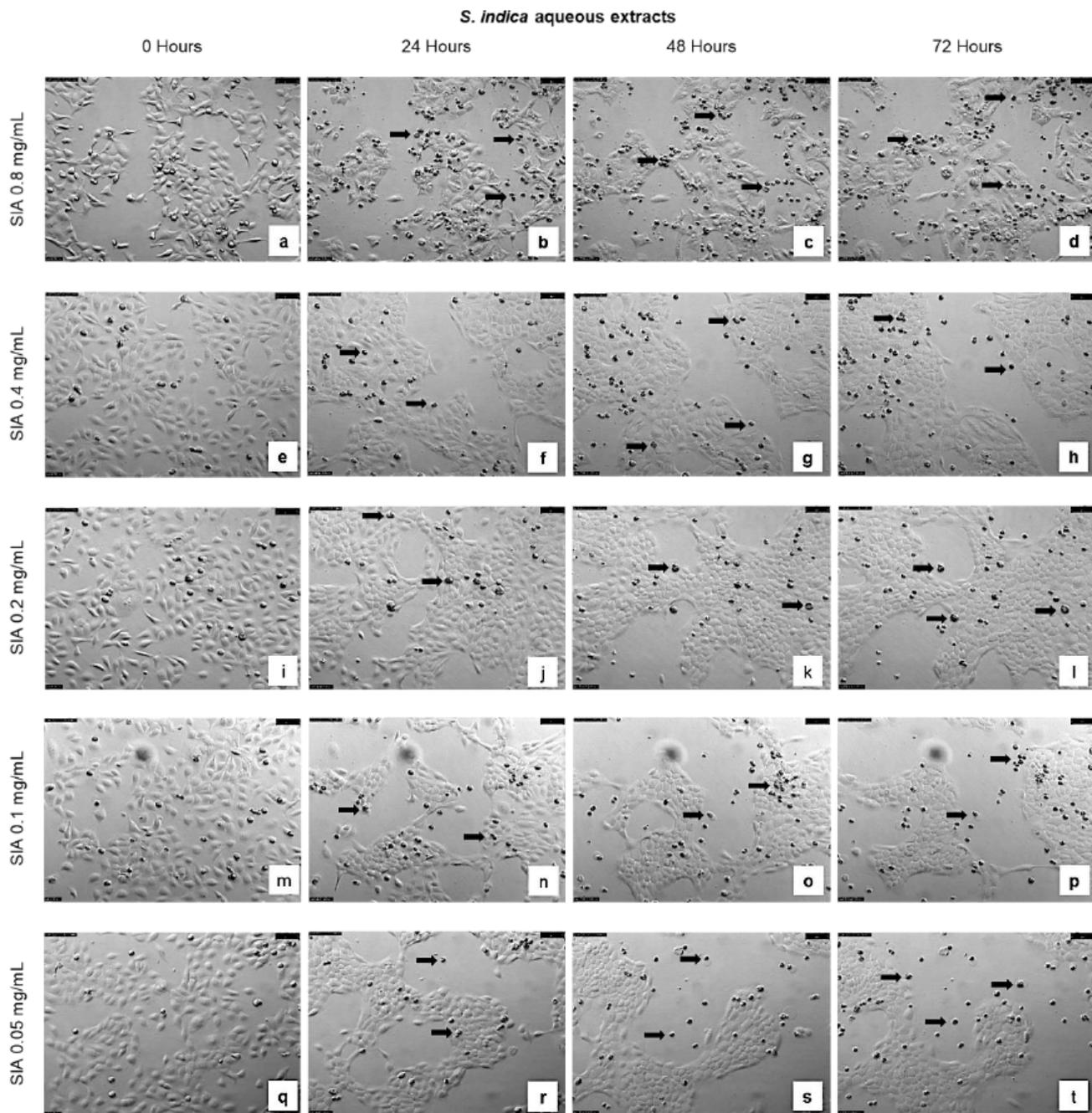


Figure 12

Proliferative effects of *S. indica* aqueous extracts (at concentrations ranging from 0.8 - 0.05 mg/mL) on human keratinocytes (HaCaT). Treated HaCaT cells were subjected to 72 hours of microscopic examination and were compared with untreated cells grown in full growth media (FGM, Fig.10 a-d) and untreated cells in serum free media (SFM, Fig.10e-h). Noticeable reduction in apoptosis was observed in HaCaT cells treated with SIA extracts within 24 - 72 hours, compared to the positive control (Fig.10 b-d). At a concentration ranging between 0.2 - 0.05 mg/mL, cells treated with SIA extracts maintained 60 % proliferation from 24 - 72 hours. SIA extract concentration of 0.8 mg/mL found to be detrimental to HaCaT cells after 24 hours. SIA: *S. indica* aqueous. (→) indicates necrotic cells. Images were captured at 1000x magnification.

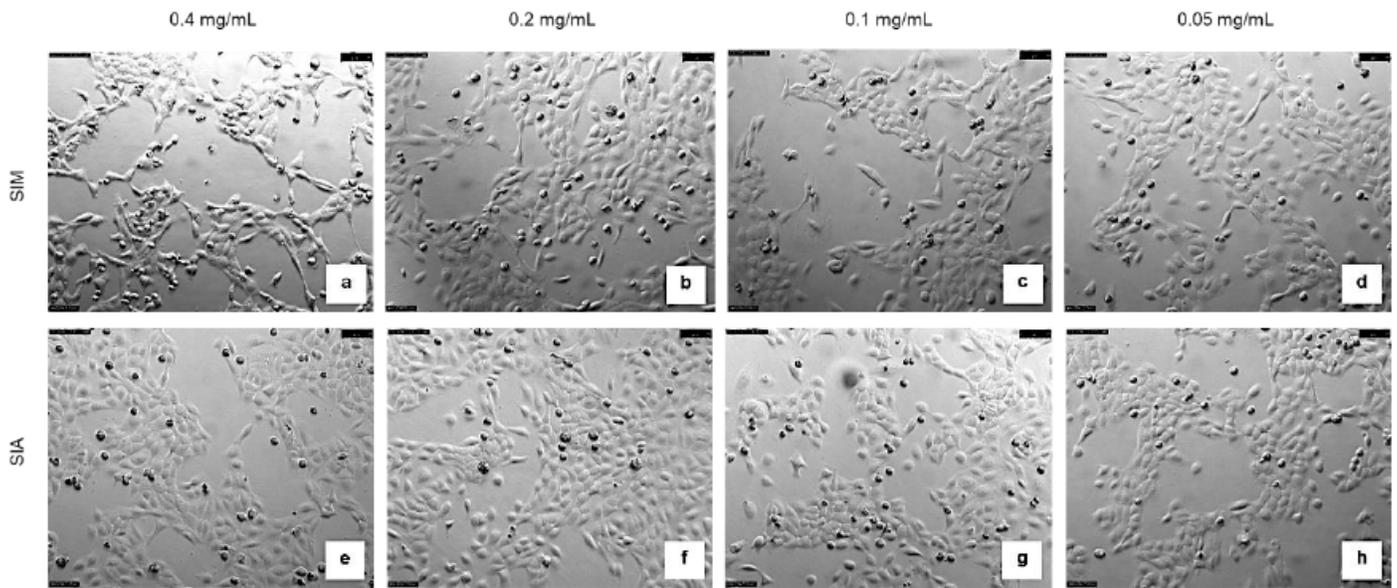


Figure 13

HaCaT cell treated with SIM extract within the concentration range 0.4 - 0.05 mg/mL (a-d), indicated high degree of motility while, SIA extract (e-h) showed reduction in motility as the concentration decreased. The comparison was based on the images obtained at 12:00 hours of the morphological assay. SIM: *S. indica* methanol and SIA: *S. indica* aqueous. Images were captured at 1000x magnification.

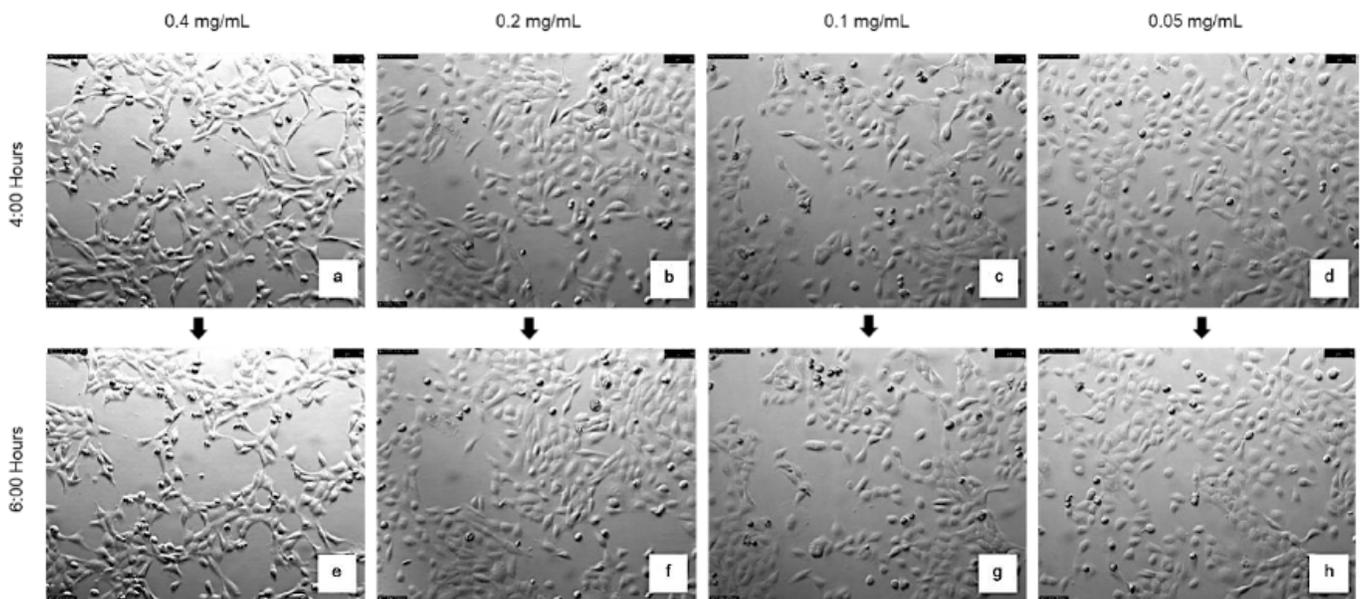
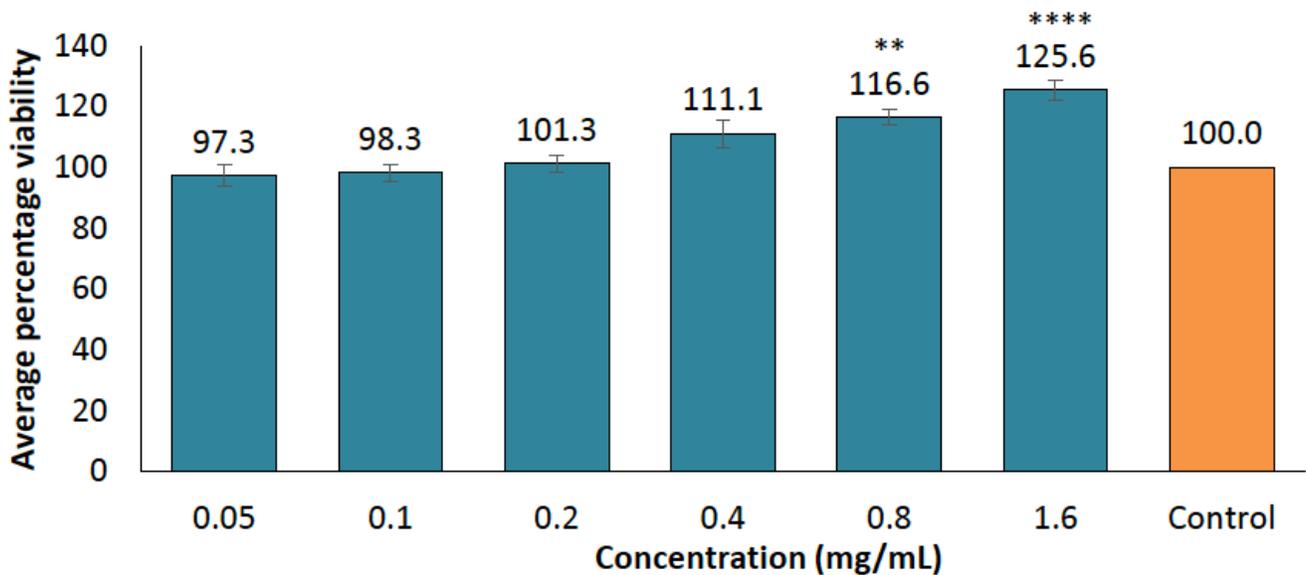


Figure 14

HaCaT cell treated with SIM extracts (0.4 - 0.05 mg/mL) indicating morphological activity producing cytoplasmic projections. The SIM extract at 4.0 mg/mL produced a high degree of morphological variation (a and e), which reduced as the concentration decreased (b and f, c and g, d and h)

consecutively). Comparison was based on the images obtained at 4:00 and 6:00 hours of the morphological assay. SIM: *S. indica* methanol. Images were captured at 1000x magnification.

(a) Relative percentage viability of HaCaT cells treated with SIM extract



(b) Relative percentage viability of HaCaT cells treated with SIA extract

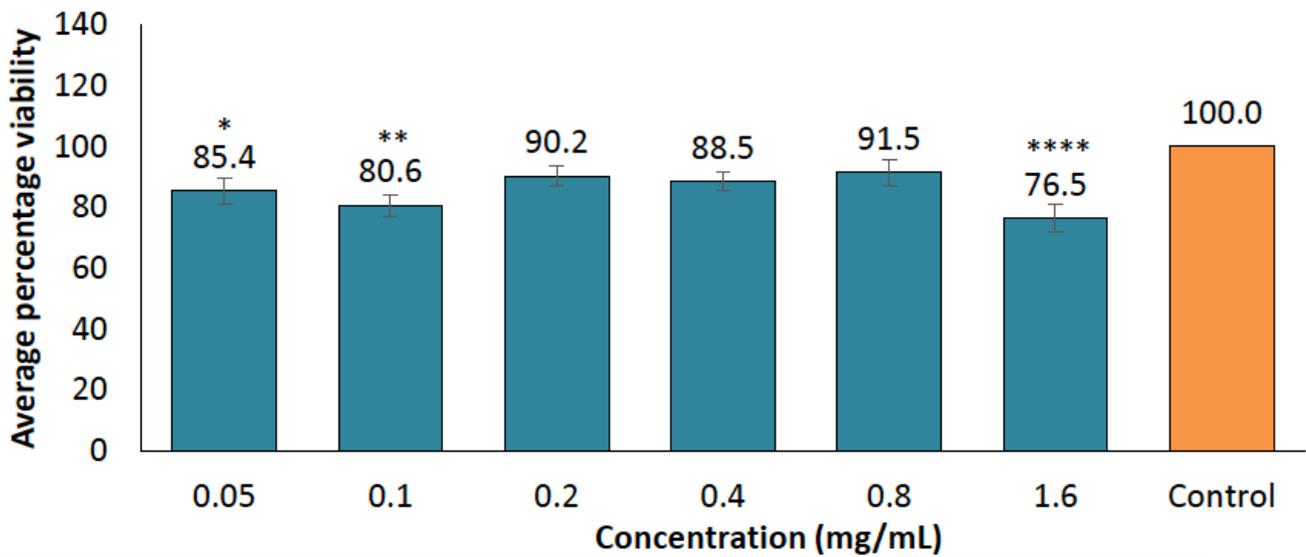
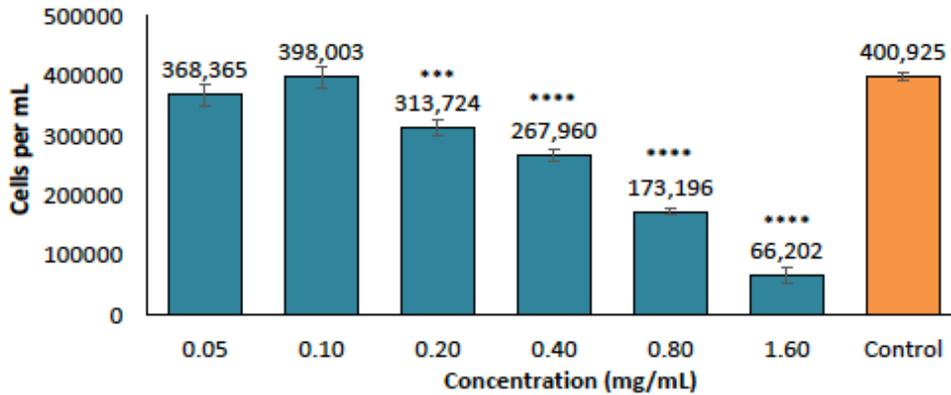


Figure 15

Relative percentage of keratinocyte viability treated with *S. indica* plant extracts: SIM (a) and SIA (b) at different concentrations (1.6 - 0.05 mg/mL) using the MTT assay. Each concentration represents the average percentage relative viability (\pm SEM). SIM extract concentrations ranging between 1.6 – 0.2 mg/mL induced higher percentage cell viability compared to positive control. Percentage cell viability induced by SIM extract at 1.6 mg/mL was significantly higher than that of the positive control. SIM extract concentrations between 0.1 - 0.05 mg/mL and all other extract treatments induced less

percentage cell viability compared to positive control. The positive control represents the untreated cells cultured in reduced serum media (RSM). Significance levels **** P<0.0001, *** P<0.001, ** P<0.01 and * P<0.05 compared to positive control. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, SIM: *S. indica* methanol, SIA: *S. indica* aqueous and RSM: reduced serum media.

(a) Proliferative effects of SIM extract on HaCaT cells



(b) Proliferative effects of SIA extract on HaCaT cells

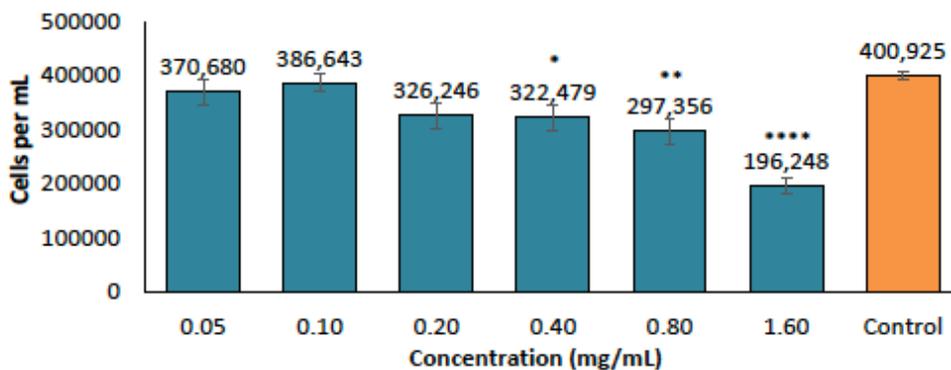


Figure 16

Viability of HaCaT cell treated with *S. indica* plant extracts; SIM (a) and SIA (b) measured by CyQUANT® assay. Values were expressed as averaged final concentrations of healthy cells (per/mL) ± SEM, classified by extract and concentration. In general, both extracts showed increase in cell concentration when the extract concentration decrease. Final cell concentrations observed in SIM extracts (1.6 – 0.4 mg/mL) and SIA extracts (1.6 – 0.8 mg/mL) treatments were significantly lower than that of the positive control. The positive control represents the untreated cells cultured in reduced serum media (RSM). Significance levels **** (P<0.0001), *** (P<0.001), ** (P<0.01) and * (P<0.05) compared to positive control. SIM: *S. indica* methanol and SIA: *S. indica* aqueous.

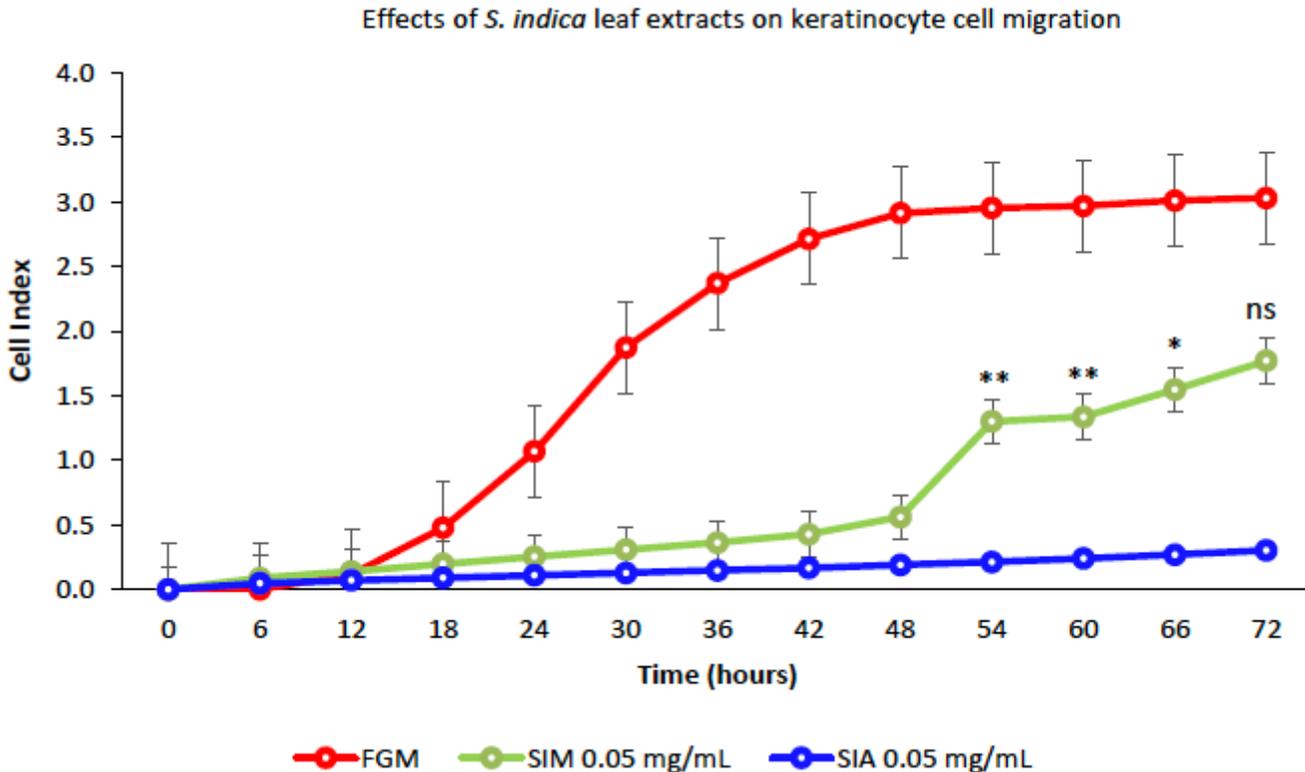


Figure 17

The cell index (CI) at 6 hour intervals (±SEM) of HaCaT cells treated with *S. indica* plant extracts at the derived optimum concentration of 0.05 mg/mL, including cells cultured in full grown media (FGM, positive control), over a 72 hour period. The results were normalised against cells cultured in serum free media (SFM, negative control). Plant extracts showed a cell index significantly lower than the positive control from 24 – 72 hours, except for SIM extract (0.05 mg/mL) which was shown to increase the cell index within 48 – 72 hours. Further, at 72 hours, the cell index of SIM extract at 0.05 mg/mL was not significantly different from the positive control. Significance levels ** (P<0.01) and * (P<0.05) compared to positive control at corresponding time intervals. CI of the SIM treated cells were significantly lower compared to those grown in FGM from 0 – 48 hours while CI of the SIA treated cells remained significantly (P<0.0001) lower compared to the positive control throughout the entire time period (0 - 72 hours). SIM: *S. indica* methanol, SIA: *S. indica* aqueous and ns: not significant.

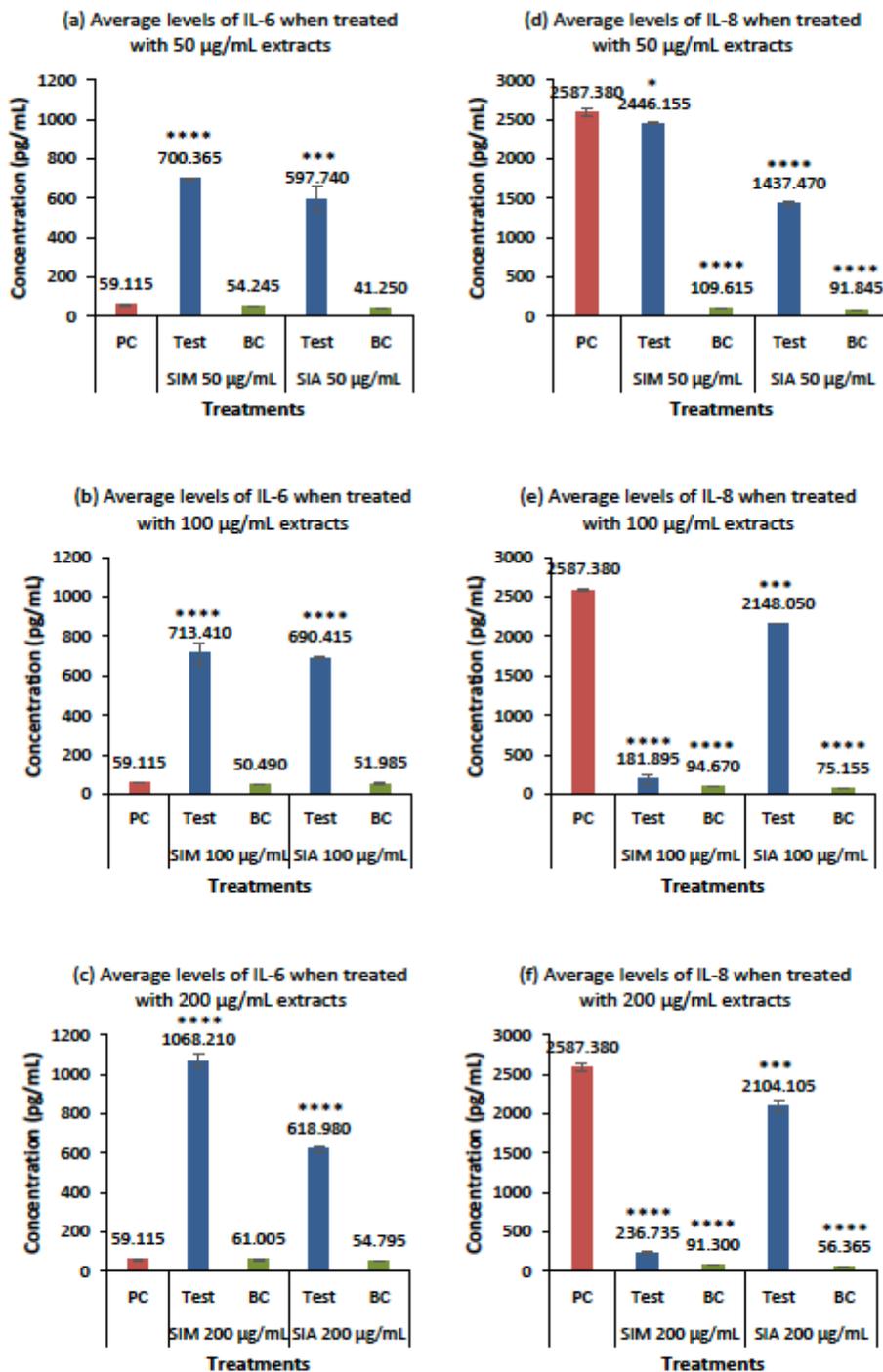


Figure 18

Average levels of interleukin (IL)-6 (a-c) and IL-8 (c-f) concentrations observed in the samples in pg/mL \pm SEM. 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. SIM: *S. indica* methanol and SIA: *S. indica* aqueous.

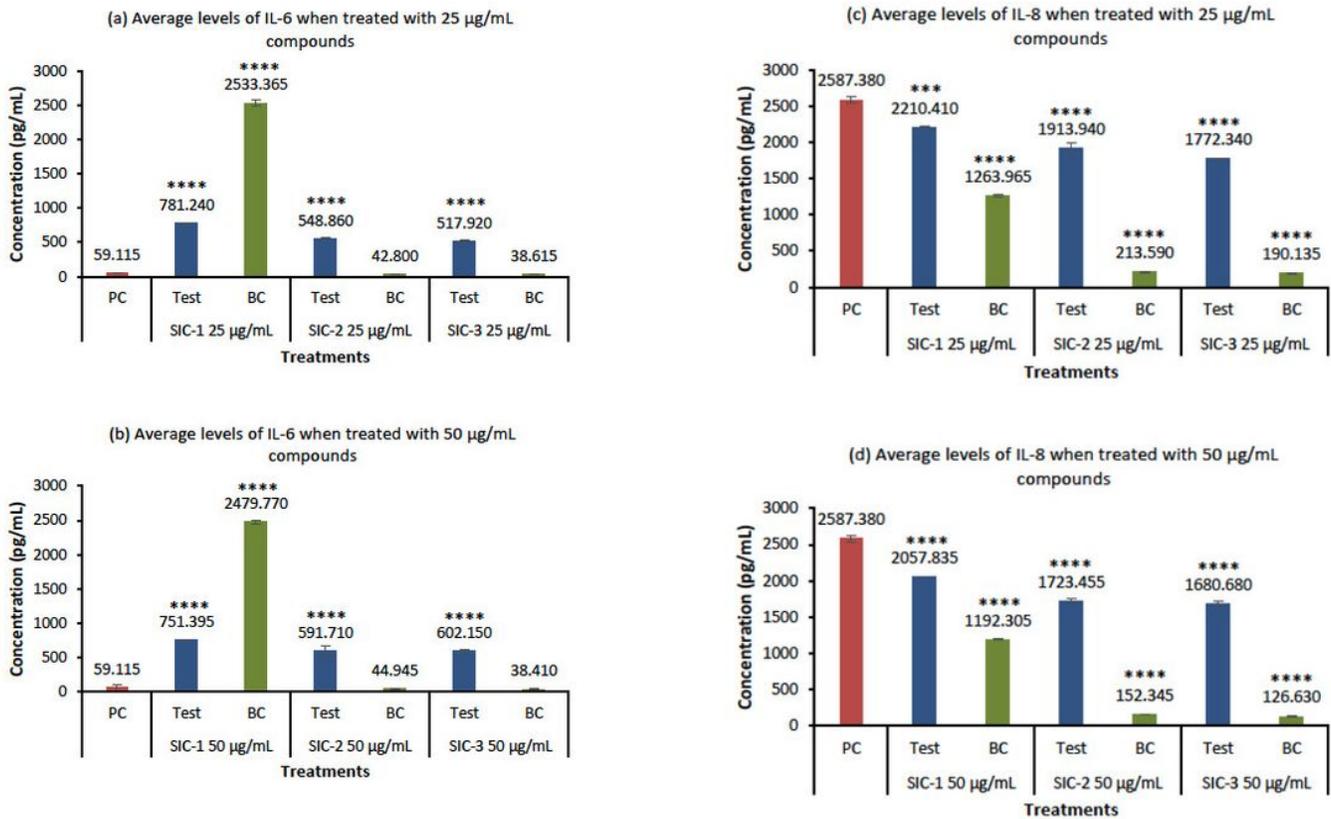


Figure 19

Average levels of interleukin (IL)-6 (a-b) and IL-8 (c-d) concentrations observed in the samples in pg/mL ± SEM. Cells pre-treated with lipopolysaccharides (LPS) followed by isolated compounds (25 and 50 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) and *** (P<0.001) compared to positive control. SIC: *S. indica* compound, SIC-1: ipolamiide, SIC-2: verbascoside and SIC-3: isoverbascoside.

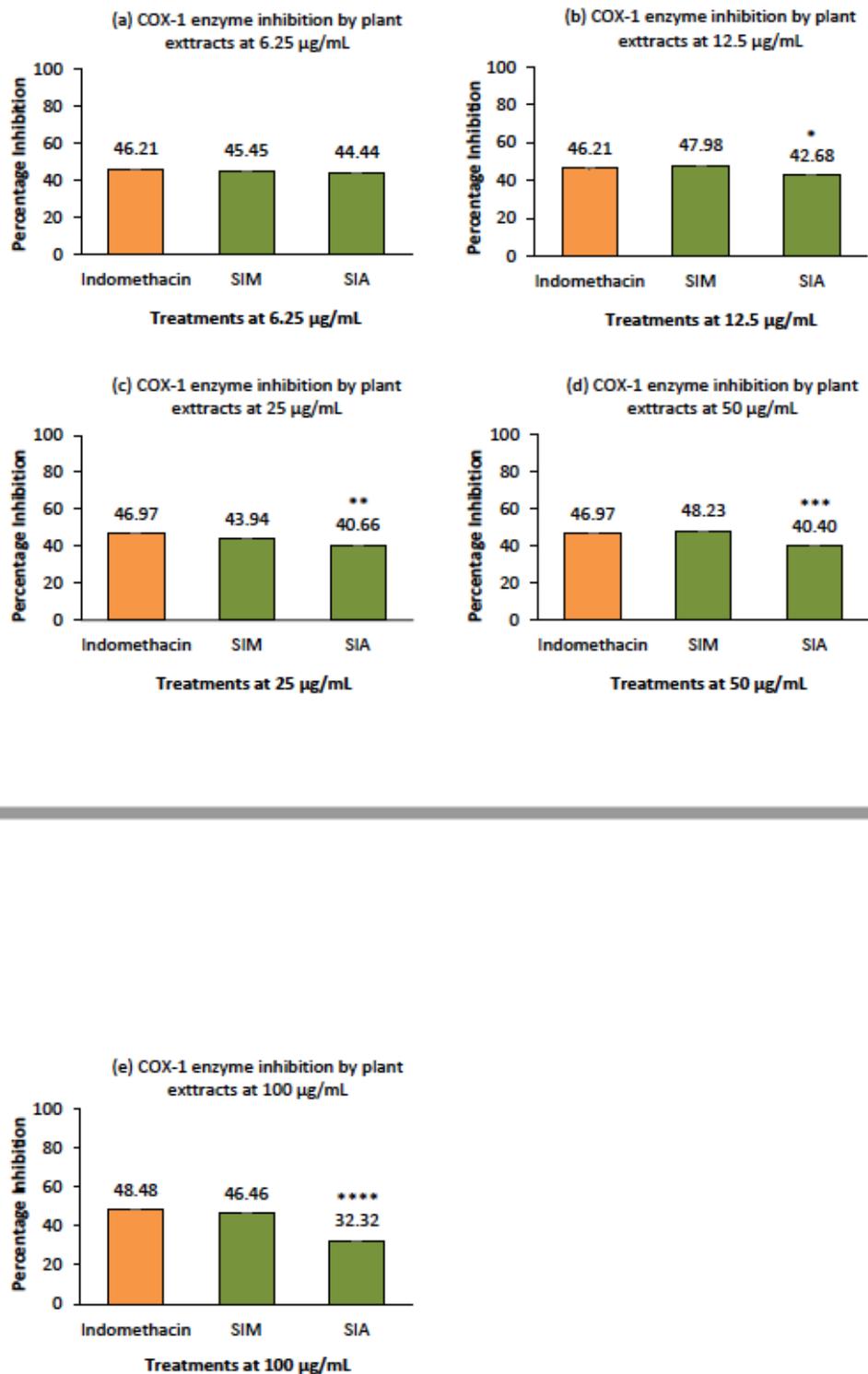


Figure 20

Cyclooxygenase (COX)-1 enzyme inhibition by plant extracts at five concentrations ranging from 6.25 – 100 µg/mL (a-e). Percentage inhibition (\pm 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. SIM: *S. indica* methanol and SIA: *S. indica* aqueous.

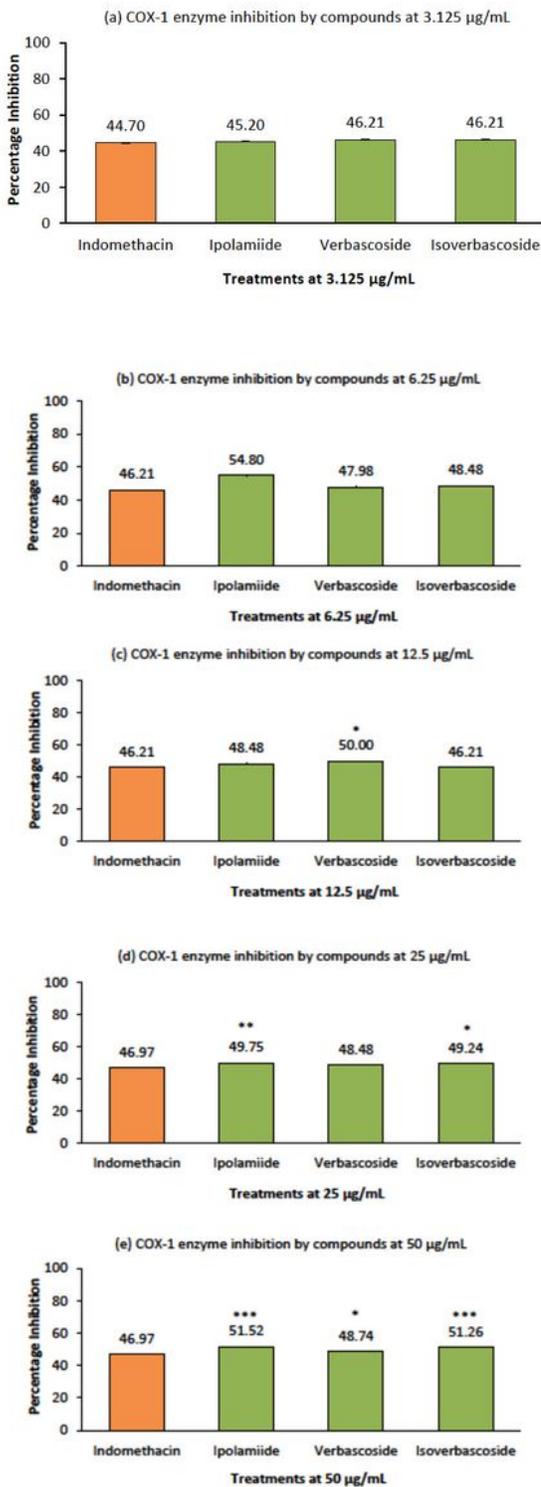


Figure 21

Cyclooxygenase (COX)-1 enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 – 50 µg/mL (a-e). Percentage inhibition (\pm 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 3.125 – 50 $\mu\text{g}/\text{mL}$ was used as a standard inhibitor for comparison. Significance levels *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$) compared to standard inhibitor at its respective concentrations. SIC: *S. indica* compound, SIC-1: ipolamiide, SIC-2: verbascoside and SIC-3: isoverbascoside.

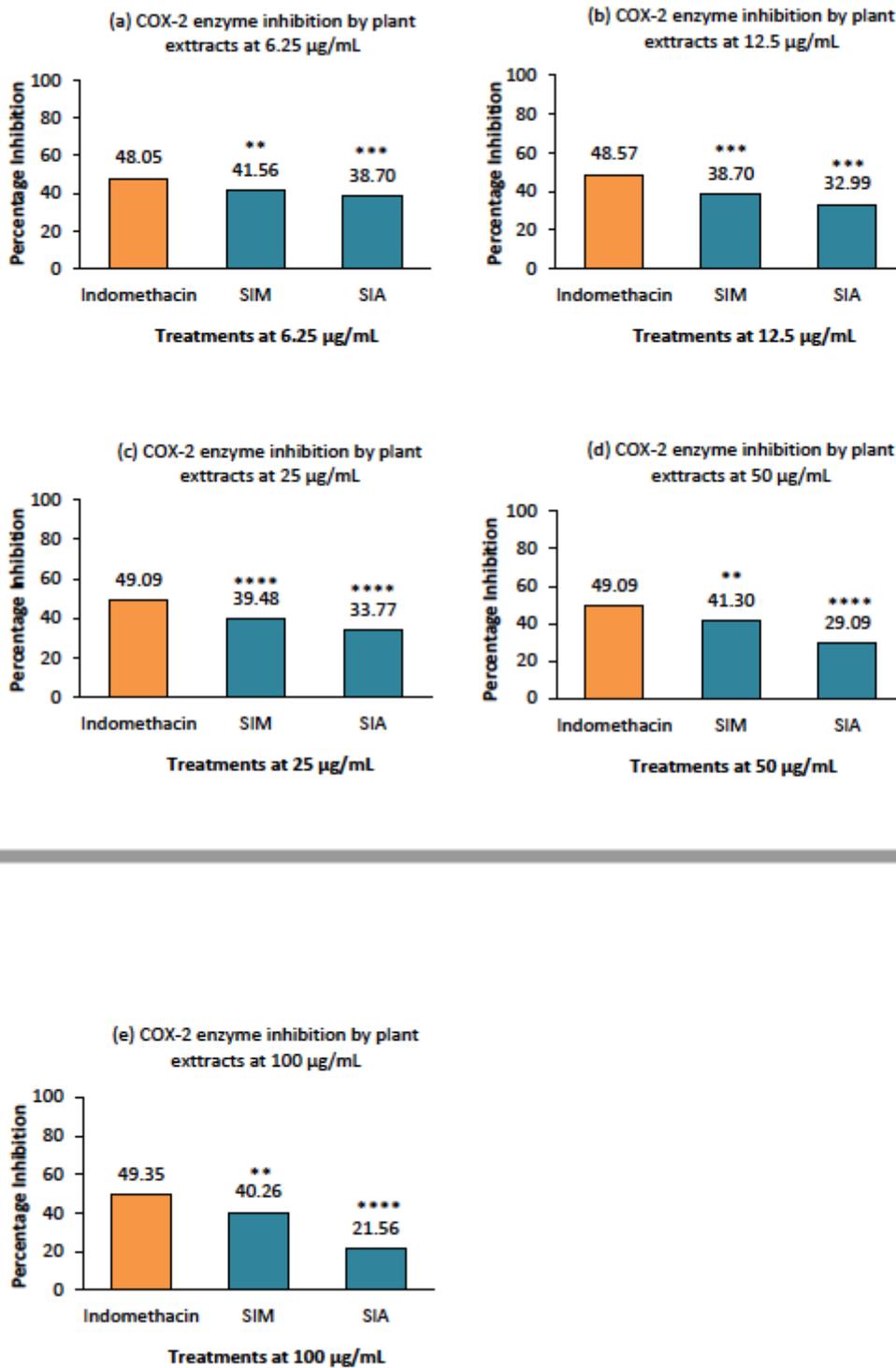


Figure 22

Cyclooxygenase (COX)-2 enzyme inhibition by plant extracts at five concentrations ranging from 6.25 – 100 µg/mL (a-e). Percentage inhibition (\pm 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) and ** (P<0.01) compared to standard inhibitor at its respective concentrations. SIM: *S. indica* methanol and SIA: *S. indica* aqueous.

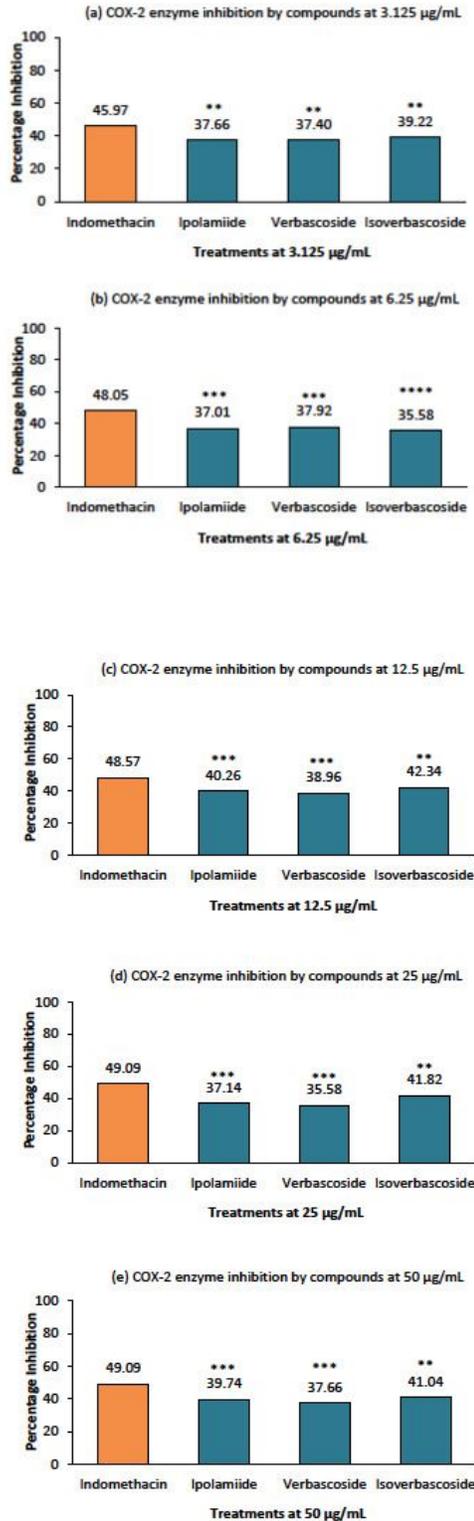


Figure 23

Cyclooxygenase (COX)-2 enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 – 50 µg/mL (a-e). Percentage inhibition (\pm 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 3.125 – 50 µg/mL was used as a standard inhibitor for comparison. Significance levels **** (P<0.0001), *** (P<0.001) and ** (P<0.01) compared to standard inhibitor at its respective concentrations. SIC: S. indica compound, SIC-1: ipolamiide, SIC-2: verbascoside and SIC-3: isoverbascoside.

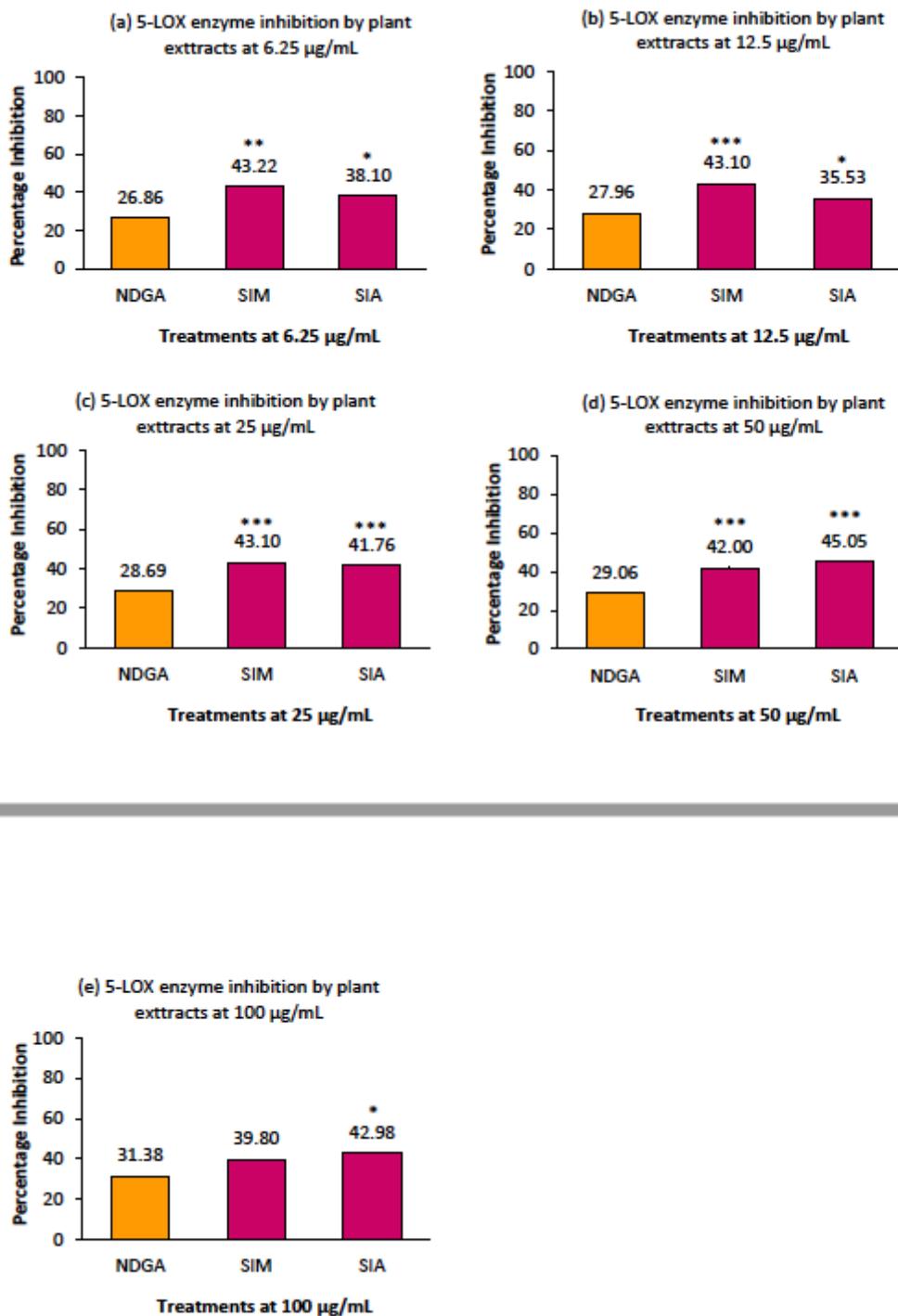


Figure 24

5-Lipoxygenase (LOX) enzyme inhibition by plant extracts at five concentrations ranging from 6.25 – 100 µg/mL (a-e). Percentage inhibition (\pm 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<0.001), ** (P<0.01) and * (P<0.05) compared to standard inhibitor at its respective concentrations. SIM: *S. indica* methanol and SIA: *S. indica* aqueous.

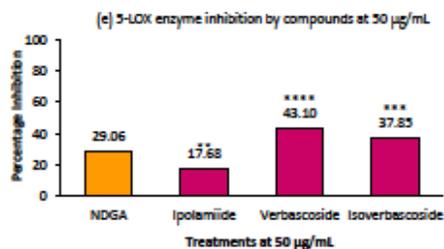
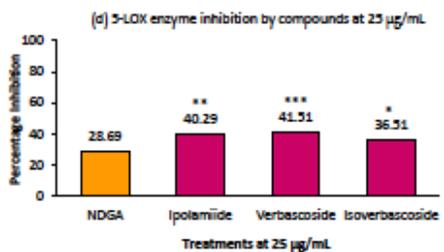
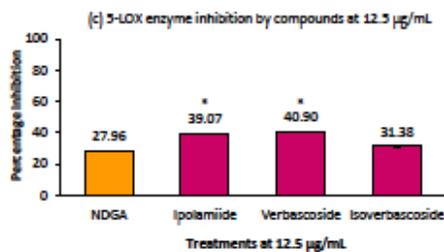
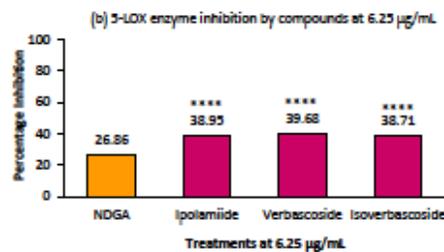
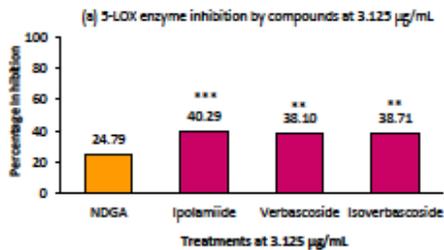


Figure 25

5-Lipoxygenase (LOX) enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 – 50 $\mu\text{g}/\text{mL}$ (a-e). Percentage inhibition (\pm 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 3.125 – 50 $\mu\text{g}/\text{mL}$ 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. SIC: *S. indica* compound, SIC-1: ipolamiide, SIC-2: verbascoside and SIC-3: iso-verbascoside.