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Synergistic-antagonistic interaction of vegetable extracts, *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora*: Wound healing, antioxidant, protectivity, and antimicrobial properties

Norfaizah Mahmud[1,2], Sujatha Ramasamy[1,2]*, Sugumaran Manickam[1,3] and Wan Abd Al Qadr Imad Wan-Mohtar[1,4]

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia.
[2]Bioactivity and Phytochemical Research Laboratory, Institute of Advance Studies, University of Malaya, 50603, Kuala Lumpur, Malaysia.
[3]Rimba Ilmu Botanic Garden, Institute of Biological Sciences, University of Malaya, 50603, Kuala Lumpur, Malaysia
[4]Functional Omics and Bioprocess Development Laboratory, Faculty of Science University of Malaya, 50603, Kuala Lumpur, Malaysia.
Email: sujatha@um.edu.my

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ABSTRACT

Aims: *Acalypha indica* (AI)*, Centella asiatica* (CA), and *Sesbania grandiflora* (SG) are vegetables commonly used in traditional medicine in Asian countries to treat skin problems. In this study, we investigated their pharmacological activities relevant to wound healing and synergistic actions to provide an insight into a promising vegetable combination as a candidate treatment for wounds.
Methodology and results: The stimulatory, antioxidant, and antibacterial activities of aqueous (A) and methanol (M) extracts of all the three vegetables were assessed alone and in combination in normal human dermal fibroblast (NHDF) cells *in vitro*. CA-A (89.52%) and the combination of AI-A+CA-A (90.76%) produced the highest percentage of wound closure. AI-A exhibited the highest total phenolic content (TPC) (82.94 mg GAE/g) and moderate reducing activity (61.63 mM Fe (II)/mg) when assessed by ferric reducing antioxidant power (FRAP) assay. Free radical scavenging activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and the combination of AI-A+CA-A exhibited scavenging activity at IC_{50} = 379.75 µg/mL and IC_{50} = 578.7 µg/mL, respectively. Pre-treatment of NHDF cells with CA-M at 100 µg/mL offered the highest protection against hydrogen peroxide. All single and combined vegetable extracts showed poor antibacterial properties against Gram negative and Gram positive bacterial species implicated in wound infection. Only AI-A+CA-A executed synergism in fibroblast migration when assessed via the combination index (CI). Furthermore, screening and identification of AI-A, CA-A, and CA-M via UHPLC (LC-MS/MS) system revealed that the major components responsible for all the tested bioactivities were phenolic groups such as simple polyphenols, flavonoids, polysaccharides, and triterpenes (asiaticoside and madecassosides).
Conclusion, significance and impact of study: The vegetable extracts of *A. indica, C. asiatica*, and *S. grandiflora* exhibited good bioactivities independently. However, only AI-A+CA-A showed synergism in combination to accelerate the migration of fibroblast and increase antioxidant activities. These findings demonstrate the potential formulation of combined vegetable extracts from the two species of *A. indica* and *C. asiatica* for optimum wound healing properties.

Keywords: Acalypha indica, *Centella asiatica*, *Sesbania grandiflora*, synergism, wound healing properties

INTRODUCTION

International Diabetes Federation (IDF) estimated that 463 million people were diagnosed with diabetes in 2019. It is predicted the number will increase by 25% (578 million people) in 2030 and will go up to 51% (700 million people) in 2045 (Saeedi *et al.*, 2019). A major concern for patients with diabetes is impaired healing of acute wounds caused by various conditions such as hyperglycaemia, neuropathy, hypoxia, and prolonged inflammation around the wounded area (Guo and Dipietro, 2010; Muniandy *et al.*, 2018). Wound healing is an orchestrated cellular and biochemical process that restores the structure, strength, and functional integrity of injured tissues. The normal response to injury happens in three main stages: 1) inflammation, 2) new tissue formation, and 3) tissue remodelling (Gurtner *et al.*, 2008; Thakur *et al.*, 2011). However, an abnormal progression

*Corresponding author

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of wound healing may result in delayed wound healing, thus leading to the formation of chronic wounds (Han and Ceilley, 2017).

The duration of complete wound repair is affected by local factors such as the size and depth of the injury site, presence of infection or tissue necrosis, and systemic factors such as age (older skin has a slower healing process), individual health status (presence of chronic illness) and nutritional condition of the body (Johnstone and Farley, 2005). In chronic wounds, vascular disruption can cause a hypoxic (oxygen depletion) microenvironment to develop in the site of wounding, affecting normal cell metabolism for the production of adenosine triphosphate (ATP) or energy, which is essential in the wound healing process (Guo and Dipietro, 2010). In addition, hypoxic conditions increase the level of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Schafer and Werner, 2008). Under normal conditions, H_2O_2 and O_2^- function as signalling molecules that stimulate cytokines and growth factors to promote the migration and proliferation of fibroblasts at the end of the inflammatory phase of the response to injury (Akbik *et al.*, 2014). However, excessive levels of ROS, including H_2O_2 and O_2^-, are considered critical causative factors in delaying the healing process (Schafer and Werner, 2008).

Another factor that can affect the process of wound healing is microbial infection. During inflammation, neutrophils and macrophages are recruited to the wounded area to remove invading microbes. However, inflammation can be prolonged if the microbial elimination process is incomplete since both bacteria and endotoxins can elevate the levels of regulators of inflammatory cytokines like interleukin-1 (IL-1) and TNF-α, resulting in wound progression into a chronic state and failure to heal (Guo and Dipietro, 2010; Wong *et al.*, 2015). Furthermore, some pathogens are resistant to existing antibiotics, although efforts are underway to identify novel therapeutic compounds to combat this issue (Taemchuay *et al.*, 2009; World Health Organization, 2017). Natural products derived from plants have been shown to have numerous biological properties (Wan-Mohtar *et al.*, 2017; Kozarski *et al.*, 2019), and traditional herbal medicine is being explored as an alternative strategy to combat resistance to antibiotics (Ncube *et al.*, 2012).

In the present study, *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora* were selected for investigation of their wound healing properties as these species of vegetables are widely consumed among Asian communities. All three vegetables have also been used in traditional practices for the treatment of various skin ailments and other illnesses. In countries such as Malaysia, Mauritius and India, *A. indica* was used to alleviate skin break out and pimples, skin infection such as scabies and dermatitis, and treatment for burns (Singh and Tewari, 2012; Lingaraju *et al.*, 2013; Zahidin *et al.*, 2017). In Malaysia, *C. asiatica* is widely consumed raw as “ulam” by the Malay community. The Chinese and the Indians consumed it as cooling drink and brain tonic, respectively. In Malay-traditional medicine, a decoction of *C. asiatica* leaves is used to cure leprosy, rheumatism and diarrhoea in children. The leaves are grounded into a paste to reduce fever. In Ayurveda and South eastern Asia medicine, whole parts of *S. grandiflora* were fully utilized to treat different diseases. The root poultice is applied to reduce inflammation, sore spots and fever (Sreelatha *et al.*, 2011). The leaves are used for the treatment of epilepsy, ulcer, night blindness and applied to sprains or bruises on the body. Tea prepared from dried leaves is claimed to have anthelmintic, antibiotic, antitumor, and contraceptive properties. However, overconsumption of the leaves might cause diarrhea. The flower of *S. grandiflora* is used as an antiseptic as well as for pain relieve. The bark is said to have antipyretic properties and is a remedy for gastric pain (Sheikh *et al.*, 2011; Sreelatha *et al.*, 2011; Hasan *et al.*, 2012).

As described above, wound healing is a multi-action process involving diverse cellular and biochemical actions. Combination of vegetables might help in the development of a drug with various action modes (Chou, 2006). The present study was therefore performed to investigate whether extracts from the three vegetables, used either alone or in combination, can promote wound healing processes. Specifically, we sought to identify the phytochemical constituents from these vegetables via ultra-high-performance liquid chromatography (UHPLC) that might be responsible for increasing the rate of fibroblast migration, exert antioxidant and protective effects against radical ions, and show antibacterial activity against selected pathogenic bacteria relevant to wound infection.

MATERIALS AND METHODS

Materials

Normal human dermal fibroblast (NHDF) (PCS-201-012) cells were obtained from the American Type Culture Collection (ATCC) (Virginia, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St. Louis USA) containing 10% foetal bovine serum (FBS) (PAA Laboratories, Austria). Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Assay plates and flasks were purchased from Thermo Scientific-Nunc (Roskilde, Denmark). Cells were grown to confluence (80%–90%) and were used in bioassays at passages 6–7 (Kim *et al.*, 2016). All solvents and chemicals used for analysis were of analytical grade.

Plant materials

Acalypha indica L. was collected from Taman OUG, Kuala Lumpur (3.07°N, 101.67°E) in March 2017; *C. asiatica* (L.) Urb. was collected from Rimba Ilmu Botanic Garden, University of Malaya (3.13°N, 101.66°E) in December 2018; and *S. grandiflora* (L.) Pers. was collected from Muar, Johor (1.96°N, 102.64°E) in November 2018. These specimens were authenticated at the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya by

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Dr. Sugumaran Manickam. The voucher numbers for *A. indica*, *C. asiatica*, and *S. grandiflora* are KLU 49900, KLU 49899, and KLU 49897, respectively.

Preparation of vegetable extract

Aqueous extraction

Fresh leaves (20 g) of *A. indica* (AI-A), *C. asiatica* (CA-A) and *S. grandiflora* (SG-A) were cut into pieces of about 1 mm^2 and heated separately at 80 °C in 500 mL of distilled water for 1 h. The solutions were filtered through filter paper (Whatman No. 1, UK), and the aqueous filtrates were collected, freeze-dried and stored in the dark at 4 °C until further use (Krishnan, 2005).

Methanol extraction

The leaves of *A. indica* (AI-M), *C. asiatica* (CA-M) and *S. grandiflora* (SG-M) were oven-dried at 35 °C for 3–5 days, grounded into powder, and soaked in methanol (MeOH) at 1:10 (w/v) for 72 h at room temperature. The filtrates were collected and excess methanol was evaporated at 40 °C under reduced pressure using a rotary evaporator. The extracts were stored at −20 °C until further use (Azis *et al.*, 2017).

Single and combined solutions of extracts

Stock solutions (20 mg/mL) of vegetable extracts (aqueous and methanol) were dissolved in sterilized distilled water, filtered, and sterilized using a 0.2 µm filter membrane (Orange Scientific, Belgium). Combined solutions of vegetable extracts were prepared at a 1:1 ratio (v/v) of the prepared stock solution.

***In vitro* wound scratch assay**

The wound healing scratch assay procedure was carried out as described previously with slight modifications (Liang *et al.*, 2007). NHDF cells were seeded (1×10^5 cells/mL) in a 24-well plate and cultured in DMEM with 10% FBS before being incubated for 24 h to allow the formation of a confluent cell monolayer. Next, the medium was discarded and fresh DMEM with 1% FBS was added to each well. The cells were incubated for another 24 h and before a small linear scratch was created in the confluent monolayer using a sterile pipette tip. The media was removed and cells were rinsed with phosphate buffered solution (pH 7.4) to remove cellular debris. Fresh DMEM with 1% FBS and 25 µg/mL of vegetable extracts was added to each well. DMEM with 10% FBS was used as a positive control and DMEM with 1% FBS (without the addition of any extracts) was used as a negative control. The distance of fibroblast cell migration towards the gap in the artificial wound was observed microscopically at 0, 24 and 48 h. The percentage of wound closure was calculated according to the following formula:

Percentage of wound closure (%) =

$$\frac{Distance\ at\ T0 - Distance\ at\ T24\ or\ T48}{Distance\ at\ T0}$$

Images of migrated cells were captured using a digital camera attached to an inverted microscope (Leica DMI 3000 B) and a Leica microsystem (Leica Application Suite, LAS V 4.0). The experiment was performed in triplicate. *In vitro* wound healing analysis in NHDF cells was also carried out using combinations of vegetable extracts at a ratio of 1:1 and concentration of 25 µg/mL.

Antioxidant properties of single and combined vegetable extracts

Chemicals and reagents

Folin-Ciocalteau (F-C) reagent, sodium bicarbonate (Na_2CO_3), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), gallic acid ($C_7H_6O_5$) and hydrogen peroxide (H_2O_2) were purchased from R & M Chemicals, UK. Ascorbic acid, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), potassium persulfate ($K_2S_2O_8$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich, St. Louis, USA.

Total phenolic content (TPC)

Total phenolic content (TPC) of single and combined vegetable extracts was determined using the F-C reagent method with slight modifications (Wan-Mohtar *et al.*, 2018; Wan-Mohtar *et al.*, 2019). In brief, 10 µL of each extract was diluted in distilled water (1 g/mL) and mixed with 25 µL of fresh F-C reagent. After 5 min, the solution was mixed with 25 µL of 20% Na_2CO_3 (w/v) solution. Distilled water was added to a final volume of 200 µL and the solution was incubated in the dark for 30 min at room temperature. Absorbance was read at 760 nm using a microplate reader. A standard curve for gallic acid was used to determine the TPC, which was expressed as gallic acid equivalent (mg GAE/g).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was adapted from a previous report (Benzie and Strain, 1996). FRAP reagent was freshly prepared (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ [10:1:1; v/v]), and 20 µL of each single and combined extract was diluted in distilled water (1 g/mL) and added with 180 µL of FRAP reagent to the wells of a 96-well plate. The plate was incubated for 30 min at room temperature in the dark. Absorbance was measured using a plate reader at 595 nm. Ferrous sulphate ($FeSO_4$) solution was used as a standard and FRAP activity was calculated as ferrous equivalent (mM FE (II)/mg). Ascorbic acid was used as a positive control.

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2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

DPPH assay was performed as described previously (Sulaiman and Ooi, 2012), with slight modifications. A 50 µL aliquot of each extract (0.31–10.0 mg/mL) was diluted in distilled water and mixed with 150 µL of 0.3 mM DPPH solution in methanol in the wells of a 96-well plate. The plate was incubated in the dark for 30 min and absorbance was measured at 517 nm. Antioxidant activity was evaluated over a range of concentrations to establish IC_{50} (the concentration that reduced DPPH absorbance by 50%). Ascorbic acid was used as a positive control.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

ABTS radical scavenging activity was determined as described previously by Kim *et al.* (2003), with minor of modifications. Briefly, 2.45 mM $K_2S_2O_8$ and 7 mM ABTS solutions were prepared and mixed together before being incubated in the dark at room temperature for 16 h before use. The absorbance of the resulting blue-green ABTS radical solution was adjusted to 0.7 ± 0.2 before use. Next, 20 µL of single and combined extracts (0.25–1.5 g/mL) was mixed with 180 µL of diluted ABTS solution and incubated at room temperature for 20 min. Absorbance was read at 734 nm using a microplate reader, with ascorbic acid as a positive reference. The scavenging ability of extracts was expressed as the IC_{50} value, which was the concentration at which 50% of ABTS radicals were scavenged.

Protective effect of single and combined vegetable extracts against hydroxyl radicals

The protective effects of single and combined vegetable extracts against hydroxyl radicals were performed as described previously by Murrell *et al.* (1990) with slight modifications. The NHDF cells were seeded at a concentration of 3×10^5 cells/mL in 96-well plates and maintained in DMEM with 10% FBS for 24 h. Two different experimental sets were performed: (a) pre-treatment of cells with single and combined vegetable extracts for 24 h before exposure to H_2O_2 (100 µM for 1 h), and (b) post-treatment of cells with single and combined vegetable extracts after H_2O_2 (100 µM for 1 h) exposure for 24 h. Ascorbic acid (100 µg/mL), an H_2O_2 scavenger, was used as a reference standard. Cells treated with H_2O_2 alone were used as negative control. After 24 h of incubation, cell viability was measured using a neutral red uptake (NRU) assay and measurement of optical density at 540 nm was performed by using a microplate reader (Mahdzir *et al.*, 2017).

Antibacterial activity assay

Antibacterial activity was determined using the Kirby–Bauer disc diffusion assay with some modifications (Klaus *et al.*, 2015; Wan-Mohtar *et al.*, 2016; CLSI, 2020). Disc diffusion testing was carried out using sterile 6-mm discs (Whatman, UK). The bacterial tested for antimicrobial activity in this study were *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Serratia marcescens*, *Staphylococcus aureus* and *Staphylococcus epidermidis.* All bacteria were obtained from the Microbiology Department, Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia. Discs were prepared using 50 µL of each vegetable extract diluted in sterile distilled water to a concentration of 200, 400, 600, or 800 mg/mL (Wan-Mohtar *et al.*, 2016). All test microorganisms were adjusted with sterile broth to 0.5 McFarland standard (approximately 1 to 2×10^8 CFU/mL), and 200 µL of 0.5 McFarland suspension was smeared on the prepared nutrient agar (NA) (Thermo Scientific-Oxoid, UK). The ready discs were placed on the surface of the inoculated NA media and incubated at 37 °C for 24 h. Vancomycin (30 µg/disc, Thermo Scientific-Oxoid, UK) was used as a positive control. After overnight incubation, the plate was observed for zones of inhibition and synergism (Vunduk *et al.*, 2019), which were measured using electronic digital callipers (Taemchuay *et al.*, 2009).

Determination of synergistic effects of combined vegetable extracts

The potential interactions of the combined vegetable extracts in each assay were analysed using the combination index (CI) model via CompuSyn software 2.0 (Biosoft, Cambridge, UK). The CI values denoted synergism if C1 < 1, additive if CI = 1, and antagonism if CI > 1 (Chou, 2006, 2010).

Compound identification through liquid chromatography mass spectrometer (LC-MS/MS) analysis

UHPLC-QTrap-MS/MS analysis

The vegetable extracts (AI-A and CA-A) were analyzed using an LC/MS-MS system equipped with the 3200 QTrap and UHPLC system (Agilent 1100 Series, California, USA). Injection of 20 µL of samples by an autosampler (Agilent 1100 G1313A, California, USA) onto a C18 column (100 mm × 3 µM × 2.0 mm) (Phenomenex Synergi, California USA), eluted with a mobile phase comprising of solvent A (0.1% formic acid with water) and solvent B (0.1%) formic acid with acetonitrile). Prior to spectral analysis (negative mode), the chromatographic separation was carried out by the gradient system, 10% of solvent B to 90% of solvent B (0.01 min to 8.0 min), hold for 3 min, and returned to original conditions (10% of solvent B) in 1 min and column re-equilibration for 5 min. Identity of the detected compound was attained by referring to the molecular ions (m/z) with reference standard of in-house library on the Agilent 1100 Series instrument.

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UHPLC-TWIMS-QTOF-MS/MS analysis

The vegetable extract (CA-M) (1 µL) was injected onto ACQUITY UPLC I-Class system (Waters Co., Massachusetts, USA), equipped with a binary pump, a vacuum degasser, an autosampler, and a column oven. The chromatographic separation of CA-M was done via ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm × 1.8 µm, Waters Co.), operated at 40 °C. A binary gradient system of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) was used as mobile phase. The separation was performed at a flow rate of 0.6 mL/min, from 1–100% of solvent B (0–18 min) and returned to original conditions (1% of solvent B, 20.0 min). Profiling of the compounds was achieved by coupling the HPLC system to an ion mobility mass spectrometer (Vion IMS) QTOF hybrid mass spectrometer (Waters Co.), equipped with electrospray ionization interphase (ESI) in the positive and negative ionization mode. The capillary voltage was adjusted to 1.50 kV, desolvation nitrogen gas (>99.5%) at 120 °C with a flow rate of 800 L/h and cone gas flow at 50 L/h. Data were obtained in the range *m/z* 50–1500 Da at 0.1 s/scan. Two independent scans at different collision energies (CE) were acquired in the run, low energy (CE of 4 eV) and high energy (10–40 eV) scans. Argon (99.999%) was used as collision-induced-dissociation (CID) gas. Identity of the detected compound was attained by referring to the molecular ions (*m/z*) with reference standard of in-house library on the ACQUITY UPLC I-Class instrument.

Statistical analysis

All measurements were carried out in triplicate, and the results were shown as mean ± S.D using GraphPad Prism software (Version 7.0) (USA). Statistical differences were analysed using one-way analysis of variance (ANOVA) followed by Dunnet's test.

RESULTS AND DISCUSSION

Methods of vegetable extraction

Extraction of phytochemicals depend on the solvent used throughout the procedure and several factors need to be considered when choosing the solvent or solvent system, i.e. the safety of the solvent, extraction or formation of unwanted compounds and solubility of the desirable compounds (Thouri *et al.*, 2017). In this study, methanol and water were used for phytochemical extraction. Methanol (organic solvent) permits maximum extraction and produces high phytochemical extraction yields. However, methanol is harmful if ingested by human and it is not suitable for consumption. Hence, the methanol must be removed from the extracts before incorporating into foods and beverages. The phytochemical groups that can be extracted by using methanol were phenolic compounds (phenols, phenolic acids, anthocyanins, and flavonoids), terpenoids (essential oils, carotenoids, diterpenoids, and triterpenoids) lipids and alkaloids (Harborne, 1999). On the other hand, water is a commonly used solvent in traditional practices for plant phytochemical extraction. It is safe for human consumption and extracted compounds are almost similar to that obtained using organic solvent with additional polysaccharides (Tiwari *et al.*, 2013; Liu *et al.*, 2015a).

Fibroblast migration

The proliferation and migration of fibroblast are vital processes in wound healing that lead to collagen synthesis, production of the extracellular matrix, the release of inflammatory mediators, and restoration of structure and function of the tissue (Singh and McNaught, 2017). In this study, the percentage of wound closure in NHDF cells was quantified via an *in vitro* scratch wound assay at 24 and 48 h post-wounding. All single and combined aqueous and methanol vegetable extracts were applied at 25 µg/mL to artificially wounded monolayer cultures of NHDF cells. As shown in Figure 1, significant migration of NHDF cells into the scratch wound area was observed in the presence of both single and combined aqueous and methanol vegetable extracts compared to the negative control.

Table 1 shows the percentage of wound closure at 24 and 48 h. For single extract treatment, the aqueous extract of *C. asiatica* (CA-A) resulted in the highest rate of

Table 1: Measurement of wound closure rates expressed as the difference between wound width at different intervals (T24 and T48).

Extracts	Percentage of wound closure[a] (%)	
	24 h	48 h
DMEM 10%	63.53 ± 3.25	92.34 ± 2.14
DMEM 1 %	41.77 ± 2.76	75.61 ± 1.02
AI-A	63.79 ± 2.63	88.79 ± 3.72
CA-A	54.42 ± 2.52	89.52 ± 4.03*
SG-A	50.78 ± 0.77	74.67 ± 5.96
AI-M	55.60 ± 3.28	77.07 ± 4.00
CA-M	62.97 ± 1.87	77.57 ± 3.17
SG-M	50.07 ± 1.65	75.12 ± 8.07
AI-A+CA-A	69.37 ± 1.17	90.76 ± 3.62*
AI-A+SG-A	55.10 ± 1.81	73.14 ± 6.12
CA-A+SG-A	51.65 ± 1.31	76.70 ± 3.92
AI-M+CA-M	61.52 ± 2.51	73.32 ± 3.19
AI-M+SG-M	54.84 ± 2.81	67.93 ± 3.84
CA-M+SG-M	53.72 ± 4.38	69.47 ± 2.53

AI-A = *A. indica* aqueous extract; CA-A = *C. asiatica* aqueous extract; SG-A = *S. grandiflora* aqueous extract; AI-M = *A. indica* methanol extract; CA-M = *C. asiatica* methanol extract; SG-M = *S. grandiflora* methanol extract

[a]The data were expressed as mean ± S.D. of triplicate values. One-way ANOVA, followed by Dunnett's test was used for statistical analysis (significant at *p<0.05 versus DMEM 1% (negative control)).

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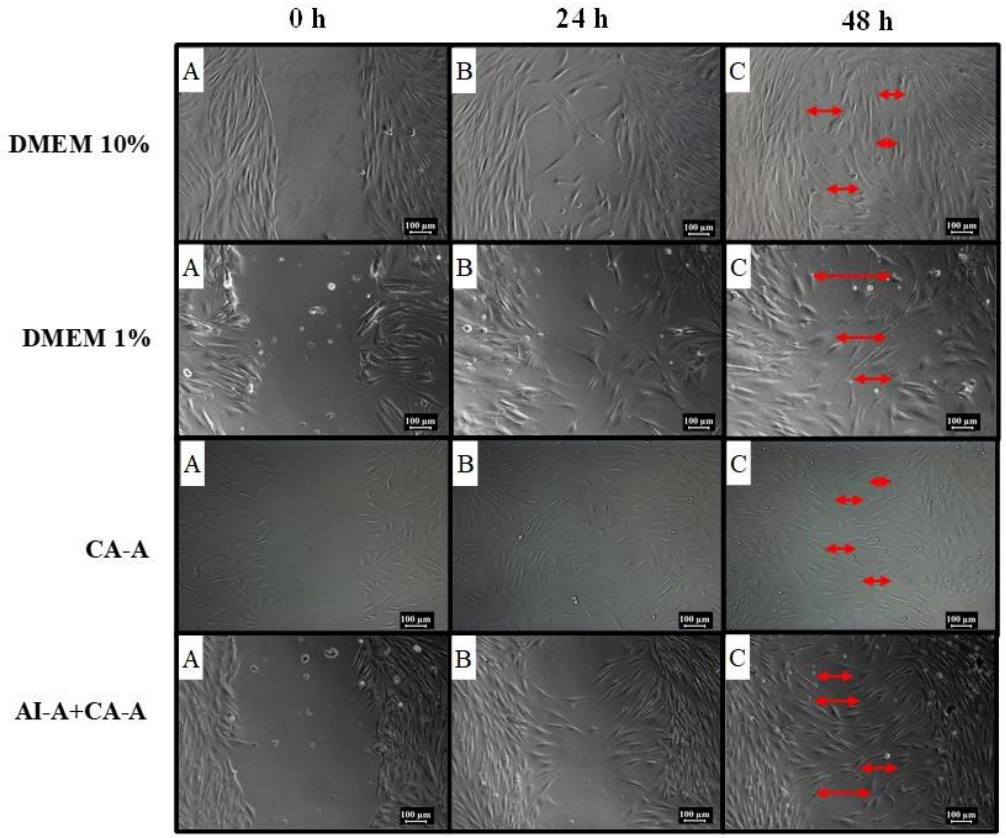

Figure 1: Migration of fibroblast treated with 25 µg/mL of single and combined aqueous vegetable extracts. Phase contrast micrographs of dermal fibroblast cells were taken at 0 (A), 24 (B), and 48 h (C). DMEM 10% served as the positive control while DMEM 1% served as the negative control. Single extract of CA-A and combined extract of AI-A+CA-A enhanced the high percentage of fibroblast migration (red arrow) compared to other vegetable extracts, 89.52 ± 4.03% and 90.76 ± 3.62% respectively. Scale bar at 100 µm, 100× magnification.

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Table 2: Antioxidative properties of single and combined vegetable extract at 1 g/mL.

Extracts	(mg GAE/g)	(mM Fe (II)/mg)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
	TPC	FRAP	DPPH	ABTS
AI-A	82.94 ± 1.95^{ef}	61.63 ± 1.48^{g}	343.46 ± 44.69^{a}	473.70 ± 17.07^{a}
CA-A	79.43 ± 2.58^{de}	39.70 ± 1.55^{e}	661.48 ± 40.98^{a}	606.73 ± 36.93^{bcd}
SG-A	61.21 ± 6.18^{b}	9.03 ± 0.35^{a}	3891.81 ± 405.34^{f}	547.13 ± 46.01^{abcd}
AI-M	44.79 ± 4.21^{a}	13.86 ± 2.28^{ab}	1840.02 ± 53.62^{c}	520.93 ± 15.22^{ab}
CA-M	72.95 ± 2.21^{cde}	17.08 ± 3.16^{b}	2557.67 ± 108.98^{d}	649.33 ± 84.85^{de}
SG-M	69.55 ± 3.62^{bcd}	13.91 ± 4.07^{ab}	7830.99 ± 120.61^{g}	519.27 ± 36.35^{ab}
AI-A+CA-A	74.25 ± 2.51^{cde}	49.55 ± 0.93^{f}	379.75 ± 16.23^{a}	578.70 ± 6.39^{bcd}
AI-A+SG-A	79.75 ± 3.89^{de}	33.76 ± 0.11^{d}	663.90 ± 41.83^{a}	544.53 ± 10.81^{abc}
CA-A+SG-A	80.40 ± 2.46^{de}	23.38 ± 0.12^{c}	1329.81 ± 76.14^{b}	642.87 ± 36.18^{cd}
AI-M+CA-M	66.90 ± 4.98^{bc}	15.29 ± 0.78^{ab}	2042.94 ± 5.37^{c}	571.10 ± 34.74^{abcd}
AI-M+SG-M	73.39 ± 6.05^{cde}	13.73 ± 0.33^{ab}	4299.38 ± 128.69^{f}	555.87 ± 22.76^{abcd}
CA-M+SG-M	92.68 ± 7.27^{f}	15.49 ± 1.02^{ab}	3347.25 ± 119.36^{e}	706.40 ± 28.12^{e}
Ascorbic acid	-	765.01 ± 19.32	28.91 ± 0.73	24.08 ± 0.80

AI-A = *A. indica* aqueous extract; CA-A = *C. asiatica* aqueous extract; SG-A = *S. grandiflora* aqueous extract; AI-M = *A. indica* methanol extract; CA-M = *C. asiatica* methanol extract; SG-M = *S. grandiflora* methanol extract.
$^{a-g}$ mean values in a column indicate significant differences at $p< 0.05$.

wound closure (89.52 ± 4.03%). For combined extract treatment, the combination of aqueous extract of *A. indica* and *C. asiatica* (AI-A+CA-A) showed the highest percentage of wound closure (90.76 ± 3.62%). A significant migration rate of rabbit corneal epithelial (RCE) cells was observed when treated with *C. asiatica* (aqueous extract) at a concentration of 62.5 parts per million (ppm) (Ruszymah *et al.*, 2012). In another study, Azis *et al.* (2017) showed that methanol fraction of *C. asiatica* extract was non-toxic to human dermal fibroblast (HDF) and human dermal keratinocytes (HaCat), and both cells were able to proliferate at a concentration of 100 µg/mL and 0.19 µg/mL.

A. indica has been widely used in folklore medicine to treat skin problems such as breakouts (pimples), scabies and dermatitis (Rahman *et al.*, 2010; Lingaraju *et al.*, 2013). In recent years, various scientific studies have investigated the wound healing properties of *A. indica*. As shown in Table 4, previous studies have reported significant wound healing activity in rats that received topical application of ethanolic extracts of *A. indica*, via increased rates of wound contraction, improvement of cell proliferation, enhanced TNF-α levels at the early stage of wound healing, and increased expression of type I and type III collagen (Reddy *et al.*, 2002; Ganeshkumar *et al.*, 2012).

Few studies have examined the wound healing activity of *S. grandiflora*, and our results showed that single (74.67%–75.12%) and combined (69.47%–76.60%) extracts of *S. grandiflora* were associated with the lowest percentage of fibroblast migration compared with the other vegetable extracts. Sheikh *et al.* (2011) reported the wound healing activity of an ethanolic extract of *S. grandiflora* flower using an *in vivo* model. In their study, the ethanolic extract was prepared as a 2% and 4% ointment and topically applied to rats. Both concentrations resulted in significant wound closure when compared with the control group (Nitrofurazone ointment, 0.2% [w/w]). However, no possible mechanism was suggested for the wound healing properties of *S. grandiflora*. Previous studies have also reported that both *A. indica* and *C. asiatica* have potent healing abilities (Shukla *et al.*, 1999b; Zahidin *et al.*, 2017). However, to the best of our knowledge, the present study is the first study to report the effects on wound healing when the extracts of these vegetables are combined. Our results indicate that CA-A and AI-A+CA-A can actively recruit cells into the wounded area and enhance proliferation of NHDF.

Antioxidant potential of vegetable extracts

The phenolic content, reducing potential and scavenging properties of the vegetable extracts were therefore evaluated. As indicated in Table 2, the most significant and highest TPC value was detected in the single extract of AI-A (82.94 ± 1.95 mg GAE/g) and the combined extract of CA-M+SG-M (92.68 ± 7.27 mg GAE/g). The aqueous and methanolic extraction methods yielded a comparable level of TPC due to the polarity of the solvent used. Both water and methanol are high polar solvents, meaning that a range of different phytochemical compounds could be extracted from the vegetables (Poovarodom *et al.*, 2010; Dirar *et al.*, 2019). However, TPC values for aqueous (82.94 ± 1.95 mg GAE/g) and methanol (44.79 ± 4.21 mg GAE/g) extracts of *A. indica* were significantly different from each other, while those for aqueous and methanol extracts of *C. asiatica* and *S. grandiflora* were not significantly different. The TPC value for AI-A was comparable with that reported previously by Marwah *et al.* (2007) (72.4 ± 4.9 mg GAE/g).

FRAP, DPPH, and ABTS assays were performed to investigate the antioxidant activities of single and combined vegetable extracts. As shown in Table 2, AI-A had the highest TPC value and thus exhibited the highest reducing potential (61.63 ± 1.48 mM Fe(II)/mg), DPPH

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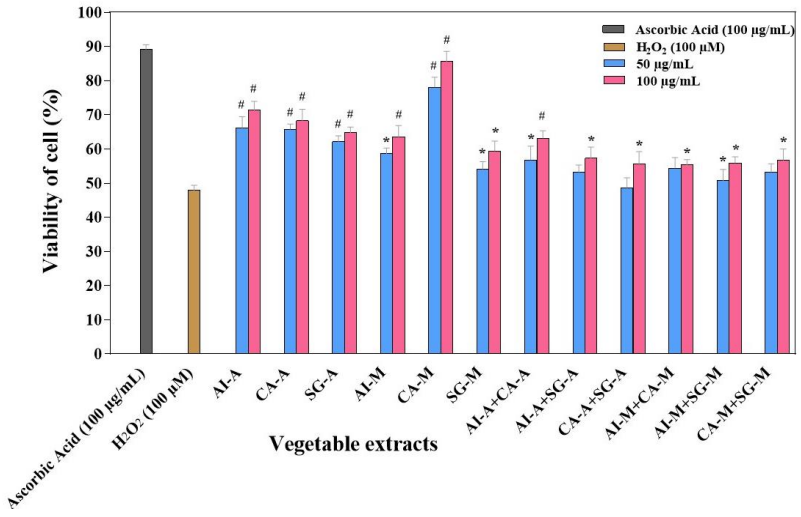

Figure 2: Protective effects of single and combined vegetable extract at 50 and 100 µg/mL. The NHDF cells were treated with the vegetable extracts for 24 h (pre-treatment), incubated in 5% CO_2 at 37 °C. Then, the cells were induced with hydrogen peroxide (H_2O_2) at concentration of 100 µM for 1 h and were assessed via Neutral Red Uptake (NRU) assay at 540 nm wavelength. The results for each point represent the mean ± SD, *n*=3. # *p*< 0.001 and **p*< 0.05 versus negative control (H_2O_2 at 100 µM).

free radical scavenging (IC_{50} = 343.46 ± 44.69 µg/mL), and ABTS free radical scavenging (IC_{50} = 473.70 ± 17.01 µg/mL). Although the combined CA-M+SG-M extract was rich in phenolic content, it did not show the highest antioxidant activities. In fact, it demonstrated low reducing activity (15.49 ± 1.02 mM Fe(II)/mg), low DPPH free radical scavenging activity (3347.25 ± 119.36 µg/mL) and moderate ABTS free radical scavenging activity (706.40 ± 28.12 µg/mL), potentially because synergistic reactions of phenolic compounds and their antioxidant qualities can vary in plants (Negi *et al.*, 2003). While the combination of AI-A+SG-A showed the highest ABTS free radical scavenging activity (IC_{50} = 544.53 ± 10.81 µg/mL), the combination AI-A+CA-A had the highest reducing (49.55 ± 0.93 mM Fe(II)/mg) and DPPH free radical scavenging (IC_{50} = 379.75 ± 16.23 µg/mL) properties. However, the antioxidant potential of the extracts was relatively lower compared with that of the positive control (ascorbic acid). High antioxidant activity is an important factor in wound management. ROS such as H_2O_2 produces free radicals that can cause biological damage by interfering with DNA structure and the subsequent production of proteins essential for wound healing. In these conditions, the antioxidant agents act as a defence mechanism against ROS toxicity and oxidative stress by 'quenching' the excess ROS (Halliwell, 2006). Isa *et al.* (2018) showed that methanol extract of *Plumeria rubra* Linn (kemboja) contains excellent sources of antioxidant compounds such as 3-*O*-caffeyolquinic acid and 5-caffeoquinic acid. Both compounds have phenolic hydroxyl groups that prone to donate a hydrogen atom or an electron to a free radical and extended conjugated aromatic system to delocalize an unpaired electron.

Protective effects of single and combined vegetable extracts

H_2O_2 can promote wound healing by acting as a signalling molecule at lower concentrations. In contrast, high levels of H_2O_2 can delay healing by causing oxidative damage (Loo *et al.*, 2012). In the present study, injury to NHDF cells was induced using 100 µM of H_2O_2. As illustrated in Figure 2, pre-treatment of NHDF cells with single and combined vegetable extracts resulted in significant protection and a markedly higher percentage of viable cells compared to post-treatment of NHDF cells with vegetable extracts (Figure 3). At a concentration of 100 µg/mL, the single extract of CA-M exhibited a significant protective effect with a high percentage of cell viability (85.56 ± 3.01%), followed by AI-A (71.35 ± 2.61%), and was comparable with the positive control (ascorbic acid, 100 µg/ml) used in this study (88.99 ± 1.77%). In the pre-treatment studies, all extract combinations provided cell protection in the range of 55.27%–63.14%. For post-treatment studies, NHDF cell injury was induced using H_2O_2 for 1 h before the addition of extracts. The survival percentage of NHDF cells for all vegetable extracts was between 46.54% and 54.41%. The viability of NHDF cells was lower compared to the pre-treatment tests and insignificant (Figure 3), most probably because H_2O_2 is known to cause oxidative damage to cells (Piah *et al.*, 2010; Adetutu *et al.*, 2011). Sharma *et al.* (2012) demonstrated that phenolic compounds from cucurbits are able to donate electrons to hydrogen peroxide to reduce it to water, thus preventing oxidative stress that causes damage to cells (Schafer and Werner, 2008; Guo and Dipietro, 2010; Loo *et al.*, 2012).

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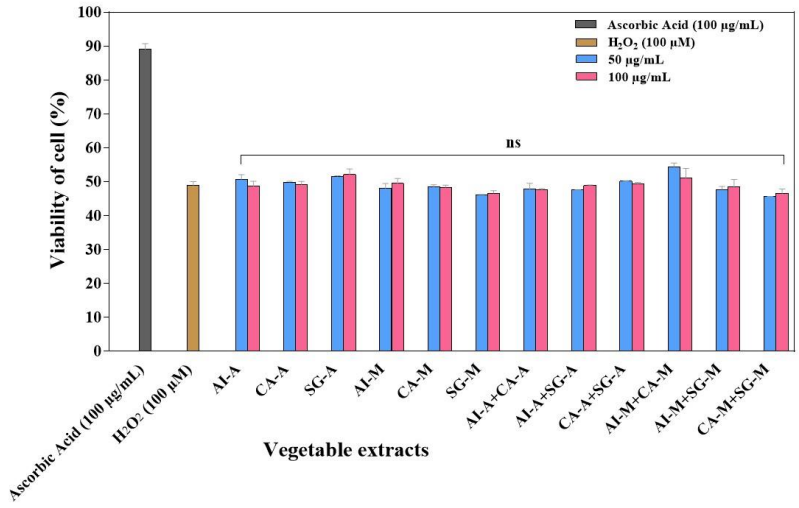

Figure 3: Protective effects of single and combined vegetable extract at 50 and 100 µg/mL. The NHDF cells were induced with hydrogen peroxide (H_2O_2) at concentration 100 µM for 1 h. Then the cells were treated with the vegetable extracts for 24 h (post-treatment), incubated in 5% CO_2 at 37 °C, and were assessed via Neutral Red Uptake (NRU) assay at 540 nm wavelength. The results for each point represent the mean ± SD, *n*=3. ns = not significant versus negative control (H_2O_2 at 100 µM).

Antibacterial properties

An essential element in wound healing is the prevention and management of infection, as infection by pathogenic bacteria (*E. coli*, *Staphylococcus*, haemolytic *Streptococci*, *Bacillus*, *Pseudomonas*, and *Proteus* species) can delay wound repair (Guo and Dipietro, 2010; Han and Ceilley, 2017). In this study, the antibacterial effects of single and combined vegetable extracts were evaluated against seven species of Gram negative and Gram positive bacteria, and the absence or presence of inhibition zones and the zone diameters were used to determine the strength of the antibacterial effects. The inhibition zone diameters are summarized in Table 3. At 800 mg/mL (dose-dependent reaction), AI-A against *E. coli* (12.0 ± 0.4 mm) and *M. luteus* (13.9 ± 0.6 mm), CA-M against *B. cereus* (10.3 ± 1.2 mm)**,** SG-M against *S. marcescens* (13.0 ± 0.5 mm) and *S. aureus* (9.7 ± 0.1 mm), AI-M+CA-M against *B. subtilis* (10.5 ± 0.4 mm), and CA-M+SG-M against *S. epidermidis* (8.5 ± 0.4 mm) demonstrated maximum inhibition zone diameters. Vancomycin (30 µg) was used as positive control and was effective against all tested bacteria except for *S. marcescens*, which is vancomycin-resistant. Interestingly, AI-A, AI-M, CA-M, SG-M, AI-A+CA-A, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M produced inhibition zones at 400–800 mg/mL (6.7–13.0 mm) against *S. marcescens*. From our results, we found that single vegetable extracts exhibited better antimicrobial activities compared to the combined vegetable extracts.

Our data are comparable with those from a previous study by Zahidin *et al.* (2017), which reported that methanol extracts of *A. indica* showed inhibitory activity against Gram positive (*B. cereus*, *B. subtilis*, and *S.* and Gram negative (*E. coli*) bacteria (Table 4). The previous study also reported that aqueous extracts of *A. indica* demonstrated inhibition of *B. subtilis*, *S. aureus*, and *Pseudomonas aeruginosa*. However, information such as inhibition methods and the preparation and concentration of extracts used in the study was not available. Saranraj *et al.* (2010) reported that ethanol and ethyl acetate extract of *A. indica* (100 mg/mL) showed antibacterial properties against *E. coli*, *S. aureus*, *B. cereus*, and *B. subtilis* (diameter zone of inhibition: 11–30 mm). In another study, ethanol and water extracts of *C. asiatica* at 800 mg/mL exhibited inhibition of *S. aureus* ATCC 25923 with a zone of inhibition of 6.44 ± 0.73 mm and 17.16 ± 1.47 mm, respectively, a finding similar to that of the current study except for a lack of inhibition by CA-A against *S. aureus* (Taemchuay *et al.*, 2009). Byakodi *et al.* (2018) reported that a methanol extract of *C. asiatica* was rich in phenolic content including flavonoid (13.2 µg/mL), phenols (43.7 µg/mL), tannins (30.09 µg/mL), and terpenoids (3.08 µg/mL). The extract exhibited antibacterial activities and showed a high zone of inhibition for *M. luteus* (15 mm). In a related study, Liu *et al.* (2015b) investigated the mode of action for the antibacterial properties of asiatic acid, a triterpenoid and one of the active phytochemical groups in *C. asiatica*, against selected foodborne bacterial pathogens such as *E. coli* O157:H7, *Salmonella typhimurium* DT104, *P aeruginosa*, *Listeria monocytogenes*, *S. aureus*, *Enterococcus faecalis*, and *Bacillus cereus*. In their study, the minimal inhibitory concentration (MIC) of asiatic acid against all tested bacteria was 20–40 µg/mL, while the minimal bactericidal concentration (MBC) was 32–52 µg/mL. Further investigation revealed that asiatic acid increased nucleotide leakage and bacterial membrane

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Table 3: Antibacterial activities of single and combined vegetable extracts.

No. / G	Bacteria	Extracts	Concentration of extracts				VANC[c] (30 µg)	Sterile disc[c]
			Diameter zone of inhibition[a,b] (mm)					
			200 mg/mL	400 mg/mL	600 mg/mL	800 mg/mL		
1 (G-)	*Escherichia coli*	AI-A	9.0 ± 0.5	9.9 ± 0.9	10.9 ± 0.9	12.0 ± 0.4	13.5 ± 0.3	6.1 ± 0.1
		AI-M	NZ	6.5 ± 0.3	6.8 ± 0.1	7.5 ± 0.2		
		CA-M	NZ	6.4 ± 0.1	6.6 ± 0.1	6.9 ± 0.2		
		SG-M	6.8 ± 0.4	7.5 ± 0.3	8.9 ± 0.5	10.0 ± 0.5		
		AI-A+CA-A	7.3 ± 0.6	7.8 ± 0.4	8.2 ± 0.4	8.9 ± 0.4		
		AI-A+SG-A	6.7 ± 0.1	8.2 ± 0.5	8.3 ± 0.3	9.3 ± 0.3		
		AI-M+CA-M	NZ	NZ	7.3 ± 0.5	8.1 ± 0.2		
		AI-M+SG-M	7.0 ± 0.4	7.9 ± 0.6	9.0 ± 0.5	10.0 ± 0.3		
		CA-M+SG-M	6.7 ± 0.3	7.5 ± 0.2	8.4 ± 0.4	9.2 ± 0.4		
2 (G-)	*Serratia marcescens*	AI-A	8.9 ± 0.9	9.7 ± 0.9	11.1 ± 0.8	12.2 ± 0.7	6.0 ± 0.0	6.2 ± 0.2
		AI-M	6.6 + 0.1	7.4 ± 0.1	7.8 ±0.3	9.1 ± 0.4		
		CA-M	NZ	6.7 ± 0.1	7.4 + 0.2	12.5 ± 0.8		
		SG-M	6.8 ± 0.1	10.8 ± 0.4	11.7 ± 0.3	13.0 ± 0.5		
		AI-A+CA-A	6.7 ± 0.3	7.3 ± 0.1	7.9 ± 0.2	8.4 ± 0.1		
		AI-M+CA-M	NZ	6.7 ± 0.3	7.2 ± 0.1	8.1 ± 0.2		
		AI-M+SG-M	NZ	6.8 ± 0.4	7.4 ± 0.1	8.8 ± 0.5		
		CA-M+SG-M	NZ	7.3 ± 0.6	8.5 ± 0.9	8.9 ± 0.1		
3 (G+)	*Bacillus subtilis*	AI-M	NZ	6.8 ± 0.1	8.1 ± 0.5	9.2 ± 0.4	16.2 ± 0.4	6.2 ± 0.1
		CA-M	NZ	NZ	6.6 ± 0.1	8.0 ± 0.2		
		SG-M	6.7 ± 0.2	8.8 ± 0.1	9.5 ± 0.2	11.8 ± 0.4		
		AI-M+CA-M	NZ	6.5 ± 0.1	7.6 ± 0.6	10.5 ± 0.4		
		AI-M+SG-M	NZ	NZ	6.7 ± 0.3	7.4 ± 0.4		
		CA-M+SG-M	NZ	NZ	6.9 ± 0.3	8.0 ± 0.1		
4 (G+)	*Bacillus cereus*	CA-A	NZ	6.6 ± 0.1	7.6 ± 0.4	7.9 ± 0.6	15.0 ± 0.2	6.1 ± 0.1
		AI-M	6.6 ± 0.1	7.7 ± 0.3	8.3 ± 0.3	9.9 ± 0.4		
		CA-M	6.4 ± 0.3	7.4 ± 0.8	8.6 ± 1.0	10.3 ± 1.2		
		SG-M	7.5 ± 0.9	8.3 ± 0.9	9.1 ± 0.4	10.1 ± 0.4		
		AI-A+CA-A	7.1 ± 0.2	7.6 ± 0.2	8.3 ± 0.3	8.7 ± 0.3		
		AI-A+SG-A	7.0 ± 0.1	7.3 ± 0.1	7.7 ± 0.2	8.4 ± 0.1		
		AI-M+CA-M	6.6 ± 0.1	6.9 ± 0.1	8.7 ± 0.3	9.9 ± 0.5		
		AI-M+SG-M	7.1 ± 0.9	7.5 ± 1.0	9.0 ± 2.4	9.6 ± 2.5		
5 (G+)	*Staphylococcus aureus*	AI-M	NZ	NZ	6.6 ± 0.1	7.2 ± 0.1	12.4 ± 0.6	6.2 ± 0.1
		CA-M	NZ	NZ	6.4 ± 0.1	6.6 ± 0.1		

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		SG-M	7.2 ± 0.2	7.4 ± 0.3	8.6 ± 0.4	9.7 ± 0.1		
		AI-M+CA-M	6.7 ± 0.2	7.0 ± 0.2	7.3 ± 0.4	8.0 ± 0.5		
		AI-M+SG-M	6.6 ± 0.2	6.8 ± 0.1	7.1 ± 0.3	7.7 ± 0.2		
6 (G+)	*Staphylococcus epidermidis*	SG-M	6.5 ± 0.1	7.7 ± 0.2	8.0 ± 0.1	8.4 ± 0.1		
		AI-A+CA-A	6.6 ± 0.1	7.1 ± 0.2	8.0 ± 0.3	8.4 ± 0.1		
		AI-M+CA-M	NZ	6.6 ± 0.1	7.1 ± 0.5	7.8 ± 0.6	13.4 ± 0.4	6.1 ± 0.1
		AI-M+SG-M	NZ	NZ	6.9 ± 0.2	7.7 ± 0.1		
		CA-M+SG-M	NZ	6.8 ± 0.1	7.7 ± 0.1	8.5 ± 0.4		
7 (G+)	*Micrococcus luteus*	AI-A	7.2 ± 0.4	9.0 ± 0.4	11.0 ± 0.4	13.9 ± 0.6		
		CA-A	6.7 ± 0.4	7.7 ± 0.3	8.2 ± 0.4	8.6 ± 0.4		
		AI-M	6.9 ± 0.2	9.0 ± 0.8	9.2 ± 0.6	11.9 ± 0.6		
		CA-M	NZ	6.7 ± 0.2	7.6 ± 0.4	9.4 ± 0.1		
		SG-M	NZ	6.9 ± 0.3	7.6 ± 0.4	9.4 ± 0.1		
		AI-A+CA-A	6.7 ± 0.2	7.3 ± 0.4	8.1 ± 0.2	9.5 ± 0.4	12.7 ± 0.2	6.2 ± 0.1
		AI-A+SG-A	NZ	NZ	6.8 ± 0.1	8.1 ± 0.3		
		CA-A+SG-A	NZ	NZ	6.7 ± 0.2	7.5 ± 0.2		
		AI-M+CA-M	NZ	6.7 ± 0.1	8.1 ± 0.3	8.8 ± 0.4		
		AI-M+SG-M	6.5 ± 0.1	7.1 ± 0.2	7.6 ± 0.4	8.4 ± 0.6		
		CA-M+SG-M	NZ	6.5 ± 0.3	7.4 ± 0.2	7.8 ± 0.1		

NZ = No Zone; AI-A = *A. indica* aqueous extract; CA-A = *C. asiatica* aqueous extract; SG-A = *S. grandiflora* aqueous extract; AI-M = *A. indica* methanol extract; CA-M = *C. asiatica* methanol extract; SG-M = *S. grandiflora* methanol extract

[a] Values represent the mean ± SD, *n*=3

[b] Sterile disc size used is 6 mm, positive antimicrobial activities were more than 6 mm.

[c] Vancomycin (VANC) and empty sterile disc were used as positive and negative control, respectively.

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Table 4: Comparison of wound healing and antibacterial activities of *A. indica*, *C. asiatica*, and *S. grandiflora* leaf extracts with literature.

Plant	Extract	Wound healing properties (*in vitro* / *in vivo*)	Antibacterial activities							References
			Bacteria							
			EC	SM	BS	BC	SA	SE	ML	
A. indica	Single AE	Percentage of *in vitro* wound closure: 73.14–90.76%	✓	✓	✗	✗	✗	✗	✓	Current study
	Combined AE		✓	✓	✗	✓	✗	✓	✓	
	Single ME	Percentage of *in vitro* wound closure: 67.93–77.07%	✓	✓	✓	✓	✓	✗	✓	
	Combined ME		✓	✓	✓	✓	✓	✓	✓	
	Single EE	Rats applied topically with 10% (w/v) *A. indica* showed complete wound healing at day 14 by enhancing proliferation of fibroblast, synthesis of TNF-α, and expression of collagen type I and III	✓	NA	✓	✓	✓	NA	NA	(Reddy *et al.*, 2002; Saranraj *et al.*, 2010; Ganeshkumar *et al.*, 2012; Zahidin *et al.*, 2017)
	Single EAE	NA	✓	NA	✓	✓	✓	NA	NA	
C. asiatica	Single AE	Percentage of *in vitro* wound closure: 76.70–90.76%	✗	✗	✗	✓	✗	✗	✓	Current study
	Combined AE		✓	✓	✗	✓	✗	✓	✓	
	Single ME	Percentage of *in vitro* wound closure: 69.47–77.57%	✓	✓	✓	✓	✓	✗	✓	
	Combined ME		✓	✓	✓	✓	✓	✓	✓	
	Single AE	A significant increment of migration rate (0.18 µm/h) of rabbit corneal epithelial cells (*in vitro* model) at a concentration up to 62.5 ppm	NA	NA	NA	NA	✓	NA	NA	(Taemchuay *et al.*, 2009; Ruszymah *et al.*, 2012; Azis *et al.*, 2017; Byakodi *et al.*, 2018)
	Single ME	Significant proliferation of fibroblast (HDF) and keratinocytes (HaCaT) at concentration of 0.2 µg/ml and 100 µg/mL	✓	NA	✓	NA	✓	NA	✓	
S. grandiflora	Single AE	Percentage of *in vitro* wound closure: 73.14–76.70%	✗	✗	✗	✗	✗	✗	✗	Current study
	Combined AE		✓	✗	✗	✓	✗	✗	✓	
	Single ME	Percentage of *in vitro* wound closure: 67.93–75.12%	✓	✓	✓	✓	✓	✓	✓	
	Combined ME		✓	✓	✓	✓	✓	✓	✓	
	Single AE	NA	✓	NA	NA	NA	✓	NA	NA	(Sheikh *et al.*, 2011; Das *et al.*, 2013; Arfan *et al.*, 2016)
	Singe EA	2% and 4% of extract ointment were applied on rats and significant wound contraction was observed at day 8.	✓	NA	✓	✓	✓	NA	NA	

ME = Methanol extract; AE = Aqueous extract; EE = Ethanol extract; EAE = Ethyl acetate extract; EC = *E. coli*; SM = *S. marcescens*; BS = *B. subtilis*; BC = *B. cereus*; SA = *S. aureus*; SE = *S. epidermidis*; ML = *M. luteus*

✓= has antibacterial activity; ✗= no antibacterial activity; NA = Not available

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damage in a dose-dependent manner, thus inhibiting the growth of the pathogenic bacteria. Phenolics also showed excellent antibacterial properties. Phenolic compounds extracted from *Paeonia suffruticosa* (common name: tree peony) altered fatty acids in the bacterial membrane and inhibited transcription of virulence factors of *S. aureus* and *E. coli* O157:H7 (Zhou *et al.*, 2019). In a different study, phenolic compounds from *Piper umbellatum* L. (common name: capeba) disrupted permeability of bacterial membranes by increasing absorption of hydrophobic antibiotics and efflux of potassium ion (K^+) thus caused leakage of nucleotides (da Silva *et al.*, 2014).

Our findings show that the methanol extract of *S. grandiflora* (SG-M) has weak wound-healing and antioxidant properties but exhibited strong antibacterial activity against all evaluated Gram negative and Gram positive bacteria. A previous study showed that 500 mg/mL ethanolic extract of *S. grandiflora* produced an inhibition zone of 9.46 mm for *E. coli*, 8.91 mm for *S. aureus*, and 9.81 mm for *P. aeruginosa* (Zarkani, 2016). The result is comparable with our finding in which the methanolic extract of *S. grandiflora* at 600 mg/mL demonstrated an inhibition zone of 8.90 mm against *E. coli* and 8.6 mm against *S. aureus*. In another study, green synthesis of silver nanoparticles (20 µg/mL) from an aqueous extract of the leaves of *S. grandiflora* showed inhibition of *S. aureus* and *Salmonella enterica* with an inhibition zone of 10.54 mm and 15.67 mm, respectively (Das *et al.*, 2013).

However, the tested concentration (200–800 mg/mL) of vegetable extracts and the reported zone of inhibition varied from the previous studies, likely because of differences in the method or solvent used for extraction (Debalke *et al.*, 2018). Our findings indicate that single and combined aqueous and methanol extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* exhibit weak antibacterial properties against pathogenic bacteria that are implicated in wound infection. Any test microbial agent must have a zone of inhibition more than 17.0 mm to be considered as sensitive and potent antimicrobial properties (CLSI, 2020). This is also in agreement with similar work which justifies our range of concentrations (Odunbaku *et al.*, 2008). Several recommendations to verify the antibacterial properties of the vegetable extracts are: i) to test on the other species of bacterial strains, ii) preparation of vegetable extracts with other solvents (wide range of polarity), and iii) antibacterial studies on different fractionates of the vegetable extracts.

Correlation between migration of fibroblast, antioxidant and protective properties

Correlation analyses (Table 5) were done to study the relationship between the rate of fibroblast migration treated with single and combined vegetable extracts with antioxidant properties that were based on TPC (phenolic content), FRAP (reducing potential), DPPH, and ABTS assay (free radical scavenging potential) and protective activities. The coefficient correlation (r) close to 1 or -1 indicates a strong correlation. There was a significant positive correlation between the rate of fibroblast with reducing potential (r = 0.8264). This suggests that antioxidant-reducing activity might contribute to the wound healing activity. Migration of fibroblast showed a moderate positive correlation, but not significant, with protectivity against H_2O_2 (r = 0.4607). Whereas, an inverse relationship (negative correlation) between migration of fibroblast with DPPH (r = -0.5622) and ABTS (r = -0.2272) free radical scavenging properties. Total of phenolic content showed no correlationship (r = 0.0852), thus might be not the main contributor for wound healing.

Table 5: Pearson's correlation coefficients (r) between wound healing and other bioactivities.

		Antioxidant potential				Protectivity against H_2O_2
		TPC	FRAP	DPPH	ABTS	
Wound healing (rate of fibroblast migration)	Pearson's correlation (r)	0.0852	0.8264*	-0.5622	-0.2272	0.4607
	Sig. (2-tailed)	0.792	< 0.001	0.057	0.478	0.132
	n	12	12	12	12	12

*****significant at $p < 0.05$
n= number of pairs

Interactions (synergism, additive, and antagonism) of the combined vegetable extracts

Drug combination study is vital to obtain synergistic therapeutic effect, dose, and reduction of toxicity or side effects, and provide alternative against drug resistance (Chou, 2006). Chou-Talalay theorem provides a quantitative definition for synergism (CI < 1), additive (CI = 1), and antagonism (CI > 1) in the combination of a drug (Chou, 2010). Table 6 summarised the interaction of the combined vegetable extracts based on the calculated CI value. Our results show that only AI-A+CA-A have synergistic effects in accelerating the migration of fibroblast and an excellent source of antioxidant (free radical scavenging activity). The combination of AI-M+CA-M and AI-M+SG-M indicated synergistic interaction to enhance their unique antioxidant properties (reducing activity). Through the interaction studies, we were able to determine that some vegetable extract executed excellent bioactivities independently. For example, CA-M offered the highest protectivity against H_2O_2. While combination of CA-M with other vegetable extract did show significant protective effects (Figure 2), however, no synergistic interaction was seen from by the CI values.

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Table 6: Interaction of the combined vegetable extracts based on the CI value executed via CompuSyn software.

Combination	Fibroblast migration	Antioxidant properties				Protectivity	Antibacterial activity
		TPC	FRAP	DPPH	ABTS		
AI-A+CA-A	CI = 0.8135 (Synergism)	CI = 1.9648 (Antagonism)	CI = 1.0916 (Antagonism)	CI = 0.5590 (Synergism*)	CI = 1.0880 (Antagonism**)	CI = 3.4768 (Antagonism)	CI > 3 (Antagonism)
AI-A+SG-A	CI = 2.3091 (Antagonism)	CI = 1.7640 (Antagonism)	CI = 1.1140 (Antagonism)	CI = 0.5133 (Synergism*)	CI = 1.0536 (Antagonism**)	CI = 6.3234 (Antagonism)	CI > 3 (Antagonism)
CA-A+SG-A	CI = 1.9419 (Antagonism)	CI = 2.0703 (Antagonism)	CI = 1.1640 (Antagonism)	CI = 0.9088 (Synergism*)	CI = 1.1176 (Antagonism**)	CI = 18.8108 (Antagonism)	CI > 3 (Antagonism)
AI-M+CA-M	CI = 1.3363 (Antagonism)	CI = 0.6412 (Synergism)	CI = 0.7921 (Synergism)	CI = 2.2907 (Antagonism)	CI = 1.1602 (Antagonism**)	CI = 5.7917 (Antagonism)	CI > 3 (Antagonism)
AI-M+SG-M	CI = 1.7447 (Antagonism)	CI = 0.9065 (Synergism)	CI = 0.7977 (Synergism)	CI = 1.4554 (Antagonism)	CI = 1.0612 (Antagonism**)	CI = 2.2579 (Antagonism)	CI > 3 (Antagonism)
CA-M+SG-M	CI = 1.6136 (Antagonism)	CI = 1.4778 (Antagonism)	CI = 1.1077 (Antagonism)	CI = 1.3424 (Antagonism)	CI = 1.1707 (Antagonism**)	CI = 4.5909 (Antagonism)	CI > 3 (Antagonism)

AI-A = *A. indica* aqueous extract; CA-A = *C. asiatica* aqueous extract; SG-A = *S. grandiflora* aqueous extract; AI-M = *A. indica* methanol extract; CA-M = *C. asiatica* methanol extract; SG-M = *S. grandiflora* methanol extract.
Synergism (CI < 1), additive (CI = 0), and antagonism (CI > 1).
*Synergistic interaction at a lower dosage (1.25–2.5 µg/mL).
**Antagonistic interaction at tested dosage (250–1500 µg/mL), but as the dosage increased, the CI values become less than 1 (CI < 1) indicate synergism at higher dosage of combined vegetable extracts.

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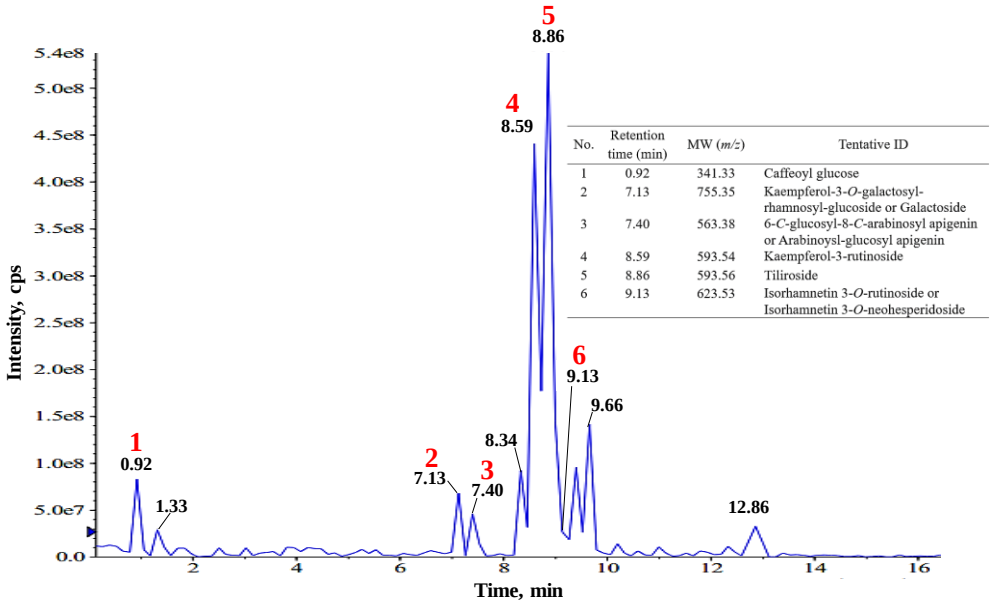
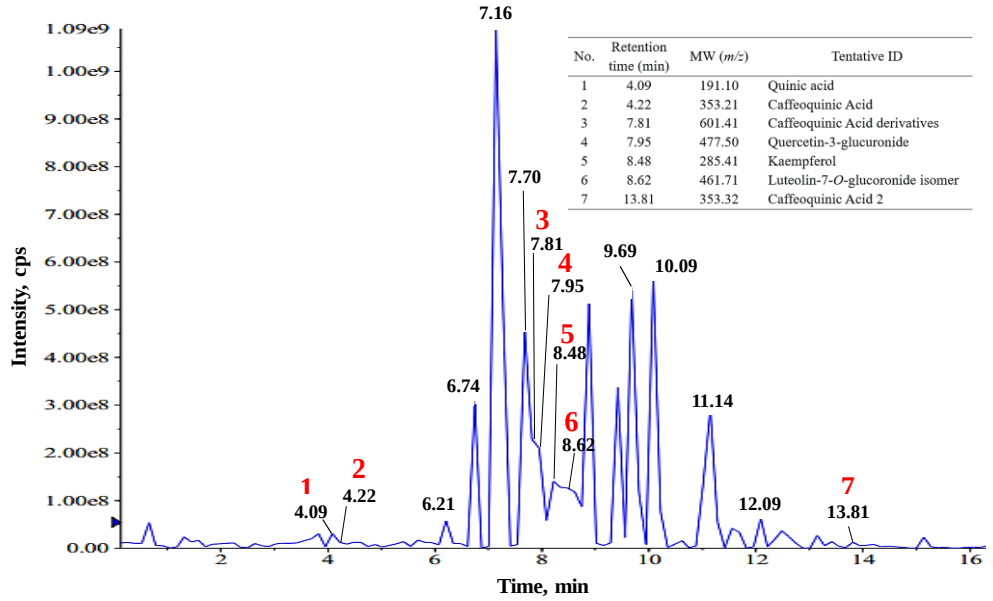


No.	Retention time (min)	MW (*m/z*)	Tentative ID
1	0.92	341.33	Caffeoyl glucose
2	7.13	755.35	Kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside or Galactoside
3	7.40	563.38	6-*C*-glucosyl-8-*C*-arabinosyl apigenin or Arabinoysl-glucosyl apigenin
4	8.59	593.54	Kaempferol-3-rutinoside
5	8.86	593.56	Tiliroside
6	9.13	623.53	Isorhamnetin 3-*O*-rutinoside or Isorhamnetin 3-*O*-neohesperidoside

Figure 4: Chromatogram of the aqueous extract of *A. indica* (AI-A) obtained by using UHPLC-QTrap-MS/MS system in the negative ionic mode.



No.	Retention time (min)	MW (*m/z*)	Tentative ID
1	4.09	191.10	Quinic acid
2	4.22	353.21	Caffeoquinic Acid
3	7.81	601.41	Caffeoquinic Acid derivatives
4	7.95	477.50	Quercetin-3-glucuronide
5	8.48	285.41	Kaempferol
6	8.62	461.71	Luteolin-7-*O*-glucoronide isomer
7	13.81	353.32	Caffeoquinic Acid 2

Figure 5: Chromatogram of the aqueous extract of *C. asiatica* (CA-A) obtained by using UHPLC-QTrap-MS/MS system in the negative ionic mode.

Identification of phytochemical compound by using the LC-MS/MS system

The investigation of the compound was performed using liquid chromatography system, UHPLC-QTrap-MS/MS and UHPLC-TWIMS-QTOF-MS/MS. Identification of compound was based on their molecular ion mass, retention time, and fragmentation pattern. Figure 4 shows the chromatogram for AI-A and six compounds were identified, caffeoyl glucose (1), kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside or galactoside (2), 6-*C*-glucosyl-8-*C*-arabinosyl apigenin or arabinoysl-glucosyl apigenin (3), kaempferol-3-rutinoside (4), tiliroside (5), and isorhamnetin 3-*O*-rutinoside or isorhamnetin 3-*O*-neohesperidoside (6). Identified compounds for CA-A is shown in Figure 5, quinic acid (1), caffeoquinic acid derivatives (2, 3, and 7), quercetin-3-glucuronide (4), kaempferol (5), and luteolin-7-*O*-glucoronide isomer (6).

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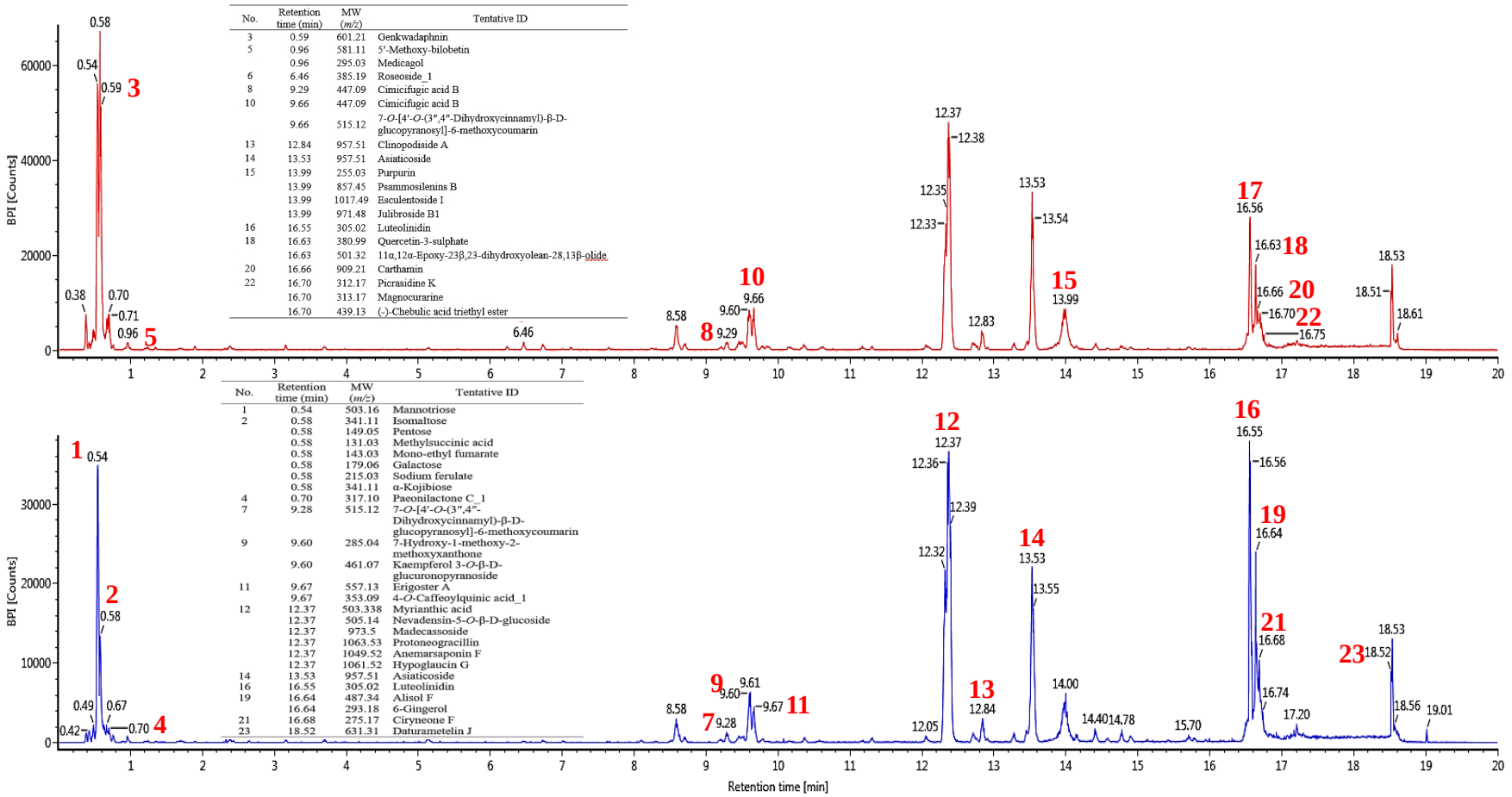

Figure 6: Chromatogram of the methanol extract of *C. asiatica* (CA-M) obtained by using UHPLC-TWIMS-QTOF-MS/MS system.

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Figure 6 shows the full chromatogram for CA-M and 23 compounds were identified: mannotriose (1), isomaltose, pentose, methylsuccinic acid, mono-ethyl fumarate, galactose, sodium ferulate, α-kojibiose (2), genkwadaphnin (3), paeonilactone C1 (4) 5′-methoxy-bilobetin, medicagol (5), roseoside 1 (6), 7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin (7), cimicifugic acid B (8), 7-hydroxy-1-methoxy-2-methoxyxanthone, kaempferol 3-*O*-β-D-glucuronopyranoside (9), 7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin (10), erigoster A (11), myrianthic acid, nevadensin-5-*O*-β-D-glucoside, madecassoside, protoneogracillin, anemarsaponin F, hypoglaucin G (12), clinopodiside A (13), asiaticoside (14), purpurin, psammosilenins B, esculentoside I, julibroside B1 (15), luteolinidin (16), pedunculagin 1, 16-oxo alisol A (17), quercetin-3-sulphate, 11α,12α-Epoxy-23β,23-dihydroxyolean-28,13β-olide (18), alisol F, 6-gingerol (19), carthamin (20), ciryneone F (21), picrasidine K, magnocurarine, (-)-Chebulic acid triethyl ester (22), and daturametelin J (23).

The main general compounds identified in AI-A, CA-A, and CA-M were polyphenols such as quinic acid derivatives, flavonols (kaempferol and quercetin), flavones (luteolin, luteolinidin, and genkwadaphin), polysaccharides (mannotriose, isomaltose, pentose, galactose, and α-kojibiose), and terpenoids (madecassoside and asiaticoside). Phenolic compounds and polysaccharides are potent agents that can contribute to the various bioactivities such as antioxidant (Ammar *et al.*, 2010; Dirar *et al.*, 2019; Güzel *et al.*, 2019), healing process (Dzialo *et al.*, 2016; Ozay *et al.*, 2018), and antimicrobial (Mani *et al.*, 2011; Bouarab-Chibane *et al.*, 2019). Asiaticoside, one of the triterpene compounds isolated from *C. asiatica*, appears to have a role in wound healing via increased type I collagen synthesis and promotion of angiogenesis (Shukla *et al.*, 1999a; Shukla *et al.*, 1999b; Ruszymah *et al.*, 2012; Azis *et al.*, 2017). Further extensive identification of the bioactive compounds should be performed by using 1D and 2D nuclear magnetic resonance (NMR) (Usuldin *et al.*, 2020).

CONCLUSION

This is the first study to investigate the wound-healing and related properties of extracts from three species of vegetables, *A. indica*, *C. asiatica*, and *S. grandiflora*. Our findings show that the combination of AI-A+CA-A demonstrated synergism that stimulated and enhanced the proliferation of NHDF cells and exhibited potent free radical scavenging activities (bifunctional), while AI-M+CA-M and AI-M+SG-M showed synergy for good reducing properties. At 100 µg/mL, CA-M protected NHDF cells against H_2O_2-induced injury, and all single and combined aqueous and methanol extracts exhibited poor antibacterial effects against selected Gram negative and Gram positive bacteria. As some of the vegetables work best on their own, a polyherbal vegetable-based treatment with multiple bioactivities would be beneficial in wound management. Based on our findings, the vegetable combinations could be consumed as supplement (functional food) in the long term to help in wound healing. However, a thorough investigation of the mechanism of action of the vegetable extracts should be performed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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