Antibacterial and Anti-inflammatory Activity of Extracts and Major Constituents Derived from *Stachytarpheta indica* Linn. Leaves and Their Potential Implications for Wound Healing

Vajira Asela Agampodi1 · Peter Katavic1 · Christopher Collet1,2 · Trudi Collet1

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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. The 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 isoverbascoside, 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.

**Keywords** Antimicrobial · MRSA · Anti-inflammatory · Cytokine · Wound healing
Introduction

A wound is defined as the interruption of the cellular and anatomic continuity of a tissue, which may occur due to physical, chemical, thermal, microbial or immunological trauma [1]. Wound healing is a complex, yet precisely coordinated interaction between inflammatory cells and mediators, with significant overlap between the different phases of wound healing [2]. Chronic wounds are identified as those that fail to transgress through the normal wound healing process and perpetually cycle within the inflammatory phase [3, 4]. Rapid wound healing is critical, as delayed healing can result in conditions such as severe oedema and chronic ulcers [5]. The prevalence of chronic wounds is predicted to rise substantially, resulting in a significant financial burden on a worldwide scale [6–8]. Moreover, a report examining global markets and growth rates by product and country for the years 2020–2027 has projected that the world market in 2027 for total wound management will be worth over US$18.7 billion [9].

Plants contain numerous biologically active compounds, many of which have antimicrobial and/or anti-inflammatory properties [10]. Further, they are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have reported medicinal qualities [11]. Potential biotherapeutic constituents can be derived from any part of the plant such as leaves, bark, flowers, stems and roots [12]. The plant kingdom represents a largely unexplored reservoir of active compounds not only for use as drugs, but also as unique templates that have the potential to serve as a starting point for synthetic analogues [13]. Currently, there are approximately 20,000 plant species used in ethnomedicine worldwide [14, 15]. The ability of various bioactive compounds to heal wounds is attributed to their antimicrobial activity, ability to increase cell proliferation, angiogenesis and collagen production [16]. In contrast, many traditionally used herbs and herbal formulations remain unexplored with regard to their efficacy against infections and the healing of wounds [17]. Recently, there has been an increased interest in identifying bioactive compounds that promote or modulate the wound healing process [18]. In general, the wound healing potential of a large number of medicinal plants and cognate bioactive components remain unknown. Thus, identification of bioactive compounds may produce novel therapeutics which are more cost effective, efficacious and have a better safety profile than those currently commercially available.

Stachytarpheta indica Linn., commonly known as “blue snakeweed”, belongs to the Family Verbenaceae [19] and grows abundantly in East Africa, tropical America and Southeast Asia [20]. S. indica has been used in traditional medicine for a multitude of conditions such as headache, alopecia, bronchitis, pruritis, diarrhoea, skin sores, dysmenorrhoea, erysipelas, inflammation, poisoning, tumours, venereal disease, cataracts, rheumatism, anti-fertility therapy and as an abortifacient [19, 21]. In Brazil, S. indica leaves are decocted into a hot tea as a remedy for fever, chronic liver problems, constipation and water retention [22] and administered for the treatment of dysentery in humans and for a similar condition in horses in Northern Nigeria [19, 23]. Moreover, the plant is also used in parts of Southern Nigeria and Peru for the treatment of Malaria [19]. Phytochemical analysis of aqueous extracts of S. indica has revealed the presence of flavonoids, terpenoids and tannins, which exert an antimicrobial effect [19, 24, 25].

There have been very few studies investigating the pharmacological properties of S. indica in relation to wound healing. At present, there are no published studies that investigate the wound healing and anti-inflammatory potential of the leaf extracts of S. indica. Hence, leaf extracts of S. indica and subsequently isolated compounds were assessed.
against a broad range of common wound-infecting microflora and also their anti-inflammatory effects, and cell proliferation ability was ascertained.

**Materials and Methods**

**Plant Material**

*S. indica* plants were commercially obtained and grown in native potting mix. Mature, healthy leaves collected from 1-year-old plants were placed in paper autoclave bags and dried at 40 °C in an oven (Hybaid, UK) for 48 h in ambient air and then ground into a fine powder using an herb grinder (WSG60 Herb Grinder, Waring, USA).

**Bacterial Culture**

*Staphylococcus aureus* (NCTC 6571), methicillin-resistant *S. aureus* (MRSA) (ATCC 33,591 and clinical isolate QUT 1113), *Bacillus cereus* (ATCC 14,579), *Klebsiella pneumoniae* (ATCC 27,736), *Escherichia coli* (ATCC 25,922), *Pseudomonas aeruginosa* (ATCC 27,853), *Bacillus subtilis* (QUT 0535), *Staphylococcus epidermidis* (QUT 0613), *Proteus vulgaris* (ATCC 7002), *Proteus mirabilis* (ATCC 6380), *Enterococcus faecalis* (QUT 1105), *Enterococcus faecium* (QUT 1101), *Acinetobacter baumannii* (ATCC 19,606), *Enterococcus gallinarum* (ATCC 49,608), *Enterococcus casseliflavus* (ATCC 700,668), *Enterococcus faecalis* (ATCC 49,532) and *Enterococcus faecium* (ATCC 700,221) were either purchased from the ATCC via In Vitro Technologies (Melbourne, Australia) or obtained from the Queensland University of Technology (Brisbane, Australia). Individual strains were streaked onto nutrient agar plates and incubated at 37 °C for 24 h, excluding *B. subtilis* which was incubated at 28 °C for the same time period. *S. pyogenes* (ATCC 19,615) was streaked onto horse blood agar and incubated at 37 °C for 24 h.

**Human Keratinocyte Cell Culture**

Human immortalized keratinocytes (HaCaT, Addexbio Technologies, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 per cent volume/volume (% v/v) 50 mL foetal bovine serum (FBS), 1% v/v (5 mL) 200 mM L-glutamine, 1% v/v (5 mL) of 50 U/mL of penicillin and 5 mg/mL streptomycin. Complete DMEM was denoted full growth media (FGM). Cells were maintained in a humidified incubator at 37 °C, 5% CO₂ and passaged at approximately 80% confluence using 1× 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 U/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).

**Extraction**

For the solvent extraction, ground plant material (20 mL of methanol per 5 g of plant material) was incubated with shaking at 100 rpm in methanol (99.8%) for 48 h at room temperature.
temperature (RT) followed by vacuum filtration. Extracts were concentrated and dried at 40 °C/2000 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 was 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 12,000 rpm for 5 min at RT. The supernatants were evaporated in a rotary vacuum concentrator (Scan Speed 40, LabGear, Australia) at 40 °C/2000 rpm until a dry residue was obtained.

**Isolation of Bioactive Compounds from S. indica Leaf Extracts**

Bioactive compounds were isolated and identified following specific and distinctive steps including fractionation and isolation of the compounds from primary methanol extracts derived from *S. indica* leaves. A 20-g sample of the dry methanol extract of *S. indica* leaves was dissolved in 100 mL methanol (99.8%) followed by the addition of 10 g of silica gel 60 Å and dried at 40 °C/100 rpm (IKA™ RV10, LabGear, Australia) to obtain a homogenous granular mixture. The mixture was transferred into a glass column containing 50 g of silica gel 60 Å conditioned with hexane (95%). Compounds were eluted with a solvent gradient consisting of 200 mL volume fractions with 10% (v/v) increments in each progressive step, starting with ethyl acetate (99.5%) then dichloromethane (99.9%) and finally methanol (99.8%). The fractions were then concentrated and dried (Scan Speed 40, LabGear, Australia) at 40 °C/2000 rpm to obtain dry residues. The 30–80% (v/v) methanol fractions derived from *S. indica* were combined and subjected to further separation of compounds using high-performance liquid chromatography (HPLC). The reverse-phase HPLC system comprised a preparative C18 column (Agilent ZORBAX™ 150 mm × 21.2 mm, 5 μm, Agilent Technologies 1260 Infinity II HPLC system). The mobile phase consisted of (A) 0.05% v/v formic acid (98%) in Milli-Q water and (B) methanol (99.8%). The gradient system 0–15 min, (B) 10–40%; 15–25 min, (B) 40–100% and 25–30 min, (B) 100–10% was applied with flow rate 20.0 mL/min. Column temperature was maintained at RT, and the sample injection volume was 900 μL. UV absorbance was monitored at 254 nm wavelength. Compound fractions corresponding to prominent peaks on the chromatograms were collected throughout each run and dried (Scan Speed 40, LabGear, Australia) at 40 °C/2000 rpm until a solid residue was obtained.

**Identification of Isolated Bioactive Compounds**

\(^1\)H and \(^{13}\)C NMR spectra were recorded at 600 MHz on Ascend™ 600 spectrometer (Bruker, Australia) with samples prepared in methanol-d4 (CD3OD). Chemical shifts (δ) were quoted in parts per million (ppm) with reference to the solvent signal. 2D experiments, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were performed. Data was analysed using TopSpin™ 4.0.2 software (Bruker, Australia). For each compound, samples were analysed on positive and negative ion modes with a range of m/z 100–800, at a scan rate of 0.5 Hz, on a LTQ XL™ ion trap (Thermo Scientific, Australia) mass spectrometer. The ion transfer tube temperature was set at 300 °C with a spray voltage of 2.0 kV, nitrogen sheath gas at 40 arbitrary units and auxiliary gas at 10 arbitrary units. The system was supported...
with Xcalibur™ 1.2 and Mass Frontier™ 5.0 software (Thermo Scientific, Australia). The ChemDraw 16.0 program (Perkin Elmer, USA) was used for the illustration of the chemical structures and molecular mass calculations of the identified compounds. The SciFinder program (Chemical Abstract Service, American Chemical Society) and the PubChem (National Institutes of Health, USA) open chemistry databases were used to examine previous literature on the identified compounds.

WDA

Fresh bacterial suspensions were prepared with sterile 0.9% w/v saline and adjusted to equate to a 0.5 McFarland standard. Mueller–Hinton (MH) agar plates (Sigma-Aldrich, Australia) were separately inoculated with 200 μL of each bacterial species using a sterile disposable spreader. Wells were aseptically punched into the agar using a 6-mm biopsy punch and filled with 80 μL of extracts prepared in Milli-Q water at 100 mg/mL. Standard antibiotic discs (Oxoid, Australia) were used as the positive controls whereby, sulfamethoxasole + trimethoprim (SXT, 25 μg each) acted as the control for both MRSA isolates, *S. epidermidis*, *P. vulgaris* and *P. mirabilis*; penicillin G (10 units) was used for *S. aureus*; erythromycin (15 μg) was used for *S. pyogenes*, *B. cereus* and *B. subtilis*; gentamicin (10 μg) was used for *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii*; teicoplanin (30 μg) was used for *E. faecalis*, *E. casseliflavus* and *E. gallinarum*; linezolid (30 μg) was used for *E. faecium*. Plates were then incubated at 37 °C for 24 h, and the subsequent zones of inhibition (radius) were measured in millimetres (mm).

MIC

MIC values of plant extracts were determined using sterile 96-well plates (ThermoFisher Scientific, Australia). Bacterial suspensions, 0.5 McFarland standard in sterile 0.9% w/v saline diluted 1:100 in MH broth were used. Concentrations ranging from 1.25 to 20 mg/mL (2 × dilutions) of plant extract in MH broth were added to the corresponding wells. Extracts prepared in MH broth were used as background control to eliminate the absorbance caused by the colour of the extracts. MH broth served as the negative control while the bacterial suspensions in MH broth provided the positive control. The plates were sealed, incubated for 24 h at 37 °C and then allowed to cool to RT. Next, 40 μL of 0.125 mg/mL iodonitrotetrazolium chloride (INT) dye was added to each plate well, incubated again for 60 min at 37 °C and then removed from the incubator to cool to RT. Colour change was determined using a plate reader at an absorbance of 550 nm. The lowest concentration of the plant extract at which the colour changed from yellow to pink was deemed the MIC value.

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% CO₂. Cells were plated at a density of 1 × 10⁵ cells/mL in a 96-well plate in FGM and allowed to attach for 24 h at 37 °C in 5% CO₂. The media was then aspirated, and cells were separately treated with a 100-μL solution of *S. indica* extracts in SFM, at concentrations ranging from 0.05 to 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% CO₂ for 72 h. Images at 1000× magnification were captured every 15 min throughout a 72-h time course. All images were analysed with LAS-X software platform (Leica Microsystems, Germany).

**Direct Cell Proliferation Assay**

The CyQUANT® direct cell proliferation assay kit (Thermo Fisher Scientific, Australia) was used to evaluate the potential effects of *S. indica* extracts on HaCaT cell viability. As per the manufacturer’s instructions, HaCaT cells cultured in FGM were seeded at a density of 1×10⁵ cells/mL in a 96-well plate (ThermoFisher, Australia) and incubated at 37 °C, 5% CO₂ for a 24-h period to allow attachment. The media was aspirated, and 100 μL of *S. indica* extracts at concentrations ranging from 0.05 to 1.6 mg/mL in RSM was 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% CO₂ for 24 h. One hundred microliters of 2× detection reagent in RSM was added to the cells and incubated for 60 min at 37 °C. A reference standard curve was also performed using nine suspensions of known cell concentrations (0 = blank control, 1.25×10⁴, 2.5×10⁴, 5.0×10⁴, 1.0×10⁵, 2.0×10⁵, 4.0×10⁵, 8.0×10⁵, 1.6×10⁶ 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).

**RTCA for Monitoring Cell Migration**

The xCELLigence RTCA dual purpose (DP) platform (ACEA Biosciences, USA) was used to examine keratinocyte migration rate, promoted by *S. indica*-derived extracts. Extracts at concentrations ranging from 0.05 to 1.6 mg/mL were prepared in SFM. The negative control was provided by SFM which minimised the influence on cell migration, while positive controls were cells in FGM, which worked as a chemo-attractant. 160 μL of each control, and plant extracts were aliquoted into the wells of the bottom chamber of the cell invasion and migration (CIM) plate. Next, 50 μL of SFM was added to all wells of the top chamber of the CIM plate. The plate was then equilibrated for 1 h in a humidified incubator at 37 °C, 5% CO₂. HaCaT cell suspension, previously passaged to 60% confluence, was serum starved in SFM for 4 h at 37 °C, 5% CO₂. After detaching and washing the cells, the final pellet was dissolved in 2 mL of SFM, and the suspension was adjusted to a concentration of 5×10⁵ cells/mL in SFM. A volume of 100 μL of the suspension was transferred into each of the wells of the top chamber of the CIM plate. After 30 min of incubation at RT, the plate was placed into the xCELLigence cradle and incubated for 72 h at 37 °C, 5% CO₂. Measurements were recorded with one sweep every 15 min throughout the entire 72-h time course. Results were analysed using the xCELLigence RTCA software program (ACEA Biosciences, USA).

**CBA for Analysing Cytokine Suppression**

A cytometric bead array (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 and isolated compounds. The protocol by Hong et al. [26] was followed in the preparation of the mammalian cells, while the implementation of the assay was executed as per the manufacturer’s instructions [27]. Five hundred microliters of HaCaT cells in FGM was seeded at 5 × 10^5 cells/mL in 24-well plates (ThermoFisher, Australia) and incubated at 37 °C, 5% CO₂ for 24 h. After incubation, the media was aspirated, and the cells were submitted to four separate treatments. Plant extracts (400, 200 and 100 μg/mL) and isolated compounds (100 and 50 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) or isolated compounds (50 and 25 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 or isolated compounds. The 1-mL suspensions treated with the extracts (200, 100 and 50 μg/mL) or isolated compounds (50 and 25 μg/mL) in RSM represented the baseline controls (BCs). The plates were then incubated at 37 °C, 5% CO₂ for 48 h. Next, the cells were removed with a disposable cell scraper and their suspensions collected and centrifuged at 300 g for 10 min 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 was normalised against the negative control and averaged. Results were analysed and processed with BD FCAP Array 3.0 (Soft Flow Limited, Hungary) software.

**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 [28] with slight modifications. Twenty microliters of samples dissolved in dimethyl sulfoxide (DMSO) was plated in triplicate at different concentrations (plant extracts 6.25–100 μg/mL and compounds 3.125–50 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. One hundred sixty microliters 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™ BMG Labtech, Germany). Concentrations of indomethacin ranging from 3.125 to 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 was determined.
with absorbance of samples relative to positive control using the formula: \((E - S)/E \times 100\), where \(E\) is the absorbance without any inhibitor and \(S\) is the absorbance of test sample.

**LOX Inhibition Assay**

The lipoxygenase (LOX) assay was conducted according to the method described by Baylac and Racine [29] and Kamatou et al. [30] 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). Twenty microliters of samples dissolved in DMSO was plated in triplicate in a 96-well microtiter plate (ThermoFisher, Australia) at different concentrations (plant extracts 6.25–100 μg/mL; compounds 3.125–50 μg/mL), followed by 160 μL of 0.1 M tris-chloride buffer pH 7.4 and maintained at 25 °C. Twenty microliters 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 to 100 μg/mL nor-dihydroguaiaretic 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™, 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 used the formula: \((E - S)/E \times 100\), 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**

**Extraction and Fractionation**

The percentage yield of compounds from *S. indica* leaves was 29.5% with methanol, while the water extract was fourfold less (7.2%). As such, only the methanolic extracts were used for fractionation.

**Isolation and Identification of Compounds**

Active fractions from the silica gel columns were further separated by semi-preparative HPLC to purify compounds for chemical identification. Similar antibacterial activity against multiple bacterial species was observed in 30–80% (v/v) methanol fractions of *S. indica*; hence, fractions were pooled for isolation processing. The most prominent peak obtained from the methanol fraction, i.e. designated compound 1, was eluted at retention
time (Rt) 10.7 min (Fig. 1) using the primary preparative HPLC method (Table 1). The peak at Rt 16.0 min was denoted compound 2. HPLC fraction 1 was further resolved using the secondary HPLC method (Table 2). Compound 3 was eluted at Rt 7.2 min (Fig. 2).

The $^{13}$C NMR spectrum (Table 3) of compound 1 (1014 mg) showed 17 carbon signals, six of which could be assigned to a β-glucopyranosyl moiety. The $^1$H NMR spectrum (Table 3) of the molecule exhibited characteristic signals for an iridoid structure (Fig. 3) and showed the existence of a methoxycarbonyl function ($\delta_H 3.74$ ppm, s) and a tertiary methyl group ($\delta_H 1.17$ ppm, s). In addition, resonances arising from two methylene groups were observed. The $^1$H NMR signal at $\delta_H 4.60$ ppm (d, J = 7.9 Hz) was assigned to the anomic proton H1′ of a β-glucopyranose unit. The resonance signal at H1 ($\delta_H 5.82$ ppm, s) was shifted down due to glycosidation, which indicated the attachment of the β-glucopyranose unit at the C1 position of the aglycone. The chemical shift and the splitting patterns of H3 ($\delta_H 7.45$ ppm, s) and H9 ($\delta_H 2.50$ ppm, s) were suggestive of C4, C5 and C8 positions to be substituted. Thus, the methoxycarbonyl group was assigned to C4, due to the highly deshielded signal of the H3 proton, and the quaternary carbon resonance at $\delta_C 71.7$ ppm was attributed to the C5 position. The multiplet signals observed at $\delta_H 2.28/1.95$ ppm and $\delta_H 2.10/1.59$ ppm could be assigned to the methylene protons at C6 ($\delta_C 38.9$ ppm) and C7 ($\delta_C 40.4$ ppm), respectively. The chemical shift of the tertiary methyl group ($\delta_H 1.17$ ppm, s) suggested its attachment at C8. COSY and HMBC correlations (Fig. 4) enabled the resonances attributed to C7 ($\delta_C 40.4$ ppm) and H7 ($\delta_H 2.10$ and

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**Table 1** Primary HPLC method used for isolation of compounds from *Stachytarpheta indica* 30–80% (v/v) methanol fractions

| Time (min) | Mobile phase (A) % | Mobile phase (B) % |
|-----------|--------------------|--------------------|
| 0         | 90                 | 10                 |
| 15        | 60                 | 40                 |
| 25        | 0                  | 100                |
| 30        | 90                 | 10                 |

Mobile phase A: 0.05% v/v formic acid in Milli-Q water, B: methanol (99.8%)
1.59 ppm), which revealed the presence of a methylene group at C7 instead of hydroxymethine. The signal at δC 171.6 ppm (C11) suggested the presence of carboxyl group at C4. Molecular ion [M+Na]+ m/z 429.33 confirmed the proposed structure which was supported with published data [31–34]. Hence, compound 1 was subsequently identified as ipolamiide (PubChem CID: 442,425), chemical formula: C18H28O10 (calculated MW 406.412 g/mol).

The 1H NMR spectrum of compound 2 (238 mg; Table 4) showed that signals were observed at δH 7.05 ppm (d, J = 2.1 Hz, H2°), 6.78 ppm (d, J = 8.2 Hz, H5°) and 6.96 ppm (dd, J = 8.2, 2.0 Hz, H6°) together with two olefinic protons at δH 6.27 ppm (d, J = 15.8 Hz, H8°) and δH 7.60 ppm (d, J = 15.6 Hz, H7°), thereby indicating the presence of a caffeoyl unit (Fig. 5). The signals observed at δH 6.70 ppm (d, J = 2.2 Hz), 6.68 ppm (d, J = 8.1 Hz), 6.57 ppm (dd, J = 8.1, 2.0 Hz) were attributed to protons H2, H5 and H6 respectively. Additionally, a methylene proton H7 at δH 2.80 ppm (ddd, J = 5.0, 7.0, 12.5 Hz) and δH 3.01 ppm (ddd, J = 2.3, 7.8, 10.3 Hz) with H8 at δH 3.73 ppm (ddd, J = 6.5, 8.0, 8.5 Hz) and 4.04 ppm (ddd, J = 2.9, 6.5, 8.1 Hz) suggested the presence of a 3,4-dihydroxyphenylethyl unit as an aglycone. The 1H NMR spectrum exhibited two anomic protons H1′ at δH 4.37 ppm (d, J = 7.9 Hz) and H1″ at 5.18 ppm (d, J = 1.9 Hz) thus indicating attachment

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**Table 2** Secondary HPLC method used for isolation of compounds from *S. indica* HPLC fraction 1

| Time (min) | Mobile phase (A) % | Mobile phase (B) % |
|------------|--------------------|--------------------|
| 0          | 88                 | 12                 |
| 5          | 88                 | 12                 |
| 6          | 0                  | 100                |
| 9          | 0                  | 100                |
| 10         | 88                 | 12                 |

Mobile phase A: 0.05% v/v formic acid in Milli-Q water, B: methanol (99.8%)
of two sugar moieties. The $^{13}$C NMR spectrum (Table 4) displayed 29 carbon resonances, of which two anomeric carbon resonances at $\delta_C$ 104.2 and 103.1 ppm could be attributed to C’ and C” respectively. The proton resonance of both sugar units was assigned with COSY and HSQC experimental results (Fig. 6). The multiplicities of the sugar protons and their coupling constants were consistent for the presence of β-glucopyranosyl and α-rhamnopyranosyl units. $^{13}$C NMR spectrum exhibited three CH$_2$ resonances at $\delta_H$ 62.4, 72.3 and 36.5 ppm, of which the first was attributed to C6’ of glucose while the latter two were assigned to C7 and C8 of the side chain of the 3,4-dihydroxyphenylethyl unit. The H4’ proton resonance of glucopyranosyl unit at $\delta_H$ 4.92 ppm (t, $J = 9.6$ Hz) was observed with a downfield shift due to esterification, indicating the site of attachment of a caffeoyl unit. The secondary methyl resonance at $\delta_H$ 1.09 ppm (d, $J = 6.2$ Hz) was indicative for the presence of a 6-deoxysugar unit rhamnose, as one of the two sugar units. Intermolecular interactions between the four molecular fragments, 3,4-dihydroxyphenylethyl alcohol, caffeic acid, glucose and rhamnose, were determined by COSY and HMBC (Fig. 6) and displayed correlations between the anomeric proton H1’ of a glucopyranosyl unit ($\delta_H$ 4.37 ppm) and the C8 ($\delta_C$ 72.3 ppm) carbon atom of aglycone (3,4-dihydroxyphenylethyl unit), which showed that the glucose unit was the core sugar within the structure. Further long-range correlations were observed between the carbonyl carbon C9° of caffeic acid ($\delta_C$ 168.4 ppm) and proton H4’ ($\delta_H$ 4.92 ppm) of glucopyranosyl unit and olefinic protons of caffeic acid (H7°/H8°) at $\delta_H$ 7.60 and 6.27 ppm, thereby confirming the attachment site on the glucose unit at C4’. Compound 2 in the negative ion mode gave a peak at m/z 623.67
Fig. 3 Chemical structure of compound 1 isolated from S. indica leaf extract. The compound was identified as ipolamide (PubChem CID: 442,425). The chemical structure was illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

Fig. 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 (blue arrow) indicate COSY correlations, red arrow indicates HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).
which corresponded to the deprotonated molecular ion [M–H]−. Based on NMR/MS data and comparison with published data [35–37] confirmed the structure of compound 2 as verbascoside (also known as acetoside) (PubChem CID 5,281,800) chemical formula: C_{29}H_{36}O_{15} (calculated MW 624.592 g/mol).

The \(^1\)H and \(^1^3\)C NMR results of compound 3 (43 mg; Table 5) showed close similarity to compound 2 in the resonance signal pattern. However, two H6' protons of a

| Moiety | Position | Carbon | \(^1^H\)-NMR | \(^1^3\)C-NMR |
|--------|----------|--------|--------------|--------------|
| Aglycone (Hydroxytyrosol) | 1 | C | – | 131.5 |
| | 2 | CH | 6.70 (d, 2.2) | 117.1 |
| | 3 | C | – | 146.1 |
| | 4 | C | – | 144.8 |
| | 5 | CH | 6.68 (d, 8.1) | 116.7 |
| | 6 | CH | 6.57 (dd, 2.0, 8.1) | 121.4 |
| | 7 | CH2 | 2.80 (ddd, 5.0, 7.0, 12.5) | 36.6 |
| | | | 3.01 (ddd, 2.3, 7.8, 10.3) | |
| | 8 | CH2 | 3.73 (ddd, 6.5, 8.0, 8.5) | 72.3 |
| | | | 4.04 (ddd, 2.9, 6.5, 8.1) | |
| Glucose | 1' | CH | 4.37 (d, 7.9) | 104.2 |
| | 2' | CH | 3.39 (dd, 9.0, 8.0) | 76.1 |
| | 3' | CH | 3.82 (t, 9.3) | 81.7 |
| | 4' | CH | 4.92 (t, 9.6) | 70.6 |
| | 5' | CH | 3.52 (m) | 76.2 |
| | 6' | CH2 | 3.54 (m) | 62.4 |
| | | | 3.62 (d, 10.5) | |
| Caffeic acid | 1° | C | – | 127.7 |
| | 2° | CH | 7.05 (d, 2.1) | 115.2 |
| | 3° | C | – | 146.9 |
| | 4° | C | – | 149.9 |
| | 5° | CH | 6.78 (d, 8.2) | 116.3 |
| | 6° | CH | 6.96 (dd, 2.0, 8.2) | 123.3 |
| | 7° | CH | 7.60 (d, 15.6) | 148.1 |
| | 8° | CH | 6.27 (d, 15.8) | 114.7 |
| | 9° | C=O | – | 168.4 |
| Rhamnose | 1" | CH | 5.19 (d, 1.9) | 103.1 |
| | 2" | CH | 3.92 (dd, 1.8, 3.1) | 72.4 |
| | 3" | CH | 3.57 (dd, 3.4, 9.6) | 70.4 |
| | 4" | CH | 3.29 (t, 9.5) | 73.8 |
| | 5" | CH | 3.55 (m) | 72.1 |
| | 6" | CH3 | 1.09 (d, 6.2) | 18.5 |

Chemical shifts (δ) and coupling constants (J, Hz) were obtained from nuclear magnetic resonance (NMR) spectra of \textit{S. indica} compound 2

\(^1\)H proton, \(^1^3\)C carbon, d doublet, dd doublet of doublets, \(ddd\) doublet of doublet of doublets, t triplet, m multiplet
glucopyranosyl unit at $\delta_H 4.38$ (d, $J = 7.8$ ppm) and $4.59$ ppm (t, $J = 7.5$ Hz) of compound 3 (Fig. 7) and H4″, H5″, and H6″ protons of the rhamnopyranosyl unit at $\delta_H 3.70$, 4.04 and 1.24 ppm exhibited higher chemical shifts, upon comparison with compound 2. In contrast, chemical shifts of the H4′ proton of glucopyranosyl unit at $\delta_H 3.42$ ppm were shown to be shielded compared to that of compound 2. Further, COSY and HMBC correlations (Fig. 8) consolidated the assignment of proton and carbon in compound 3 and suggested that the 3,4-dihydroxyphenylethyl unit was

Fig. 5 Chemical structure of compound 2 isolated from *S. indica* leaf extract. The compound was identified as verbascoside (also known as acetoside) (PubChem CID 5,281,800). The chemical structure illustrated with ChemDraw 16.0 program (Perkin Elmer, USA)

Fig. 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: blue arrow indicates COSY correlations; red arrow indicates HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA)
attached to glucopyranosyl unit at C6′ instead of C4′. Compound 3 in the negative ion mode gave a peak at m/z 623.58 which corresponded to the deprotonated molecular ion [M–H]−. Taken together, the NMR/MS data and published literature [37, 38] confirmed the structure of compound 3 to be isoverbascoside (also known as isoacteoside) (PubChem CID: 6,476,333) chemical formula: C29H36O15 (calculated MW 624.592 g/mol), which is an isomer of verbascoside.

### Table 5

| Moiety       | Position | Carbon | 1H-NMR   | 13C-NMR   |
|--------------|----------|--------|----------|-----------|
| Aglycone     | 1        | C      | –        | 131.5     |
|              | (Hydroxytyrosol) | 2 | CH      | 6.69 (d, 2.5) | 117.2     |
|              | 3        | C      | –        | 146.2     |
|              | 4        | C      | –        | 144.7     |
|              | 5        | CH     | 6.67 (d, 8.0) | 116.3     |
|              | 6        | CH     | 6.57 (dd, 2.1, 8.1) | 121.3     |
|              | 7        | CH2    | 2.81 (m) | 36.6      |
|              |          |        | 3.19 (m) |           |
|              |          |        | 3.73 (m) |           |
| Glucose      | 1′       | CH     | 4.37 (d, 8.0) | 104.3     |
|              | 2′       | CH     | 3.39 (m) | 76.2      |
|              | 3′       | CH     | 3.81 (t, 9.4, 3.2) | 81.3     |
|              | 4′       | CH     | 3.42 (m) | 70.6      |
|              | 5′       | CH     | 3.54 (m) | 76.1      |
|              | 6′       | CH2    | 4.38 (d, 7.8) | 65.4     |
|              |          |        | 4.59 (t, 7.5) |           |
| Caffeic acid | 1°       | C      | –        | 127.7     |
|              | 2°       | CH     | 7.05 (d, 2.0) | 115.2     |
|              | 3°       | C      | –        | 146.9     |
|              | 4°       | C      | –        | 149.8     |
|              | 5°       | CH     | 6.78 (d, 8.3) | 116.5     |
|              | 6°       | CH     | 6.95 (dd, 2.1, 8.3) | 123.3     |
|              | 7°       | CH     | 7.59 (d, 15.8) | 148.1     |
|              | 8°       | CH     | 6.28 (d, 16.1) | 114.7     |
|              | 9°       | C=O    | –        | 164.7     |
| Rhamnose     | 1″       | CH     | 5.19 (s) | 102.7     |
|              | 2″       | CH     | 3.93 (m) | 72.4      |
|              | 3″       | CH     | 3.57 (m) | 70.4      |
|              | 4″       | CH     | 3.70 (m) | 73.8      |
|              | 5″       | CH     | 4.04 (m) | 72.1      |
|              | 6″       | CH3    | 1.24 (d, 6.5) | 18.0      |

Chemical shifts (δ) and coupling constants (J, Hz) were obtained from nuclear magnetic resonance (NMR) spectra of S. indica compound 3. 1H proton, 13C carbon, s singlet, d doublet, dd doublet of doublets, t triplet, m multiplet.
Fig. 7 Chemical structure of compound 3 isolated from *S. indica* leaf extract. The compound was identified as isoverbascoside (also known as isoacetoside) (PubChem CID 6,476,333). The chemical structure illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).

Fig. 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: blue arrow indicates COSY correlations; red arrow indicates HMBC correlations. Illustrated with ChemDraw 16.0 program (Perkin Elmer, USA).
Antibacterial Activity

Methanol and aqueous extracts of *S. indica* leaves at a concentration of 100 mg/mL elicited an antimicrobial effect against four of the 19 bacteria screened, i.e. *S. aureus*, MRSA clinical isolates (ATCC 33,591 and QUT 1113) and *S. epidermidis* (Table 6). The growth of all gram-negative bacteria tested was impervious to *S. indica* leaf extracts regardless of extraction method. Of the four bacteria shown to be susceptible to the aqueous extract, both MRSA isolates exhibited a similar degree of inhibition with annular radii 6.22 mm ± 0.22 (*P* < 0.0001) and 5.67 mm ± 0.17 (*P* < 0.0001) compared to the standard antibiotic SXT control (8.17 mm ± 0.17 and 7.25 mm ± 0.13, respectively).

The methanol extract was fractionated using a solvent gradient in a silica gel column and produced 30 fractions. Based on initial activity of the plant extract, the fractions were screened against four bacterial species (Table 7). It was evident that 30–80% (v/v) methanol fractions of *S. Indica* all inhibited the growth of the bacterial species tested (Fig. 9). Although the 30–40% (v/v) ethyl acetate fractions of *S. indica* also inhibited the growth of all the bacteria, the zones of inhibition were considerably smaller compared to the methanol fractions. In contrast, none of the dichloromethane fractions elicited any antibacterial effects. In general, the activity was relatively uniform across 30–80% (v/v) methanol fractions of *S. indica*; hence, they were pooled to form a single fraction which was used for the isolation of compounds in subsequent stages.

| Bacterial strain | Standard antibiotic | Methanol extract | Aqueous extract |
|------------------|---------------------|------------------|-----------------|
| *S. aureus* (NCTC 6571) | 19.33 ± 0.38 | 3.56 ± 0.34 | 5.89 ± 0.23 |
| MRSA (ATCC 33,591) | 8.17 ± 0.16 | 3.22 ± 0.32 | 6.22 ± 0.22 |
| MRSA (QUT 1113) | 7.25 ± 0.13 | 2.67 ± 0.17 | 5.62 ± 0.17 |
| *S. epidermidis* (QUT 0613) | 10.11 ± 0.43 | 3.33 ± 0.17 | 5.67 ± 0.29 |
| *B. cereus* (ATCC 14,579) | 8.92 ± 0.57 | – | – |
| *B. subtilis* (QUT 0535) | 8.33 ± 0.68 | – | – |
| *S. pyogenes* (ATCC 19,615) | 7.58 ± 0.25 | – | – |
| *E. faecium* (QUT 1101) | 7.89 ± 0.10 | – | – |
| *E. faecium* (ATCC 700,221) | 9.33 ± 0.61 | – | – |
| *E. faecalis* (QUT 1105) | 4.22 ± 0.31 | – | – |
| *E. faecalis* (ATCC 49,532) | 3.78 ± 0.14 | – | – |
| *E. gallinarum* (ATCC 49,608) | 4.67 ± 0.16 | – | – |
| *E. casseliflavus* (ATCC 700,668) | 4.00 ± 0.22 | – | – |
| *E. coli* (ATCC 25,922) | 3.92 ± 0.14 | – | – |
| *P. aeruginosa* (ATCC 27,853) | 5.08 ± 0.08 | – | – |
| *K. pneumoniae* (ATCC 27,736) | 4.00 ± 0.17 | – | – |
| *A. baumannii* (ATCC 19,606) | 5.22 ± 0.14 | – | – |
| *P. vulgaris* (ATCC 7002) | 8.44 ± 0.39 | – | – |
| *P. mirabilis* (ATCC 6380) | 7.44 ± 0.32 | – | – |

Zones of inhibition (radius) were measured in millimetres (mm) and expressed as mean ± SEM. Negative control: sterile Milli-Q water (0 ± 0.0 mm). (–) indicates no activity.
**Table 7** Minimum inhibitory concentrations (MIC) of *S. indica* extracts (mg/mL)

| Bacterial strain                  | Methanol extract | Aqueous extract |
|-----------------------------------|-----------------|-----------------|
| *S. aureus* (NCTC 6571)          | 3.75            | 0.625           |
| MRSA (ATCC 33,591)               | 3.75            | 0.625           |
| MRSA (QUT 1113)                  | 5.00            | 1.25            |
| *S. epidermidis* (QUT 0613)      | 3.75            | 1.25            |

**Fig. 9** Antibacterial activity of *S. indica* leaf extract fractions at 10 mg/mL concentration. Averaged annular radii of clear zones ± 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.
The process of compound isolation from active (and pooled) methanolic fractions utilised a bio-guided method in which the focus was based on activity. Isolated compounds were screened against a scheme of 10 bacteria, including five gram-positive (S. aureus — NCTC 6571, MRSA — ATCC 33,591, MRSA — QUT 1113, B. cereus — ATCC 14,579 and S. epidermidis — QUT 0613) and five gram-negative (E. coli — ATCC 25,922, P. aeruginosa — ATCC 27,853, K. pneumoniae — ATCC 27,736, P. vulgaris — ATCC 7002 and A. baumannii — ATCC 19,606) species to determine their MIC values (Table 8). Verbascoside and isoverbascoside isolated from S. indica were found to produce an antibacterial effect while ipolamiide did not. Verbascoside showed strong antibacterial activity against three gram-positive bacteria, i.e. S. aureus, MRSA (ATCC 33,591) and S. epidermidis, as the growth was halted at a concentration of 9.77 μg/mL. Moreover, MRSA (QUT 1113) and P. vulgaris (gram-negative) were also susceptible to the compound, although to a lesser degree as growth inhibition occurred at 312.5 μg/mL. The antimicrobial efficacy of isoverbascoside was limited to gram-positive bacteria, which included S. epidermidis (9.77 μg/mL), S. aureus and both clinical isolates of MRSA (625 μg/mL).

**Morphological Responses of the Human Keratinocyte Cells**

HaCaT cells separately treated with the plant extracts at concentrations ranging from 0.05 to 0.80 mg/mL were compared with both negative and positive controls. Images captured at 15-min intervals over the period of 72 h for each extract were examined at all concentrations and evaluated for proliferation rate, motility, morphological changes and potential apoptotic effects. Broad, pale colour cells were identified as live cells while the dark, almost circular-shaped granules were identified as dead cells. Overall, the keratinocytes demonstrated a morphological change post-treatment and presented with elongation of the cell, to a broader polygonal shape within the first 48 h of the experiment. Moreover, the live elongated cells were observed to move over the surface of the bottom plate while producing cytoplasmic protrusions. Results obtained from the morphological assays clearly indicated that keratinocytes grown in FGM proliferate rapidly within the first 24 h and achieved full confluence (Fig. 10b, c). However, within the

| Bacterial species | Isolated bioactive compounds | Ipolamiide | Verbascoside | Isoverbascoside |
|-------------------|-----------------------------|------------|--------------|----------------|
| Gram-positive     | S. aureus (NCTC 6571)       | –          | 9.77         | 625            |
|                   | MRSA (ATCC 33,591)          | –          | 9.77         | 625            |
|                   | MRSA (QUT 1113)             | –          | 312.5        | 625            |
|                   | B. cereus (ATCC 14,579)     | –          | –            | –              |
|                   | S. epidermidis (QUT 0613)   | –          | 9.77         | 9.77           |
| Gram-negative     | E. coli (ATCC 25,922)       | –          | –            | –              |
|                   | P. aeruginosa (ATCC 27,853) | –          | 1250         | –              |
|                   | K. pneumoniae (ATCC 27,736) | –          | –            | –              |
|                   | P. vulgaris (ATCC 7002)     | –          | 312.5        | –              |
|                   | A. baumannii (ATCC 19,606)  | –          | 1250         | –              |

(–) indicates no activity
course of the next 48 h, rapid apoptosis in positive control cells was observed. In contrast, the cells grown in SFM did not reach 100% confluence even after 72 h (Fig. 10e-h). At 24 h, apoptotic cells were evident in the negative control, which continued to increase in number for the duration of the experiment.

In general, the _S. indica_ methanol (SIM) and _S. indica_ aqueous (SIA) extracts were found to induce cell proliferation (Figs. 11 and 12). Furthermore, an inverse relationship was shown as the concentration of plant extract was decreased (0.8 to 0.05 mg/mL), keratinocyte proliferation increased (Figs. 11a-t and 12a-t). Although the SIM and SIA extracts did not elicit 100% cellular confluence at any of the tested concentrations, a substantial reduction in apoptosis compared with FGM treated cells was observed at 72 h (Figs. 11i, p, t and 10d). Moreover, a concentration of 0.05 mg/mL of the SIM extract only enhanced proliferation by 50% at 24 h (Fig. 11r), which was maintained throughout the remainder of the experimentation (Fig. 11s, t). In contrast, concentrations of the SIA extract equivalent and below 0.2 mg/mL improved confluency by an additional 10% at 24 h (Fig. 12 j, n and r). The SIM extract at 0.8 mg/mL completely killed the HaCaT cells within the first 24 h (Fig. 11a, b), while the same concentration of the SIA extract was found to be detrimental to HaCaT cells in the final 48 h of testing (Fig. 12c, d). As such, a concentration of 0.8 mg/mL _S. indica_ extracts imposed a negative effect on the HaCaT cells, thereby reducing cell proliferation.

Overall, keratinocytes treated with extracts derived from _S. indica_ leaves showed a greater level of motility and morphological changes (Figs. 11a-t and 12a-t) compared to cells grown in both control treatments (Fig. 10a-h). Further, the motility of HaCaT cells was high upon treatment with the SIM extract at concentrations ranging between 0.4 and 0.05 mg/mL (Fig. 13a-d). In contrast, increased cellular motility was evident at 0.4–0.2 mg/mL concentrations of the SIA extract (Fig. 13e, f). Enhancement of morphological changes gradually reduced as the concentration of the SIM extracts decreased (Fig. 14a–e, b–f, c–g and d–f).

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**Fig. 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 h microscopic examination, and images were captured every 15 min. Arrow indicates necrotic cells. Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% v/v (50 mL) foetal bovine serum (FBS), 1% v/v (5 mL) 200 mM l-glutamine and 1% v/v (5 mL) of 50 U/mL of penicillin and 5 mg/mL streptomycin was denoted FGM. Pure DMEM, without any additions, was designated as SFM. Images were captured at 1000× magnification.
Plant extracts prepared from *S. indica* were tested on HaCaT cells, with the number of live cells calculated using the standard curve. An overall trend showed that keratinocyte cell numbers increased in response to a decrease in plant extract concentration (Fig. 13a, b). However, none of the plant extract treatments increased the number of cells beyond that of the control. In fact, treatments with the plant extracts at various concentrations (SIM 1.6 mg/mL ($P < 0.0001$), SIM 0.8 mg/mL ($P < 0.0001$), SIM 0.4 mg/mL ($P < 0.0001$), SIM 0.2 mg/mL ($P = 0.0010$), SIA 1.6 mg/mL ($P < 0.0001$), SIA 0.8 mg/mL ($P = 0.0011$) and SIA 0.4 mg/mL ($P = 0.0316$)), all resulted in a significant reduction in cell number.

**Fig. 11** Proliferative effects of *S. indica* methanol extracts (at concentrations ranging from 0.8 to 0.05 mg/mL) post-treatment. Treated HaCaT cells were subjected to 72 h microscopic examination and were compared with untreated cells grown in full growth media (FGM: Fig. 10a-d) and untreated cells in serum free media (SFM: Fig. 10e-h). Noticeable reduction in apoptosis was observed in HaCaT cells treated with SIM extracts within 24–72 h, compared to positive control (Fig. 10b-d). 0.05 mg/mL SIM extract maintained 50% proliferation from 24 to 72 h. 0.8 mg/mL SIM extract completely killed HaCaT cells within 24 h. SIM *S. indica* methanol. Arrow indicates necrotic cells. Images were captured at 1000× magnification.
compared to the treatment control (Fig. 13a, b). For both *S. indica* extracts, the optimum concentration for maximum cell viability was found to be 0.1 mg/mL.

**Keratinocyte Cell Migration**

In the preliminary study, it was identified that a concentration of 0.05 mg/mL was the optimum dosage for testing cell migration in HaCaT cells using the xCELLigence system. HaCaT cells grown in FGM (positive control) showed the highest CI between 12
and 72 h (Fig. 14). Final results of the cells treated with plant extracts at 0.05 mg/mL showed a moderate level of cell migration compared to the positive control (Fig. 14). Cells dosed with the SIM extracts achieved a higher overall CI compared to cells treated with SIA. However, none of the keratinocytes treated with extract, regardless of concentration, were able to enhance cellular migration to the same level achieved by those cells grown in FGM. The $P$ values were significant because the CI of the plant extract–treated cells were lower than the positive control; hence, no significant enhancement of cell migration was observed. However, after 72 h, the SIM extract, at a concentration of 0.05 mg/mL, showed an increase in CI, which was less than the positive control, although was not significantly different (Fig. 14).
Inhibition of Cytokine Expression

Using the CBA method, plant extracts and isolated compounds were evaluated 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 measurable. The cytokine levels of the test sample were found to be greater than the relevant BCs subsequent to individual treatment with plant extracts at all concentrations, hence indicating they were not strong inducers of IL-6 and IL-8 (Fig. 15a-f) secretion compared to the positive control; therefore, they were not pro-inflammatory. Furthermore, the plant extracts were not able to suppress IL-6 production (Fig. 15a-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. Lower IL-6 concentrations were observed in HaCaT cells treated with the SIA extract while the SIM extract caused an elevation in the production of IL-6 as extract concentrations increased. In general, all plant extracts at 50 μg/mL were able to suppress IL-8 production (Fig. 15d-f) in LPS-stimulated HaCaT cells compared to the positive control, therefore demonstrating an anti-inflammatory effect. The SIM extract at concentrations of 200 μg/mL ($P < 0.0001$) and 100 μg/mL ($P < 0.0001$) was the most effective at suppressing IL-8 (Fig. 15e, f).

Almost all compounds tested, irrespective of concentration, failed to suppress IL-6 secretion (Fig. 16a, b). Two compounds isolated from *S. indica*, i.e. verbascoside and isoverbascoside, showed that their cytokine levels in the test sample were greater than the relevant BCs at both concentrations, thus signifying that neither were strong inducers of IL-6 (Fig. 16a, b). However, ipolamiide at 25 and 50 μg/mL in both test and BC treatments (Fig. 16a, b) was shown to stimulate the production of IL-6 compared to the positive control ($P < 0.0001$), thereby indicating a pro-inflammatory effect. All the BCs of the tested compounds showed a significant ($P < 0.0001$ and ipolamiide 25 μg/mL test treatment $P < 0.001$) reduction in the production of IL-8 compared to the positive control, suggesting that they are not strong inducers of the cytokine (Fig. 16c, d). Isoverbascoside was the most effective compound in suppressing IL-8 at 25 μg/mL ($P < 0.0001$) and 50 μg/mL ($P < 0.0001$) in the test samples. Ipolamiide at 50 μg/mL and verbascoside at 25 and 50 μg/mL in the test samples showed a significant ($P < 0.0001$) reduction in IL-8 levels compared to the positive control. All three compounds also suppressed IL-8 expression in a dose-dependent manner.

Cyclooxygenase Inhibition

Overall, the inhibition pattern of the two COX enzymes by plant extracts and their isolated compounds was found to be similar. Moreover, the percentage inhibition of COX-1 enzyme (Figs. 17 and 18) was slightly high compared to that of COX-2 (Figs. 19 and 20). At higher concentrations of the plant extracts (greater than 100 μg/mL), both COX-1 and COX-2 enzymes responded poorly. As such, the experiments were undertaken using concentrations ranging between 6.25 and 100 μg/mL.

Almost all extracts and individual compounds imposed a 40–60% inhibition of COX-1 compared to the indomethacin standards (Figs. 17 and 18). Treatment with SIA
extracts showed a decreasing trend in COX-1 inhibition upon an increase in concentration (Fig. 17a-e). Ipolamiide, verbascoside and isoverbascoside showed better COX-1 inhibition compared to indomethacin at all concentrations tested (Fig. 18a-e). Ipolamiide at 6.25 and 50 µg/mL, verbascoside at 12.5 µg/mL and isoverbascoside at 50 µg/mL all imposed over 50% inhibition of COX-1 (Fig. 18a-e).

The extracts and isolated compounds produced a 30–50% inhibition of COX-2 compared to the indomethacin standards (Figs. 19 and 20). Moreover, indomethacin was superior to almost all plant extracts and compounds with regard to inhibition of the COX-2 enzyme regardless of concentrations tested. SIA extracts showed a decreasing trend in COX-2 inhibition upon an increase in extract concentration.

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![Fig. 15 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](image-url)
Lipoxygenase Inhibition

Extracts and isolated compounds were shown to inhibit the 5-LOX enzyme by 30–50% compared to the NDGA standards (Fig. 21). Both plant extracts were superior to the positive control at inhibiting the enzyme at all concentrations tested (Fig. 21a-e). The SIM extract elicited significant inhibition of 5-LOX at 6.25 µg/mL (43.22%, $P = 0.0027$), 12.5 µg/mL (43.10%, $P = 0.0007$), 25 µg/mL (43.10%, $P = 0.0001$) and 50 µg/mL (42.00%, $P = 0.0001$), compared to the standard. However, the inhibitory potential of 5-LOX by the SIM extract showed a decreasing trend when extract concentration increased (Fig. 21a-e). The highest percentage of 5-LOX inhibition was observed by the SIA extract at a concentration of 50 µg/mL (45.05%). This result was significantly ($P < 0.0001$) greater than that of NDGA at the same concentration. Moreover, a moderate level of 5-LOX inhibition was evident upon treatment with 25 µg/mL of the SIA extract (41.51%, $P = 0.0006$) and 50 µg/mL (43.10%, $P < 0.0001$), compared to the control (Fig. 22 a, b, d and e). Ipolamiide

![Effects of S. indica leaf extracts on keratinocyte cell migration](image)

**Fig. 16** The cell index (CI) at 6-h 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-h 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 to 72 h, except for SIM extract (0.05 mg/mL) which was shown to increase the cell index within 48–72 h. Further, at 72 h, 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. CIs of the SIM-treated cells were significantly lower compared to those grown in FGM from 0 to 48 h 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 h). SIM S. indica methanol, SIA S. indica aqueous and ns not significant

**Lipoxygenase Inhibition**

Extracts and isolated compounds were shown to inhibit the 5-LOX enzyme by 30–50% compared to the NDGA standards (Fig. 21). Both plant extracts were superior to the positive control at inhibiting the enzyme at all concentrations tested (Fig. 21a-e). The SIM extract elicited significant inhibition of 5-LOX at 6.25 µg/mL (43.22%, $P = 0.0027$), 12.5 µg/mL (43.10%, $P = 0.0007$), 25 µg/mL (43.10%, $P = 0.0001$) and 50 µg/mL (42.00%, $P = 0.0001$), compared to the standard. However, the inhibitory potential of 5-LOX by the SIM extract showed a decreasing trend when extract concentration increased (Fig. 21a-e). The highest percentage of 5-LOX inhibition was observed by the SIA extract at a concentration of 50 µg/mL (45.05%). This result was significantly ($P < 0.0001$) greater than that of NDGA at the same concentration. Moreover, a moderate level of 5-LOX inhibition was evident upon treatment with 25 µg/mL of the SIA extract (41.76%, $P = 0.0002$).

At 3.125 µg/mL (Fig. 22 a), significant inhibition of the 5-LOX enzyme was observed upon treatment with ipolamiide (40.29%, $P = 0.0009$) and isoverbascoside (38.71%, $P = 0.0019$), compared to NDGA at the same concentration (26.86%). Verbacoside demonstrated a strong inhibitory effect against the 5-LOX enzyme at 3.125 µg/mL (38.10%, $P = 0.0025$), 6.25 µg/mL (39.68%, $P < 0.0001$), 12.5 µg/mL (40.90%, $P = 0.0205$), 25 µg/mL (41.51%, $P = 0.0006$) and 50 µg/mL (43.10%, $P < 0.0001$), compared to the control (Fig. 22 a, b, d and e). Ipolamiide
and verbascoside were superior to NDGA with regard to 5-LOX inhibition at all concentrations tested (Fig. 22a–e). Verbascoside also showed an increasing trend of enzymatic inhibition upon an increase of compound concentration.

Fig. 17 Average levels of interleukin (IL)-6 (a–c) and IL-8 (c–f) concentrations observed in the samples in pg/mL ± SEM. Cells pre-treated with lipopolysaccharides (LPSs) 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 (BCs). 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
Discussion

Upon comparison with published peer-reviewed data, the structure of compound 1 was confirmed as ipolamiide, an iridoid glycoside [39, 40]. Structurally, iridoids are cyclopentano pyran monoterpenoids, and chemotaxonomically, they provide a structural link between terpenes and alkaloids [41]. Ipolamiide has previously been isolated and identified from aerial parts of *Stachytarpheta cayennensis* [42], *Stachytarpheta urticaefolia* [43], several *Phlomis* spp [40, 44], *Wiedemannia orientalis* [45] and is also reportedly present in plants belonging to the Family *Verbenaceae* [45]. Although there is no evidence of antibacterial activity, the anti-inflammatory effects of ipolamiide have been demonstrated in several studies [40, 43, 46]. Ipolamiide was isolated from *S. indica* by Tantisewie and Sticher [47] and Roengsumaran et al. [48], although there was no indication of biological activities. However, the biosynthesis of ipolamiide has also been reported [49].

Comparison of the NMR data of compounds 2 and 3 with the current literature [50] confirmed the structures of verbascoside and isoverbascoside [51–53], respectively. Phenylethanoid glycosides, such as verbascoside, are naturally occurring water-soluble compounds and widely distributed in the plant kingdom with remarkable biological properties [54]. Verbascoside was first isolated from *Verbascum sinuatum* in 1963 [54]. Its isomer, isoverbascoside, co-exists in many plant species, such as *Cistanches* spp., *Castilleja* spp. and *Plantago* spp. [55]. The compound has been previously isolated from the seeds of *Plantago psyllium* [51], the Chinese herb *Pedicularis longiflora* [56], *Stachytarpheta cayennensis* roots [57] and aerial parts [42], *Leonurus sibiricus* root extracts [58], *Buddleia davidii* meristematic cells [59] and an Australian native herb *Prostanthera rotundifolia*.
Fig. 19 Cyclooxygenase (COX)-1 enzyme inhibition by plant extracts at five concentrations ranging from 6.25 to 100 µg/mL (a–e). 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 to 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.
Fig. 20 Cyclooxygenase (COX)-1 enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 to 50 µg/mL (a–e). 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 3.125 to 50 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. SIC S. indica compound, SIC-1 ipolamiide, SIC-2 verbascoside and SIC-3 isoverbascoside
Fig. 21 Cyclooxygenase (COX)-2 enzyme inhibition by plant extracts at five concentrations ranging from 6.25 to 100 µg/mL (a–e). 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 to 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.
Fig. 22 Cyclooxygenase (COX)-2 enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 to 50 µg/mL (a–e). 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 3.125 to 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.
Although verbascoside and isoverbascoside have the same molecular formula, their chemical structures differ in the specific structural arrangement of certain protons and carbons. The major difference between the two isomers is the attachment of the glucose moiety at positions C4 and C6. Verbascoside has been reported to have antioxidative, anti-inflammatory, anti-nociceptive, anti-metastatic, hepatoprotective and cytoprotective properties [54, 55, 61].

Compounds ipolamiide and verbascoside were identified as major constituents of the SIM extract. Unfortunately, given their high proportions contained within the extract, this impeded the opportunity to isolate other compounds, as the remaining compounds which were present in the initial fraction were in relatively minute quantities. Ipolamiide, verbascoside and isoverbascoside have been isolated from *Stachytarpheta* spp. and various plants belonging to other genera [33–35, 45, 57, 60]. However, this project is the first to identify all three compounds from *S. indica*.

The SIA extract elicited superior inhibition against all gram-positive *Staphylococci* spp. tested. In contrast, a study by Princely et al. [25] reported that an ethanol extract (1 μg/disc) obtained from the aerial parts of *S. indica* produced greater zones of inhibition against *S. aureus* (19.3 mm ± 0.1) compared with the aqueous extract at an identical concentration. Moreover, the same study demonstrated inhibition of *E. coli* (17.3 mm ± 0.1), *B. subtilis* (18.1 mm ± 0.2) and *P. aeruginosa* (16.2 mm ± 0.29). Although the study by Princely and colleagues [25] showed greater antimicrobial activity compared to our results, the authors did not measure the annular radius of the inhibition zone as undertaken in our study, but instead, measured the diameter. Further, all extracts were resuspended in dimethyl sulfoxide (DMSO) prior to testing. Although DMSO is a highly polar and stable substance which can be used to re-solubilise dried extracts, given its known bactericidal effects [62–64], the addition of DMSO to the plant extracts would clearly distort and misrepresent the actual antibacterial potency of the plant.

The SIA extract elicited the greatest antimicrobial effect when compared to the methanol extract (SIM). This finding indicates that the act of boiling (100 ºC) the extract did not destroy the obviously thermostable compounds, but instead enhanced their extraction from the plant material. It is also interesting to note that the extracts obtained from the leaves were only antibacterial against gram-positive bacteria. This suggests that compounds extracted solely from the leaves, irrespective of solvent used, are able to penetrate the peptidoglycan cell wall of gram-positive bacteria [65, 66] and subsequently exert their bactericidal effects, unlike gram-negative bacteria, which in addition to the same murein cell wall also contain an outer membrane [65, 66], which appears to be impervious to *S. indica* leaf-derived compounds.

In this current study, verbascoside inhibited the growth of seven bacteria including both gram-positive and gram-negative species. Compared to isoverbascoside, the MIC values of verbascoside against the bacterial species belonging to the *Staphylococcus* genera were shown to be almost 64 times lower (excluding MRSA clinical isolate QUT 113), thereby indicating a superior antimicrobial effect. Verbascoside inhibited bacterial species *S. aureus* (NCTC 6571), MRSA (ATCC 33,591) and *S. epidermidis* (QUT 0613) at 9.77 μg/mL. In a previous study, verbascoside isolated from *Stachytarpheta cayennensis* (concentration range 32–63 μg/mL) inhibited *S. pyogenes*, *S. epidermidis* and *S. aureus* [57]. Rigano et al. [67] reported the MIC values of verbascoside were 16 μg/mL and 32 μg/mL against *S. aureus* (ATCC 13,709) and *S. epidermidis* (ATCC 10,875), respectively. In contrast, Nazemiyeh et al. [68] reported a MIC value of 128 μg/mL against *S. aureus* (ATCC 25,923) for the same compound. In comparison, the results for our current study showed verbascoside to be more effective as it was also able to inhibit three gram-negative
bacteria, i.e. *P. vulgaris* (312.5 μg/mL), *P. aeruginosa* and *A. baumannii* (both at 1,250 μg/mL). Conversely, Rigano et al. [67] reported that verbascoside inhibited *P. vulgaris* at 4 μg/mL and *P. aeruginosa* at 32 μg/mL, which are lower than the values generated in this current study. The same study by Rigano and colleagues [44] also reported that *E. coli* was inhibited by verbascoside at a concentration of 2 μg/mL. However, this result was in direct contrast to our study as verbascoside was shown to be totally ineffective at inhibiting the growth of *E. coli*. In the study conducted by Barnes et al. [69], verbascoside isolated from Australian native plant *Eremophila microtheca* was inactive against numerous *S. aureus* bacterial strains. This result contradicts those of the current project as well as various other studies as *S. aureus* has always been reported as a highly susceptible species to that particular compound [68, 70]. However, Barnes and colleagues [69] demonstrated that *E. coli* was not inhibited by verbascoside, which is consistent with our results. In our current study, isoverbascoside was found to inhibit only four gram-positive bacteria, all belonging to the *Staphylococcus* genera, including the two MRSA species. Moreover, *S. epidermidis* was the most sensitive to isoverbascoside, with a MIC value of 9.77 μg/mL. The exact mechanism of how these compounds exert their antibacterial activity is still poorly understood. Avila and colleagues [70] have reported that verbascoside interferes with protein synthesis in *S. aureus* by obstructing leucine absorption in the cell membrane, which eventually inhibits bacterial growth. In a separate study, Funes and colleagues [71] postulate that verbascoside’s ability of inhibiting *S. aureus* is attributed to its ability to disturb the phospholipid-water interface, specifically targeting phosphatidylglycerol, an abundant and common lipid within the bacterial cell membrane of gram-positive bacteria. This further supports the results observed in our study.

The ability of a plant extract to alter cellular morphology, interfere with cell adhesion and affect cell proliferation or cell differentiation is extremely important as the information helps provide greater understanding of the in vitro effects, including the potential cytotoxic nature of the plant in question. Differential interference contrast (DIC) microscopy is a non-invasive microscopic technique, which visualizes live cells without alteration [72]. Hence the DIC microscope technique was used to visualize the morphological effects of keratinocytes in response to plant extract treatments. It was clear that higher concentrations of the SIM extracts (0.8–0.2 mg/mL) induced an intense morphological reaction, i.e. producing cytoplasmic projections within the cells during the initial stages of the assay (Fig. 11a-l). Concurrently, the cells became extremely active and motile, suggesting their metabolic activity was extremely high and produced as a direct result of treatment. However, the CyQUANT® assay showed a low number of viable cells in those treated with plant extracts compared to the positive control (Fig. 13a-d). This evidence suggests that the SIM extracts have the ability to induce an exhaustive metabolic rate in HaCaT cells (within the concentration range 0.8–0.2 mg/mL), resulting in cellular necrosis. However, further investigation is warranted to determine the exact mechanism of action and identify the compound/s responsible for the effect as they may have potential as a novel anti-cancer therapy, since cancer is merely uncontrolled cellular replication.

The xCELLigence assay is a valuable tool in validating the cell migration potential of compounds and extracts as this cellular process is associated with wound closure, which occurs during the proliferation phase of the wound healing process. Moreover, it mimics the natural process to some extent and measures the rate of cell migration. The xCELLigence RTCA system measures the variation of the electronic impedance between micro-electrodes placed on the bottom surface of the upper chamber via relative surface contact over time. This variation in electrical impedance is represented by a unit-less parameter, cell index (CI), and is dependent upon the number of cells attached to the wells of the upper
chamber. The gold electrodes embedded in the base of the wells monitor electrical resistance and generate an output presented as a CI. Our results indicate that the SIM extract at a concentration of 0.05 mg/mL was shown to be superior compared to the SIA treatment, although both can impose strong negative effects on HaCaT cells at concentrations higher than 0.2 mg/mL. During this process, the extracts induced an intense metabolic reaction in the keratinocytes, resulting in the production of a large number of cytoplasmic projections, which became actively motile. When keratinocytes migrate across the wound bed, the cells become flat and elongated with long cytoplasmic projections, called lamellipodia [73]. In our current study, similar activity was observed under in vitro conditions in those cells treated with S. indica extracts (SIM and SIA) (0.8–0.4 mg/mL). It has been reported that the soluble N-terminal ectodomain of the amyloid-β precursor protein (sAPP) acts as an epidermal growth factor, which also exerts a chemotactic effect on keratinocytes [74]. Thus, it might be involved in guiding keratinocytes towards the wound bed during epidermal wound healing as they are capable of producing sAPP [74, 75]. Based on the current results, it is reasonable to theorise that S. indica extracts might have the ability to induce keratinocytes to produce sAPP, hence improving cell migration in wounds. It could be an interesting influence of the extracts on keratinocytes despite their negative effect at concentrations greater than 0.2 mg/mL. However, further investigation is required to identify and confirm the underlying principle of this effect.

To our knowledge, this study is the first to successfully evaluate the effects of S. indica leaf extracts on human keratinocytes (HaCaT), with regard to cell viability, proliferation, cytotoxicity and migration potential. In general, S. indica extracts showed the ability to induce morphological changes and increase HaCaT cell motility (Fig. 23a-h and 24). However, concentrations between 0.8 and 0.2 mg/mL exerted an apoptotic effect, while lower concentrations (0.1–0.05 mg/mL) improved cell viability and proliferation.

In our study, both extracts and individual compounds were able to suppress IL-8 levels compared to the positive control. However, all BCs (cells only treated with extract) and individual compounds did not induce IL-8 production compared to the positive control, thus indicating that S. indica plant extracts (SIM and SIA) and derived compounds had no pro-inflammatory effect. The SIM extract at 100–200 μg/mL was shown to significantly downregulate IL-8 (P < 0.0001) compared to the positive control. Moreover, the SIA extract at 50–200 μg/mL was found to have a moderate effect on the regulation of IL-6 levels upon treatment with the test samples. These results further support prior reports regarding the use of S. indica as an alternative medicine for the treatment of inflammation [19, 21]. Moreover, both the SIM (6.25 μg/mL, P = 0.0027) and SIA (50 μg/mL, P < 0.0001) extracts were shown to significantly inhibit the 5-LOX enzyme compared to NDGA. To the best of our knowledge, this is the first study to report the anti-inflammatory effects of extracts and compounds derived from S. indica.

The anti-inflammatory activity of the isolated compounds verbascoside, iso-verbascoside and ipolamiide has previously been reported [45, 76, 77]. The anti-inflammatory activity of verbascoside has been confirmed in vitro, as the compound was shown to significantly reduce the release of pro-inflammatory chemokines in human myelomonocytic leukaemia cells (THP-1) [76] and in human umbilical vein endothelial cells (HUVEC) [78]. High levels of anti-inflammatory activity produced post-stimulation with verbascoside (in human keratinocytes) were previously reported by Pastore and colleagues [79]. Furthermore, the compound was also shown to be an effective inhibitor of IL-8 at 50 mM (31.23 mg/mL) as secretion of the cytokine was reduced by more than 90% [80]. Lee et al. [77] studied the inflammatory effect of Paulownia tomentosa extracts enriched with verbascoside and isoverbascoside, in LPS-stimulated macrophages (RAW 264.7), and found
Fig. 23 5-Lipoxygenase (LOX) enzyme inhibition by plant extracts at five concentrations ranging from 6.25 to 100 μg/mL (a–e). 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 to 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.
Fig. 24 5-Lipoxygenase (LOX) enzyme inhibition by isolated compounds at five concentrations ranging from 3.125 to 50 µg/mL (a–e). 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 3.125 to 50 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. SIC S. indica compound, SIC-1 ipolamiide, SIC-2 verbascoside and SIC-3 iso-verbascoside
that the production of IL-6 decreased. In a separate study, treatment with verbascoside at 25 μg/mL considerably inhibited LPS induction of IL-6 in microglial murine cells [81]. In this current project, verbascoside and isoverbascoside were able to suppress IL-6 production in BCs compared to the positive control, thus indicating strong anti-inflammatory activity. The two isomeric compounds were also shown to reduce IL-8 production in both test samples (cells stimulated with LPS prior to addition of the compound) and BCs (compound only added to cells), thereby confirming their powerful anti-inflammatory effect. Moreover, verbascoside also elicited strong inhibition of both the COX-1 (12.5 μg/mL, 50%) and 5-LOX enzymes (50 μg/mL, 43.10%).

Ipolamiide isolated from *Stachytarpheta cayennensis* exerted significant anti-inflammatory activity by inhibiting the formation of carrageenan-induced oedema in rats [45, 82]. The activity of ipolamiide was previously predicted by the use of computer-aided molecular modelling [40]. In our project, we discovered that verbascoside, isoverbascoside and ipolamiide were unable to inhibit IL-6 secretion; however, all inhibited the expression of IL-8 in human keratinocytes induced with LPS. Our results indicate that the BCs of ipolamiide at a concentration of 25–50 μg/mL induced IL-6 production, thereby indicating a pro-inflammatory effect. This finding directly contradicts its known anti-inflammatory effects [45]. However, ipolamiide was also shown to significantly reduce the level of IL-8 (*P* < 0.0001) in both test samples and BCs upon comparison to the positive control. Moreover, the compound also inhibited the COX-1 (6.25 μg/mL, 54.80%) and 5-LOX enzymes (3.125 μg/mL, 40.29%) compared to the respective controls at the same concentrations.

**Conclusion**

In this project, *S. indica* plant extracts and isolated compounds exerted antibacterial effects, improved cell viability and migration under in vitro conditions, were not cytotoxic to human-derived cells and elicited significant anti-inflammatory activity by inhibiting IL-8 and pro-inflammatory cytokines. Therefore, our results have scientifically validated the antibacterial, anti-inflammatory and regenerative effects of *S. indica* plant extracts and isolated compounds, which may have therapeutic potential for the treatment of chronic wounds.

**Author Contribution** VA and TC contributed to the study conception and design. VA conducted the experiments and the research work. PK analysed and interpreted NMR data. Analysis and interpretation of experimental data and preparation of manuscript were done by VA. TC and CC reviewed and edited the manuscript. All the authors read and approved the manuscript.

**Data availability** Not applicable.

**Declarations**

**Ethics Approval** The management and use of the bio-specimens, Human immortalized keratinocyte cell line (HaCaT, Catalogue No.T0020001, Addexbio Technologies, USA) was accorded by the Human Research Ethics Committee, Queensland University of Technology, Brisbane, Australia (approval number: 1700001128).

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

**Competing Interests** The authors declare no competing interests.
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**Authors and Affiliations**

**Vajira Asela Agampodi**1,2 **·** **Peter Katavic**1 **·** **Christopher Collet**1,2 **·** **Trudi Collet**1

1 Innovative Medicines Group, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Brisbane, QLD 4059, Australia

2 School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, 2 George Street, Brisbane, QLD 4000, Australia