Malaysian Journal of Microbiology, Vol 16(5) 2020, pp. 382-395
DOI: http://dx.doi.org/10.21161/mjm.190682

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Synergistic-antagonistic interaction of traditional herbs *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora*: In antifungal-demelanising activities and nitric oxide immunomodulatory responses

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Received 19 December 2019; Received in revised form 30 March 2020; Accepted 4 May 2020

ABSTRACT

Aims: The oriental-based herbs *Acalypha indica* (AI), *Centella asiatica* (CA), and *Sesbania grandiflora* (SG) possess a broad range of undisclosed therapeutic activities which are edible and easily available throughout the year. To convert the herb extracts into a potential drug form, aqueous (A) and methanol (M) extracts of herbs were assessed alone and in combination for their antifungal-demelanising activity and nitric oxide (NO) immunomodulatory responses. A new bioactive synergistic and antagonistic assessments approach was made on these herbs to identify which extract combination qualifies as a natural drug candidate.
Methodology and results: Via micro-dilution technique, methanol extract of *A. indica* (AI-M) showed the strongest antifungal activity against *Aspergillus niger*, with a minimum inhibitory concentration (MIC) of 50 mg/mL and a minimum fungicidal concentration (MFC) of 100 mg/mL. Sublethal (50 mg/mL) and subinhibitory (25 mg/mL) doses of AI-M produced the optimal black pigmentation reduction to demelanise *A. niger*. The combinations AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M showed similar antifungal activities (MIC = 100 mg/mL). At 500 µg/mL, CA-A and the combination CA-A+SG-A successfully induced RAW264.7 cells to produce NO at 17.85 µM and 40.84 µM, respectively. The combination of herbs extract showed synergistic interaction towards stimulation of NO production. In contrast, they demonstrated antagonism towards antifungal-demelanising properties. Compound identification of AI-M, SG-M, and SG-A were performed using a UHPLC-QTrap-MS/MS system, which detected phenolic compounds from various groups (cinnamic acids, benzoic acids, and flavonoids).
Conclusion, significance and impact of study: The combination of herb extracts showed better stimulation of NO production while the single herb extracts demonstrated good antifungal-demelanising activity. These findings help in the selection of herbs combination for potential natural drug discovery. A good combination of herbs demonstrated synergism to execute better bioactivities compared to individual herb extracts.

Keywords: Acalypha indica, antifungal activity, *Centella asiatica*, *Sesbania grandiflora*, synergistic interaction

INTRODUCTION

In recent years, cost-effective and accessible alternatives have been increasingly investigated for the treatment of various ailments especially from non-animal functional materials (Wan-Mohtar *et al.*, 2018; Kozarski *et al.*, 2019; Ring *et al.*, 2019; Vunduk *et al.*, 2019). Locally available edible plant materials can provide nutritional and pharmaceutical benefits, and many Asian populations regularly consume healthy and medically important plants such as curry leaf, spinach, mint, and coriander, which are inexpensive and available throughout the year (Raju *et al.*, 2007). Vegetables eaten cooked or raw in salads are considered part of a traditional healthy diet enriched with polyphenols, which are natural sources of antioxidants, vitamins, minerals, and fibre (Ong, 2004; Abas *et al.*, 2006; Sulaiman *et al.*, 2011).

A plant may exhibit unique phytochemical compounds according to their specific species or genera, and traditional practitioners thus combine different plants to obtain maximum therapeutic effectiveness in a process known as polyherbal formulation (Wink, 2003;

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Parasuraman *et al.*, 2014; Baharon *et al.*, 2019; Koh *et al.*, 2019). For example, Triphala is a well-known Ayurvedic polyherbal compound used in skincare and consisting of three myrobylan fruits, *Embelica officinalis*, *Terminalia chebula*, and *T. belerica*. Triphala polyherbal is rich in phenolics such as ellagic acid, gallic acid, and chebulinic acid, and these compounds exhibit bioactive properties such as antioxidant, antipyretic, anti-inflammatory, antibacterial, wound-healing, and anticancer activities (Varma *et al.*, 2016).

Acalypha indica L. (Indian acalypha), *Centella asiatica* (L.) Urb. (pennywort), and *Sesbania grandiflora* (L.) Pers. (hummingbird) are traditional herbs widely consumed in Asia. These herbs have been used in traditional practices for different therapeutic purposes such as wound healing (Marwah *et al.*, 2007; Ganeshkumar *et al.*, 2012; Azis *et al.*, 2017), antimicrobial, antioxidant (Taemchuay *et al.*, 2009; Sivaraj *et al.*, 2014; Byakodi *et al.*, 2018), and anticancer (Park *et al.*, 2005; Sreelatha *et al.*, 2011; Sivaraj *et al.*, 2014) activities. The herbs enriched with phenolic compounds that are responsible for their medicinal values (Arun *et al.*, 2014; Rattanakom and Yasurin, 2015; Zahidin *et al.*, 2017).

There is considerable interest in the use of oriental herbs in the development of microbial drugs, particularly as pathogens continue to develop resistance towards conventional drugs. Liu *et al.* (2016) reported that MCR-1 gene mutation in *Escherichia coli* showed resistance towards synthetic drug peptidic colistins (the drug used as a last resort to kill the pathogen). There is, therefore, a need to identify alternative treatments from medicinal herbs since natural products are one of the important pools for the discovery of new drugs to overcome the antimicrobial resistance and treatment of various human disease (Taemchuay *et al.*, 2009; Atanasov *et al.*, 2015; Newman and Cragg, 2016).

According to Chou (2006), a combination of herbs might be the alternative treatment for single target or disease with multiple modes of action and decrease the development of antimicrobial drug resistance. A single extract of *A. indica*, *C. asiatica*, and *S. grandiflora* has been extensively studied for various bioactivities compared to combined herb extracts. Because of that, we investigated and compared the bioactivities of the single and combined extracts of the selected herbs.

A previous study reported that standard antifungal therapy using voriconazole failed to treat aspergillosis in a kidney transplant patient (Pilmis *et al.*, 2018). In this case, *Aspergillus fumigatus* isolated from the wound acquired azole resistance, thus explaining the treatment failure. Aspergillosis caused by fungi from *Aspergillus* sp. is typically observed in immunocompromised patients including recipients of organ transplants, patients undergoing chemotherapy, and patients with human immunodeficiency virus (HIV) (Lutz *et al.*, 2003; Prasanna *et al.*, 2016). In rare cases, immunocompetent patients are also at risk of developing aspergillosis. One example is the infection of skin lesions with *Aspergillus niger* in an immunocompetent 45-year-old male from Kancheepuram district, India (Prasanna *et al.*, 2016).

Nitric oxide (NO) is a vital factor in mammalian immune systems. It is produced by the conversion of the amino acid L-arginine into NO, catalysed by the NO synthase family of enzymes (Wilson and Garthwaite, 2009). NO serves as a critical signalling molecule in immune cells, specifically macrophages, which play an essential role in the defence mechanism against pathogens (Schairer *et al.*, 2012). By stimulating NO production, the defense system of immunocompetent patients may be improved, thus reducing the risk of pathogen infection. According to Tapsell *et al.* (2006), the consumption of herbs is regarded as necessary for a healthy diet and can boost the immune system, thus the aim of the present study was to determine whether single and combined extracts derived from *A. indica*, *C. asiatica*, and *S. grandiflora* could significantly catalyse the production of NO by RAW264.7. The RAW264.7 cell line is derived from a mouse tumor induced with Abelson leukemia virus and has been widely utilised in the investigation of macrophage cellular physiology to understand the mechanism of inflammation in the human immune system (Maurya *et al.*, 2013). The present study investigated the antifungal-demelanising activity of the extracts, their capacity for NO stimulation, as well as their possible synergy interaction, and sought to identify the compounds responsible for these bioactivities. To the best of our knowledge, this is the first study in which combined extracts from the three species were investigated for their synergism and polyherbal properties.

MATERIALS AND METHODS

Materials

RAW264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC) (Virginia, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) containing 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 µg/mL amphotericin B (PAA Lab, Austria) in a humidified incubator at 37 °C and 5% CO_2. Cell culture 96-well plates and flasks were purchased from Nunc (Denmark). All chemicals and solvents used for analysis were of analytical grade. *A. niger* was obtained from the Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia and used for screening studies. Potato dextrose agar (PDA) (Sigma-Aldrich, Dorset UK) was used for maintenance of the *A. niger* culture at 4 °C (Supramani *et al.*, 2019).

Preparation of herb extracts

Aqueous extraction

Fresh leaves (20 g) of *A. indica* (AI-A), *C. asiatica* (CA-A), and *S. grandiflora* (SG-A) were cut into small pieces by using a sharp blade (<3 mm^2) and were heated separately at 80 °C in 500 mL of distilled water for 1 h. The solutions were filtered through filter paper (Whatman

Malays. J. Microbiol. Vol 16(5) 2020, pp. 382-395
DOI: http://dx.doi.org/10.21161/mjm.190682

No. 1) and the resulting 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 dried in an oven at 35 °C for 3–5 days. The dried leaves were ground into a powder and soaked separately in methanol (MeOH) at a ratio of 1:10 (w/v) for 72 h at room temperature. The MeOH filtrates were collected and excess solvent was evaporated to dryness under reduced pressure using a rotary evaporator at 40 °C, resulting in dark-greenish MeOH extracts. The herb extracts were stored at −20 °C until further use (Azis *et al*., 2017).

Preparation of single and combination herb extracts

Stock solutions of herb aqueous extracts were dissolved in sterile distilled water, filtered, and sterilised through a 0.2 µm filter cap (Orange Scientific, Belgium). Stock solutions of methanol extracts were dissolved in sterile distilled water and filtered via filter paper (Whatman No.1). Combinations of herb extracts were prepared in a 1:1 ratio (v/v) of the stock solution.

Antifungal-demelanising activity of herb extracts

The antifungal-demelanising activity of single and combined herb extracts was evaluated using the micro-dilution technique based on Heleno *et al.* (2013) and Wan-Mohtar *et al.* (2017) with slight modification. Single and combined herb extracts were dissolved in sterile distilled water and added to potato-dextrose broth (Oxoid, UK) containing fungal spore suspensions. Samples were visualised by light microscopy (Leica DM1000), and the lowest extract concentration that resulted in no visible growth (no formation of mycelium) was defined as the MIC.

On the other hand, minimum fungicidal concentrations (MFCs) were identified using a serial sub-cultivation of 2 µL of herb extracts dissolved in medium for 72 h of cultivation and subsequently transferred into 96-well plates containing 100 µL of broth per well. The plates were incubated for 72 h at 28 °C and the lowest concentration of herb extract that resulted in no visible growth was defined as the MFC, representing 99.5% killing of the inoculum. Fluconazole (2 mg/mL) was used as a positive control and 5% of DMSO was used as a negative control in these assessments. The demelanising activity of single and combined herb extracts against *A. niger* was further evaluated in 6-well plates to determine the minimum demelanising concentration (MDC), as according to Wan-Mohtar *et al.* (2017) including the sublethal and subinhibitory concentration necessary to provoke demelanisation in fungus during 72 h. Treated and untreated *A. niger* cultivations were observed by light microscopy (Leica DM1000). 5% of DMSO was used as a negative control. The lowest concentration that resulted in demelanisation of conidia and fungal hyphae was considered the MDC.

Fractional Inhibitory Concentration Indices (FIC) for combination herbs study

The fractional inhibitory concentration indices (FIC) calculated is limited to combinations with two extracts only. FIC index expresses the interaction of two or more agents in which the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently. The FIC was calculated as the MIC of the combination (MIC_{AB}) divided by the MIC of each component extract, as shown by this formula:

$$FIC_{AB} = \frac{MIC_{AB}}{MIC_A} + \frac{MIC_{AB}}{MIC_B}$$

The FIC index was calculated as the sum of each component FIC in a combination and interpreted as either synergistic (≤0.5), additive (0.5-1.0), indifferent (1.0-4.0), or antagonistic (≥4.0) (Schelz *et al*., 2006).

Measurement of nitric oxide production

RAW264.7 cells were detached from the culture flask using a cell scraper and centrifuged at 100 × *g* for 10 min at 4 °C. The cells were then seeded at approximately 5×10^5 cells/mL into a 96-well plate (200 µL of cell suspension in each well) and incubated at 37 °C for 24 h at 5% CO_2. The culture media was replaced with fresh DMEM supplemented with 10% FBS and cells were stimulated with extracts, with 1 µg/mL of lipopolysaccharide (LPS) from *E. coli* 0111:B4 used as a positive control. Cells were subsequently incubated for 24 h at 37 °C and 5% CO_2. Nitric oxide production in the culture medium was measured by the Griess reaction using sodium nitrite as the standard (0-100 µM), and 100 µL of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphtyl) ethylenediamide dihydrochloride) was mixed with 100 µL of cell supernatant. A microplate reader was used to measure the absorbance of samples at 540 nm. Nitrite concentration was expressed as µmol/mL by using the predetermined sodium nitrite calibration curve (Wan-Mohtar *et al.*, 2016).

Synergy behaviour of combined herbs towards stimulation of nitric oxide production

Synergism action of the combined herb extracts was determined via Compusyn software 2.0 (Biosoft, Cambridge) based on combination index (CI) value. CI <1 denoted synergism, CI = 1 denoted additive, and CI >1 denoted antagonism (Chou, 2006; 2010).

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Identification of compounds via UHPLC-QTrap-MS/MS analysis

Compounds present in the herb extracts were identified using a liquid chromatography UHPLC-QTrap-MS/MS system equipped with a 3200-hybrid trap (triple quad tandem mass spectrometer) (Agilent 1100 Series, California, USA). Herb extracts (AI-M, SG-M, and SG-M) were filtered using a 0.45 μM nylon filter prior to injection, and 20 μL of the sample was injected using an autosampler (Agilent 1100 G1313A, California, USA). The samples were run through a C18 column (100 mm × 3 μM × 2.0 mm) (Phenomenex Synergi, California, USA) and elution system (mobile phase) composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Chromatographic separation (in the negative mode) was performed with a gradient system as follows: 10% of solvent B to 90% solvent B (0.01 min to 8.0 min), hold for 3 min, and return to original conditions (10% of solvent B) for 1 min and column re-equilibration for 5 min. Compound identities were confirmed by comparison the mass of molecular ions (*m/z*) to the standard and correlation with the reported literature.

Statistical analysis

Each measurement was carried out in triplicate and results shown as mean ± standard deviation using GraphPad Prism 5 software (Version 5.04). Statistical differences were analysed using one-way analysis of variance (ANOVA) followed by Tukey test.

RESULTS AND DISCUSSION

Antifungal activity

The antifungal activity of single and combined herb extracts is shown in Table 1. Only methanol extracts (single and combined) exhibited antifungal properties, among which AI-M showed the highest and significant (p <0.001) antifungal activity with a MIC of 50 mg/mL and MFC of 100 mg/mL. Single extracts of CA-M and SG-M and combination extracts of AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M had a similar MIC value of 100 mg/mL and MFC values of 200 mg/mL. The MICs for all single and combined aqueous herb extracts were greater than 300 mg/mL. Our findings showed that the MIC and MFC of the single herb extracts were lower than the combination. This explains that the combination of herb extracts exhibited weak or almost similar antifungal effect as the single herb extracts.

Demelanising activity

The demelanising activity of single (Figure 1) and combined (Figure 2) herb methanol extracts was evaluated against *A. niger* at the concentrations of 6.25–200 mg/mL, and the results showed that the demelanising activity was dose-dependent. Greater numbers of black heads (conidiophores) were observed with decreasing herb extract concentration, indicating lower demelanising activity. The results were reported as MDCs, defined as the sublethal and subinhibitory concentrations required to cause demelanisation of fungus over 72 h (Heleno *et al.*, 2013). For single extracts (Figure 1), the subinhibitory and sublethal concentrations for AI-M were 25 mg/mL and 50 mg/mL, respectively. On the other hand, subinhibitory concentrations of 50 mg/mL and sublethal concentrations of 100 mg/mL were observed for CA-M, SG-M, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M. Significant morphological changes and depigmentation associated with melanisation of *A. niger* are shown in Figure 3. Demelanised cultures of tested fungi displayed markedly with fewer heads (Figure 3A and 3B) compared with untreated cultures (Figure 3C). The demelanisation and reduction of heads of *A. niger* were observed by light microscopy (Figure 3D-F).

Table 1: Antifungal activity, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) of single and combined herb extracts (mg/mL).

Extracts	*A. niger*	
	MIC (mg/mL)	MFC (mg/mL)
AI-A	>300	NA
CA-A	>300	NA
SG-A	>300	NA
AI-M	50*	100*
CA-M	100	200
SG-M	100	200
AI-A+CA-A	>300	NA
AI-A+SG-A	>300	NA
CA-A+SG-A	>300	NA
AI-M+CA-M	100	200
AI-M+SG-M	100	200
CA-M+SG-M	100	200
Fluconazole (positive control)	1	2
DMSO (negative control)	NA	

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; NA: Not available
*p<0.001

The green-colored chromoprotein or black-insoluble pigment contained in the heads of conidiophores of some *Aspergillus* and *Penicillium* species belong to the group of melanin pigments (Heleno *et al.*, 2013). Melanin can contribute to fungal virulence and survival by functioning as a 'shield' for fungal (Gómez and Nosanchuk, 2003; Rosa *et al.*, 2010). In the current study, methanol extracts (single and combined) displayed a sublethal and subinhibitory MDC, indicating that they may be directly involved in the suppression or modification of the demelanisation mechanism. The demelanisation results reported here are of significance because the MDC (sublethal) for the fungus was at small doses of extract compared with the inhibitory and fungicidal doses for

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(5) 2020, pp. 382-395
DOI: http://dx.doi.org/10.21161/mjm.190682

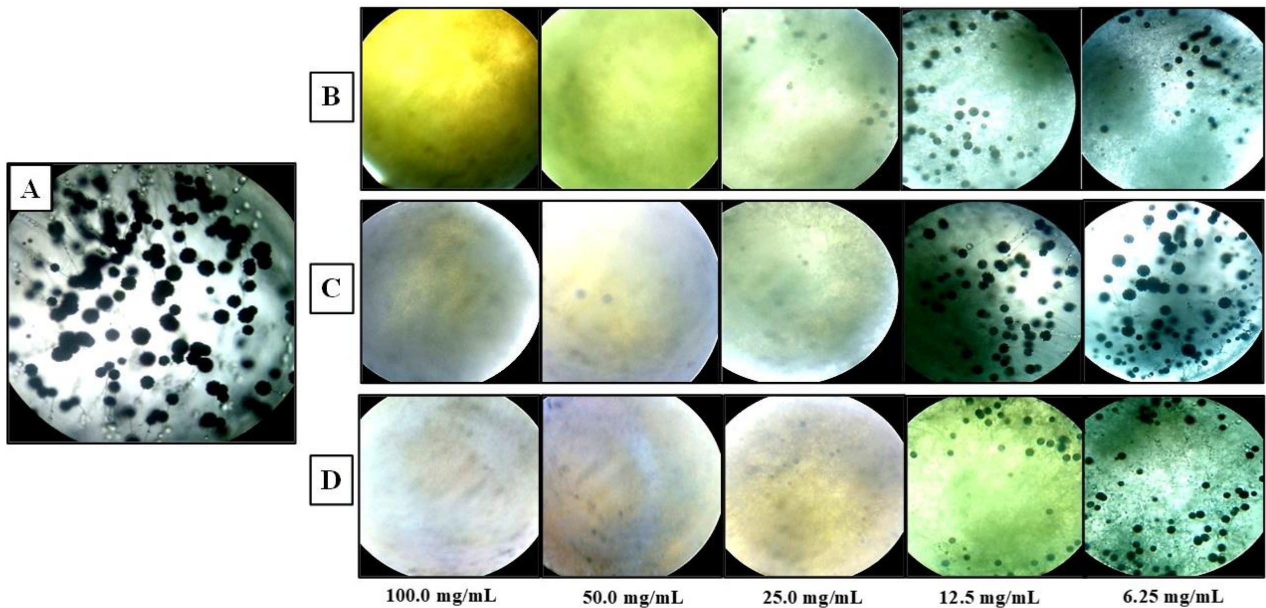

Figure 1: Demelanising properties for single herb extracts at concentration 6.25-100 mg/mL on *A. niger*. (A) Culture without any treatment as control; (B) Culture treated with AI-M; (C) Culture treated with CA-M; and (D) Culture treated with SG-M. *Images were obtained under light microscopy (10× magnification).

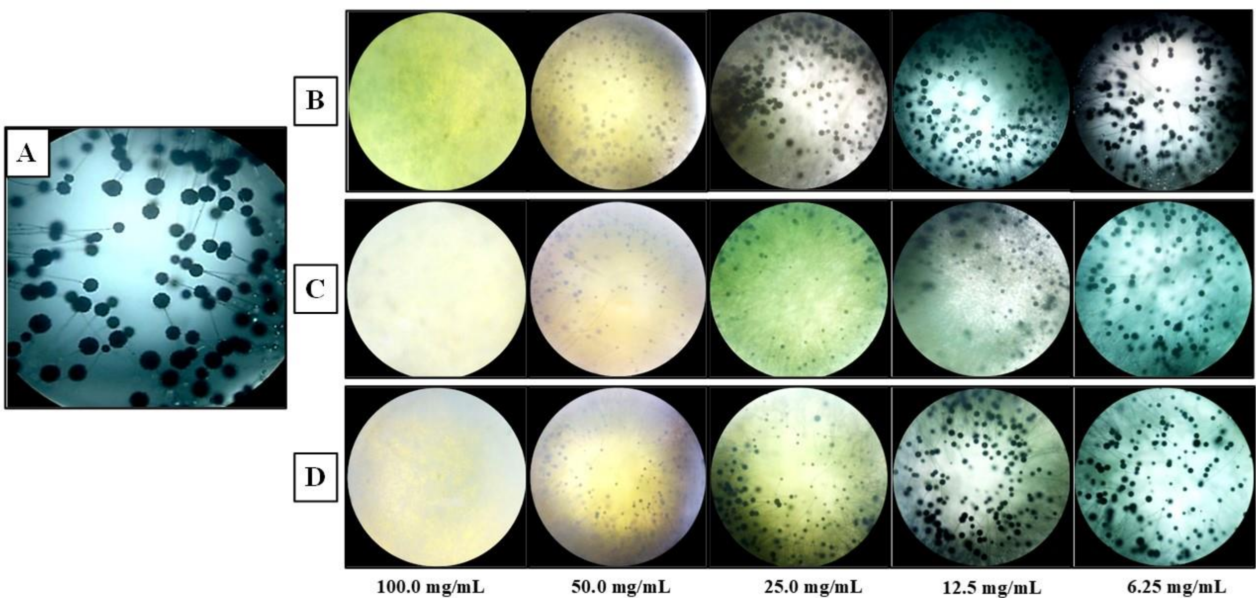

Figure 2: Demelanising properties of combined herb extracts at concentration 6.25-100 mg/mL on *A. niger*. (A) Culture without any treatment as control; (B) Culture treated with AI-M+CA-M; (C) Culture treated with AI-M+SG-M; and (D) Culture treated with CA-M+SG-M. *Images were obtained under light microscopy (10× magnification).

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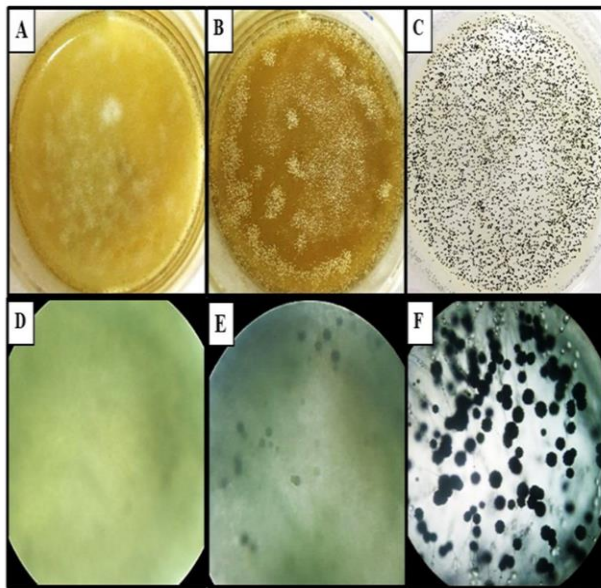

Figure 3: Antifungal-demelanising activity of different concentration of methanol extract of *A. indica* (AI-M). (A) Demelanised mycelium of *A. niger* at 50 mg/mL of AI-M; (B) Mycelium of *A. niger* treated with AI-M at 25 mg/mL; (C) Normal mycelium of *A. niger* without any treatment; (D) Culture of *A. niger* with few number of heads treated with AI-M 100 mg/mL; (E) Culture of *A. niger* with small number of heads treated with 12.5 mg/mL of AI-M; (F) Typical culture of *A. niger* with numerous heads; (D-F) Images were obtained under light microscopy (10× magnification). Lesser black heads (conidiophores) indicates stronger demelanising properties.

each of the tested herb extracts.

Production of nitric oxide by stimulated-RAW264.7 cells

The single and combined herb extracts induced nitric dependent manner in comparison with untreated controls (Figure 4). In general, aqueous extracts stimulated more NO production compared with the methanol extracts. Only AI-A and CA-A significantly (p<0.0001) stimulated NO production. At 100 µg/mL, AI-A and CA-A produced 4.57 µM and 12.36 µM of NO, respectively. At 500 µg/mL, AI-A and CA-A produced 5.52 µM and 17.85 µM of NO production, respectively. AI-M stimulated the production of 3.11 µM NO at 100 mg/mL and 7.66 µM NO at 500 mg/mL. However, no significant NO production was observed when RAW264.7 cells were treated with SG-A, CA-M, or SG-M (1.10–3.13 µM). NO production by single herb extracts was ranked as follows: CA-A > AI-A > AI-M.

Interestingly, all combined herb extracts stimulated NO production by RAW264.7 cells. At 100 µg/mL, only AI-A+CA-A (13.65 µM) and CA-A+SG-A (13.10 µM) significantly stimulated the production of NO. At 500 µg/mL, AI-A+CA-A (38.89 µM) and CA-A+SG-A (40.84 µM) stimulated the highest production of NO followed by AI-A+SG-A (15.03 µM), AI-M+CA-M (7.28 µM), AI-M+SG-M (6.07 µM), and CA-M+SG-M (5.96 µM). RAW264.7 cells treated with the combination of AI-A+CA-A and CA-A+SG-A produced more NO compared with the positive control, LPS (1 µg/mL = 30.28 µM). NO production by combined herb extracts was ranked as follows: CA-A+SG-A > AI-A+CA-A > AI-A+SG-A > AI-M+CA-M > AI-M+SG-M > CA-M+SG-M. From our results, both aqueous and methanol herb extracts showed the different potential of NO stimulatory. Here, the methanol herb extracts displayed weak NO stimulatory compared to the aqueous herbs extracts and this might be due to the different phytochemical compounds present in each herb extract from different extraction solvent (Thouri *et al*., 2017).

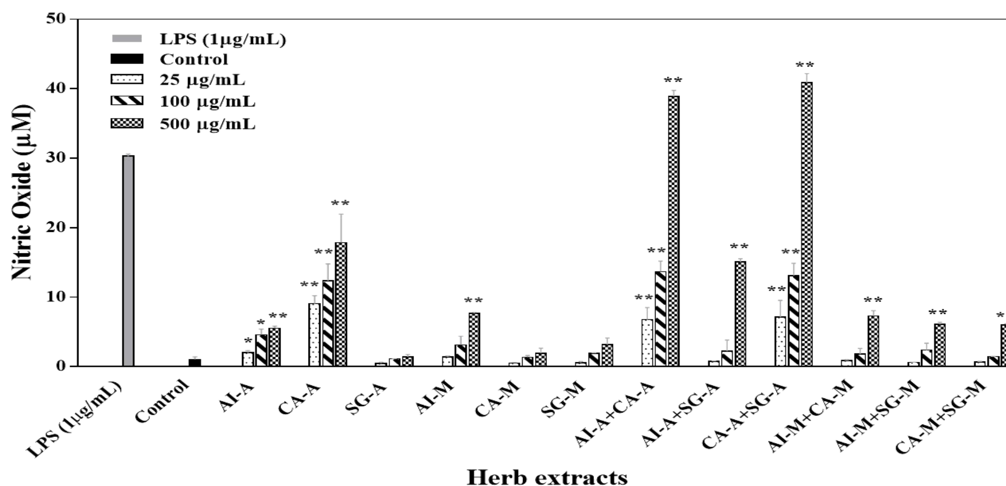

Figure 4: Production of nitric oxide (NO) by RAW 264.7 macrophage cell line treated with single and combined herb extracts. *p<0.001, **p<0.0001 versus positive control (LPS = 1 µg/mL).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(5) 2020, pp. 382-395
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Table 2: Interaction of the combined herb extracts towards the stimulation of nitric oxide (NO) production based on the fractional inhibition concentration (FIC) index and combination index (CI).

Combination	Antifungal		Stimulation of NO		
	FIC value	Interaction	Dose (µg/mL)	CI value	Interaction
AI-A+CA-A	NA	NA	25	0.9638	Synergism
			50	0.5046	
			100	0.0961	
AI-A+SG-A	NA	NA	25	2.4621	Antagonism
			50	1.2000	
			100	0.1501	Synergism
CA-A+SG-A	NA	NA	25	0.7859	Synergism
			50	0.4786	
			100	0.0661	
AI-M+CA-M	3	Indifference	25	1.0177	Antagonism
			50	1.0999	
			100	0.6495	Synergism
AI-M+SG-M	3	Indifference	25	1.5298	Antagonism
			50	0.9938	Synergism
			100	0.9130	
CA-M+SG-M	2	Indifference	25	0.8876	Synergism
			50	0.9080	
			100	0.4895	

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; NA: Not available
Synergism: (FIC ≤0.5) or (CI <1); Additive: (0.5< FIC <1) or (CI =0); Indifferent: (1< FIC <4); Antagonism: (FIC ≥4.0) or (CI >1)

Based on Schairer *et al.* (2012), the function of NO is determined by its concentration. At low concentrations (<1 µM), NO serves as an intracellular signal to control the activity and growth of immune cells. At higher concentrations (>1 µM), NO acts as a ‘pathogen hunter’ in which it covalently binds proteins, DNA, and lipids, thus killing or inhibiting pathogens. Inflammation is a vital phase in the wound healing process in which macrophages remove pathogens regulated by NO. However, excess NO can lead to prolonged inflammation and a delayed healing process. A previous study by Punturee *et al.* (2004) reported both inhibitory and stimulatory activities of ethanol and aqueous extract of *C. asiatica*, respectively, on NO production in J774.2 mouse macrophages. These findings agree with the results of the current study, which shows increased production of NO when RAW264.7 macrophage cells were stimulated with aqueous extracts and no significant effect following treatment with methanol extracts. The RAW264.7 and J774.2 are the *in vitro* model that widely used to study inflammation with different mechanism of action (Cabral *et al.*, 2018). The infected RAW264.7 cells were able to produce more than 30-fold TNF-α (pro-inflammatory cytokines) and induce higher inflammation response compared to the J774.2 cells (Lindmark *et al.*, 2004; El Aamri *et al.*, 2015). Further studies are required to study the mechanism of *A. indica*, *C. asiatica*, and *S. grandiflora* for modulation of NO production to facilitate the development of novel immunomodulatory drugs.

Analysis of synergic behaviour of the combined herb extracts

Table 2 shows the FIC values for antifungal properties of the combined herb extracts. The AI-M+CA-M (FIC = 3), AI-M+SG-M (FIC = 3) and CA-M+SG-M (FIC = 2) demonstrated indifferent interaction between the combined herbs since all the FIC values were more than 1. Our results demonstrated that the execution of antifungal properties of herb extracts was better independently, compared to the combined extracts. Meanwhile, analysis of synergistic interaction of the combined herb extracts on stimulation of NO production also shown in Table 2. As the concentration of extract increased (25, 50, and 100 µg/mL), AI-A+CA-A, CA-A+SG-A, CA-M+SG-M demonstrated synergism (CI <1) at all combination data points. AI-A+SG-A and AI-M+CA-M showed antagonism (CI >1) at 25–50 µg/mL and synergism (CI <1) at 100 µg/mL). AI-M+SG-M displayed antagonistic (CI >1) interaction at 25 µg/mL and synergism (CI <1) at 50–100 µg/mL.

Nowadays, there is an increase of interest in the combination therapy studies for the treatment of diseases such as cancer, acquired immunodeficiency syndrome (AIDS), and pulmonary tuberculosis (Mitchison, 2012; Schrijvers and Debyser, 2012; Che *et al.*, 2013; Maio *et al.*, 2013). Synergistic behaviour analyses of medicinal herbs might lead towards a better formulation of treatment, which reduces the dosage of drugs and eliminate the need of taking more than one drugs at a time (Chou,

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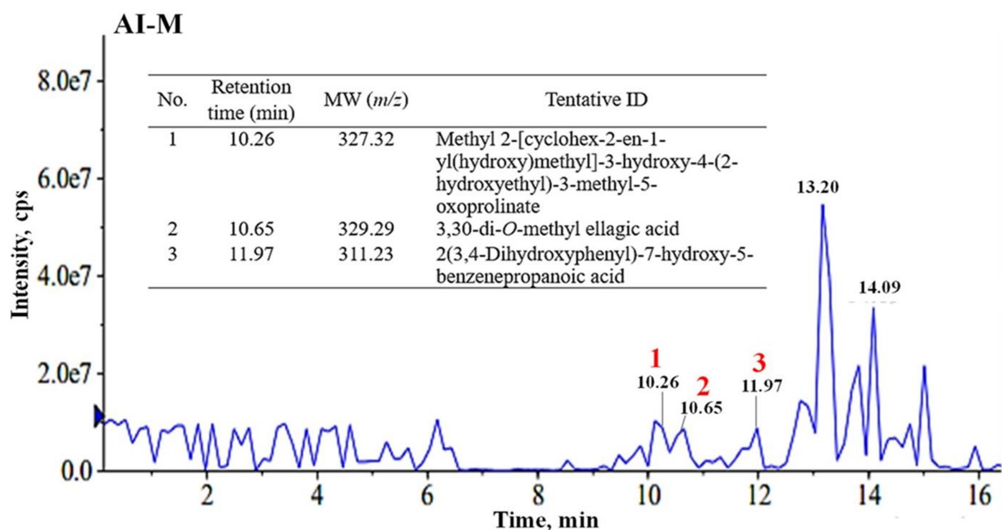

Figure 5: UHPLC chromatogram of methanol extract of *A. indica* (AI-M).

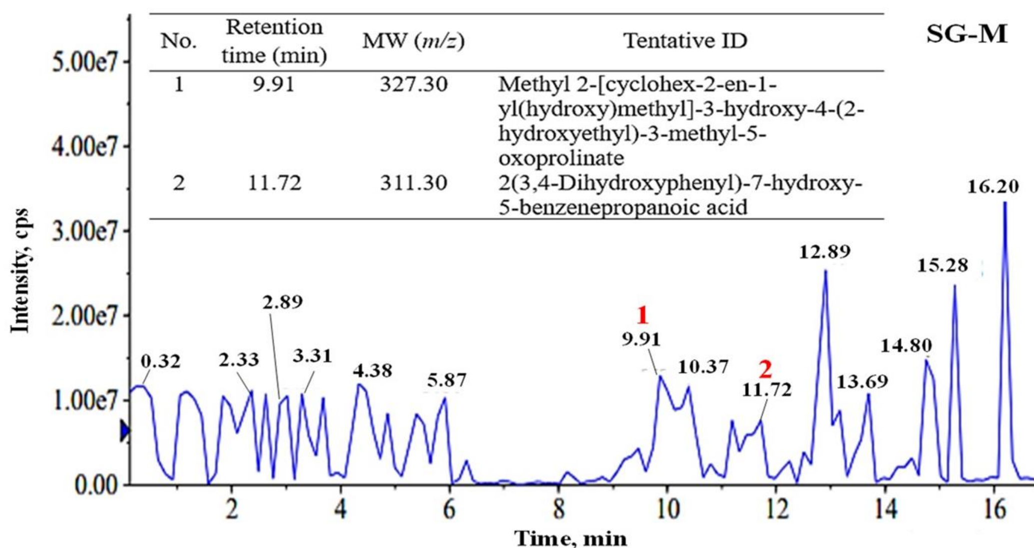

Figure 6: UHPLC chromatogram of methanol extract of *S. grandiflora* (SG-M).

2010; Parasuraman *et al.*, 2014). In this study, the herb extracts have antifungal and NO stimulatory properties hence could be utilized as a supplement to maintain healthy body immune system.

Phytochemical identification using UHPLC-QTrap-MS/MS

Compounds present in the herb extracts were identified using a liquid chromatography UHPLC-QTrap-MS/MS system based on mass of molecular ions, retention time, and fragmentation pattern. In this study, we only identify the compounds in AI-M, SG-M, and SG-A which was significant for both of our antifungal-demelanising and NO stimulation bioactivities. Figure 5 shows the full chromatographic separation of AI-M, with three compounds identified: methyl 2-[cyclohex-2-en-1-yl (hydroxy) methyl]-3-hydroxy-4 -(2-hydroxyethyl)-3-methyl-5-oxoprolinate, 3,30-di-*O*-methyl ellagic acid, and 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid. For SG-M, two compounds were identified (Figure 6) which are methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate and 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid. Meanwhile, SG-A contains caffeoyl glucose, ferulic acid, ferulic acid derivatives, kaempferol-rhamnose-

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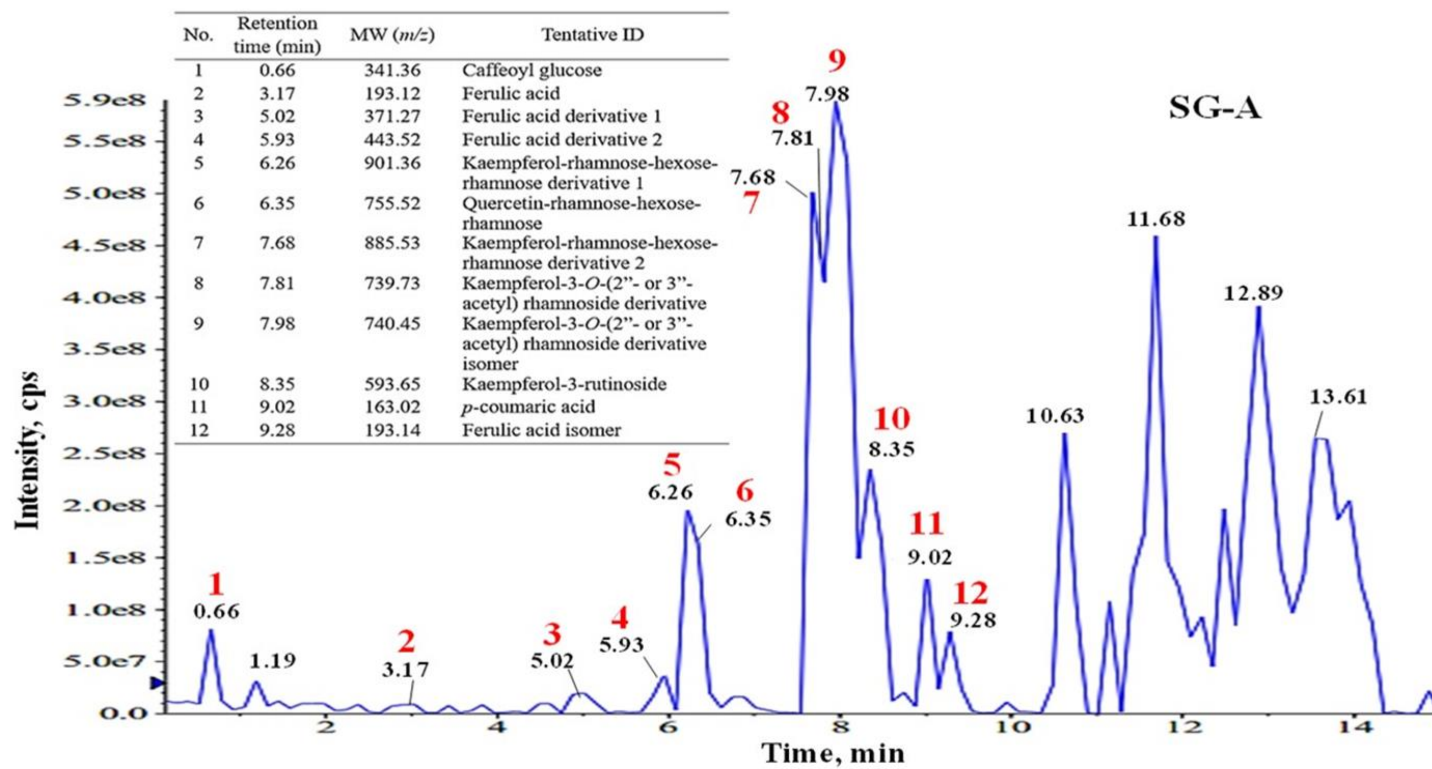
DOI: http://dx.doi.org/10.21161/mjm.190682



No.	Retention time (min)	MW (*m/z*)	Tentative ID
1	0.66	341.36	Caffeoyl glucose
2	3.17	193.12	Ferulic acid
3	5.02	371.27	Ferulic acid derivative 1
4	5.93	443.52	Ferulic acid derivative 2
5	6.26	901.36	Kaempferol-rhamnose-hexose-rhamnose derivative 1
6	6.35	755.52	Quercetin-rhamnose-hexose-rhamnose
7	7.68	885.53	Kaempferol-rhamnose-hexose-rhamnose derivative 2
8	7.81	739.73	Kaempferol-3-*O*-(2''- or 3''-acetyl) rhamnoside derivative
9	7.98	740.45	Kaempferol-3-*O*-(2''- or 3''-acetyl) rhamnoside derivative isomer
10	8.35	593.65	Kaempferol-3-rutinoside
11	9.02	163.02	*p*-coumaric acid
12	9.28	193.14	Ferulic acid isomer

Figure 7: UHPLC chromatogram of aqueous extract of *S. grandiflora* (SG-A).

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(5) 2020, pp. 382-395
DOI: http://dx.doi.org/10.21161/mjm.190682

Table 3: Comparison of antifungal-demelanised, nitric oxide activity, and synergy studies of *A. indica*, *C. asiatica*, and *S. grandiflora* leaves with the literature.

Plant	Extract / compound	Tested fungus	Antifungal MIC (mg/mL)	Demelanising properties	Nitric oxide (µM)	Synergism studies		References
						Antifungal	NO stimulation#	
A. indica	Single ME	*A. niger*	50	+	7.66	NA	NA	Current study
	Combined ME		100	+	6.07 – 7.28	Indifferent	Synergism	
	Single AE		>300	-	5.51	NA	NA	
	Combined AE		>300	-	15.03 – 38.89	NA	Synergism	
	Singe EA	*Aspergillus flavus*	100	NA	NA	NA	NA	(Solomon *et al.*, 2005; Sakthi *et al.*, 2011)
		A. fumigatus	100					
		A. niger	NA					
		Candida albicans	100					
		Candida glabrata	100					
		Penicillium chrysogenum	100					
	Single EAE	*A. flavus*	100	NA	NA	NA	NA	
		A. fumigatus	100					
		A. niger	NA					
		C. albicans	100					
		C. glabrata	NA					
		P. chrysogenum	NA					
C. asiatica	Single ME	*A. niger*	100	+	1.91	NA	NA	Current study
	Combined ME		100	+	5.96 – 7.28	Indifferent	Synergism	
	Single AE		>300	-	17.85	NA	NA	
	Combined AE		>300	-	38.89 – 40.84	NA	Synergism	
	Single ME (whole plant)	*A. niger*	66	NA	<0.2	NA	NA	(Punturee *et al.*, 2004; Restuati and Diningrat, 2018)
		Penicillium expansum	0					
		Fusarium oxysporum	96					
	Single AE (whole plant)	NA	NA	NA	1.6	NA	NA	(Punturee *et al.*, 2004)
S. grandiflora	Single ME	*A. niger*	100	+	3.13	NA	NA	Current study
	Combined ME		100	+	5.96 – 6.07	Indifferent		
	Single AE		>300	-	1.38	NA		
	Combined AE		>300	-	15.03 – 40.84	NA		

AE: Aqueous extract; ME: Methanol extract; EAE: Ethyl acetate extract;
NA: Not available; (+): demonstrate demelanising activity; (-): no demelanising activity
#NO stimulation: interaction at herb extract concentration of 100 µg/mL

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

-hexose-rhamnose derivatives, quercetin-rhamnose-hexose-rhamnose, kaempferol-3-O-(2”- or 3”-acetyl) rhamnoside derivatives, kaempferol-3-rutinoside, p-coumaric acid, and ferulic acid isomer (Figure 7).

From our study, the major compounds in the extracts were phenolic compounds, consisting of various groups such as the simple polyphenols cinnamic acids (ferulic acid and ferulic acid derivatives) and benzoic acids (2 (3, 4-dihydroxyphenyl) -7- hydroxy- 5-benzenepropanoic acid), and flavonoids (kaempferol and quercetin derivatives). Some of the detected compounds were reported by previous studies (Arun *et al*., 2014; Zahidin *et al*., 2017; Supritha and Radha, 2018). It has been reported that plant phenolic compounds have various therapeutic activities such as antimicrobial and immunostimulant properties (Maas *et al*., 1991; Harborne, 1994; Grigore, 2017; Supritha and Radha, 2018). Therefore, in previous studies the identified flavonoids such as 5’-methoxy-bilobetin (Krauze-Baranowska and Wiwart, 2003), kaempferol derivatives, and tiliroside (Zhang *et al*., 2013), quercetin-3-glucoronide (Santos *et al*., 2018), and ferulic acids (Huang *et al*., 1988) may be contributed to the antifungal-demelanising effects against *A. niger*. While kaempferol-3-rutinoside**,** isorhamnetin 3-*O*-rutinoside (Kim *et al*., 2016), genkwadaphnin (Kang *et al*., 2014; Yoo *et al*., 2016), asiaticoside, and madecassoside (Punturee *et al*., 2004) may be responsible for immunostimulatory properties observed in the present study.

Comparison of antifungal demelanising and NO activity of *A. indica*, *C. asiatica*, and *S. grandiflora* with results from previous studies

The current results indicate that methanol extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* have potent antifungal properties in comparison with aqueous extracts, in agreement with the results of previous studies as shown in Table 3. However, comparative analyses of antifungal activity are unavailable as there are very few studies up to date have investigated the antifungal properties of these three herbs. Previous studies by Solomon *et al.* (2005) and Sakthi *et al.* (2011) have reported that ethanol and ethyl acetate extracts of *A. indica* did not show antifungal effects towards *A. niger*. In contrast, AI-M showed inhibitory effects (MIC = 50 mg/mL), suggesting that the compound may be suitable for the treatment of aspergillosis and wound infections caused by *A. niger* (Pasqualotto and Denning, 2006). This discrepancy might be attributable to differences in the extraction method and the variety of phenolic groups and structures responsible for the numerous therapeutic effects of each plant, including antifungal activity (Harborne, 1994). To the best of our knowledge, our study is the first to report antifungal activity for combinations of *A. indica*, *C. asiatica*, and *S. grandiflora* extracts. From the data, the single AI-M extract appeared to exhibit the most potent antifungal-demelanising activity among the evaluated extracts.

Among the three herbs used in this study, the immunomodulatory effects of *C. asiatica* have been extensively studied compared to *A. indica* and *S. grandiflora.* From literature survey, no combination and synergism studies have been done to investigate the immunomodulatory activities of these herbs. As shown in Table 3, a previous study showed that J774.2 mouse macrophages treated with water and methanol extract of *C. asiatica* produced 1.6 μM and <0.2 μM of NO, respectively (Punturee *et al*., 2004). However, in the present study, RAW264.7 mouse macrophages treated with aqueous extract of *C. asiatica* produced increased levels of NO. NO production may vary by cell line and investigated compound or extract. Methanol herb extracts stimulated low NO production compared with aqueous extracts in both studies, and NO production from RAW264.7 cells was increased following treatment with aqueous herb extracts.

CONCLUSION

In conclusion, single and combined herb extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* exhibited considerable antifungal-demelanising effects against *A. niger* and significant immunomodulatory activities in RAW264.7 macrophage cells. These findings suggest that single and combined herb extracts may have the potential for the development of antimicrobial-synergistic and host-immunomodulatory drugs to treat new types of infections. Further studies should be done to study the specific mechanism of these herbs for their antifungal and inflammatory effect.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by a grant from Institute of Research Management and Monitoring (IPPP) University of Malaya with project number PG110-2016A, BKP-073-2017, and FP066-2018A.

REFERENCES

Abas, F., Lajis, N. H., Israf, D., Khozirah, S. and Umi Kalsom, Y. (2006). Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. *Food Chemistry* **95(4), 566-573.**

Arun, A., Karthikeyan, P., Sagadevan, P., Umamaheswari, R. and Peo, R. R. (2014). Phytochemical screening of *Sesbania grandiflora* (Linn). *International Journal of Bioscience and Nanosciences* **1(2), 33-36.**

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E.-M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M. and Stuppner, H. (2015). Discovery and resupply

of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances* **33(8), 1582-1614.**

Azis, H., Taher, M., Ahmed, A., Sulaiman, W., Susanti, D., Chowdhury, S. and Zakaria, Z. (2017). *In vitro* and *in vivo* wound healing studies of methanolic fraction of *Centella asiatica* extract. *South African Journal of Botany* **108, 163-174.**

Baharon, M. N., Zulkifli, N. S. A., Baharom, N. A. and Yunus, S. M. (2019). Antifungal activity of selected plant extracts against *Curvularia* sp. infecting local purple sweet potato (*Ipomoea batatas*). *Malaysian Journal of Microbiology* **15(4), 342-345.**

Byakodi, M. K., Bagewadi, Z. K. and Muddapur, U. M. (2018). Phytoconstituents profiling and evaluation of antimicrobial and antioxidant attributes of methanolic extract of *Centella asiatica*. *Research Journal of Pharmaceutical Biological and Chemical Sciences* **9(3), 493-500.**

Cabral, G. R. A., Wang, Z. T., Sibley, L. D. and DaMatta, R. A. (2018). Inhibition of nitric oxide production in activated macrophages caused by *Toxoplasma gondii* infection occurs by distinct mechanisms in different mouse macrophage cell lines. *Frontiers in Microbiology* **9, 1-11.**

Che, C. T., Wang, Z. J., Chow, M. S. and Lam, C. W. (2013). Herb-herb combination for therapeutic enhancement and advancement: Theory, practice and future perspectives. *Molecules* **18(5), 5125-5141.**

Chou, T. C. (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews* **58(3), 621-681.**

Chou, T. C. (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Research* **70(2), 440-446.**

El Aamri, F., Remuzgo-Martinez, S., Acosta, F., Real, F., Ramos-Vivas, J., Icardo, J. M. and Padilla, D. (2015). Interactions of *Streptococcus iniae* with phagocytic cell line. *Microbes and Infection* **17(4), 258-265.**

Ganeshkumar, M., Ponrasu, T., Krithika, R., Iyappan, K., Gayathri, V. S. and Suguna, L. (2012). Topical application of *Acalypha indica* accelerates rat cutaneous wound healing by up-regulating the expression of Type I and III collagen. *Journal of Ethnopharmacology* **142(1), 14-22.**

Gómez, B. L. and Nosanchuk, J. D. (2003). Melanin and fungi. *Current Opinion in Infectious Diseases* **16(2), 91-96.**

Grigore, A. (2017). Plant phenolic compounds as immunomodulatory agents. *In:* Phenolic Compounds: Biological Activity. Soto-Hernandez, M., Palma-Tenango, M. and Garcia-Mateos, M. R. (eds.). IntechOpen, London, UK. **pp. 75-98.**

Harborne, J. B. (1994). Do natural plant phenols play a role in ecology? *Acta Horticulturae* **381, 36-43.**

Heleno, S. A., Ferreira, I. C., Esteves, A. P., Ciric, A., Glamoclija, J., Martins, A., Sokovic, M. and Queiroz, M. J. (2013). Antimicrobial and demelanizing activity of *Ganoderma lucidum* extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. *Food and Chemical Toxicology* **58, 95-100.**

Huang, M. T., Smart, R. C., Wong, C. Q. and Conney, A. H. (1988). Inhibitory effect of curcumin, chlorogenic acid, caffeic adic, and ferulic acid on tumor promotion in mouse skin by 12-*O* tetradecanoylphorbol-13-acetate. *Cancer Research* **48(21), 5941-5946.**

Kang, H. B., Ahn, K. S., Oh, S. R. and Kim, J. W. (2014). Genkwadaphnin induces IFN-gamma via PKD1/NF-kappa B/STAT1 dependent pathway in NK-92 cells. *PLoS ONE* **9(12), e115146.**

Kim, G. T., Tran, N. K. S., Choi, E. H., Song, Y. J., Song, J. H., Shim, S. M. *et al.* (2016). Immunomodulatory efficacy of standardized *Annona muricata* (Graviola) leaf extract via activation of mitogen-activated protein kinase pathways in RAW 264.7 macrophages. *Evidence-Based Complementary and Alternative Medicine* **2016, Article ID 2905127.**

Koh, S. P., Sharifudin, S. A., Abdullah, R., Hamid, N. S. A., Mirad, R. and Mustaffa, R. (2019). Antimicrobial efficacy of fermented mango leaves beverage towards selected foodborne pathogens. *Malaysian Journal of Microbiology* **15(4), 320-326.**

Kozarski, M., Klaus, A., Jakovljević, D., Todorović, N., Wan Abd Al Qadr Imad, W.-M. and Nikšić, M. (2019). *Ganoderma lucidum* as a cosmeceutical: Antiradical potential and inhibitory effect on hyperpigmentation and skin extracellular matrix degradation enzymes. *Archives of Biological Sciences* **71(2), 253-264.**

Krauze-Baranowska, M. and Wiwart, M. (2003). Antifungal activity of biflavones from *Taxus baccata* and *Ginkgo biloba*. *Journal of Biosciences* **58(1-2), 65-69.**

Krishnan, P. (2005). *In vitro* wound healing effects of single and combined African herbal medicines. PhD. Thesis. Durham University, UK.

Lindmark, H., Rosengren, B., Hurt-Camejo, E. and Bruder, C. E. (2004). Gene expression profiling shows that macrophages derived from mouse embryonic stem cells is an improved *in vitro* model for studies of vascular disease. *Experimental Cell Research* **300(2), 335-344.**

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., Doi, Y., Tian, G. B., Dong, B. L., Huang, X. H., Yu, L. F., Gu, D. X., Ren, H. W., Chen, X. J., Lv, L. C., He, D. D., Zhou, H. W., Liang, Z. S., Liu, J. H. and Shen, J. Z. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infectious Diseases* **16(2), 161-168.**

Lutz, B. D., Jin, J., Rinaldi, M. G., Wickes, B. L. and Huycke, M. M. (2003). Outbreak of invasive *Aspergillus* infection in surgical patients, associated with a contaminated air-handling system. *Clinical Infectious Diseases* **37(6), 786-793.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Maas, J. L., Galletta, G. J. and Stoner, G. D. (1991). Ellagic acid, an anticarcinogen in fruits, especially in strawberries: A review. *HortScience* **26(1), 10-14.**

Maio, M. D., Costanzo, R., Giordano, P., Piccirillo, M. C., Sandomenico, C., Montanino, A., Carillio, G., Muto, P., Jones, D. R. and Daniele, G. (2013). Integrated therapeutic approaches in the treatment of locally advanced non-small cell lung cancer. *Anti-Cancer Agents in Medicinal Chemistry* **13(6), 844-851.**

Marwah, R. G., Fatope, M. O., Al Mahrooqi, R., Varma, G. B., Al Abadi, H. and Al-Burtamani, S. K. S. (2007). Antioxidant capacity of some edible and wound healing plants in Oman. *Food Chemistry* **101(2), 465-470.**

Maurya, M. R., Gupta, S., Li, X., Fahy, E., Dinasarapu, A. R., Sud, M., Brown, H. A., Glass, C. K., Murphy, R. C. and Russell, D. W. (2013). Analysis of inflammatory and lipid metabolic networks across RAW264.7 and thioglycolate-elicited macrophages. *Journal of Lipid Research* **54(9), 2525-2542.**

Mitchison, D. A. (2012). Prevention of drug resistance by combined drug treatment of tuberculosis. *In:* Antibiotic Resistance. Handbook of Experimental Pharmacology. Coates, A. (eds.). Springer, Berlin, Heidelberg. **pp. 87-98.**

Newman, D. J. and Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. *Journal of Natural Products* **79(3), 629-661.**

Ong, H. C. (2004). Buah: Khasiat makanan & ubatan. Utusan Publications, Malaysia.

Parasuraman, S., Thing, G. S. and Dhanaraj, S. A. (2014). Polyherbal formulation: Concept of ayurveda. *Pharmacognosy Reviews* **8(16), 73-80.**

Park, B. C., Bosire, K. O., Lee, E.-S., Lee, Y. S. and Kim, J.-A. (2005). Asiatic acid induces apoptosis in SK-MEL-2 human melanoma cells. *Cancer Letters* **218(1), 81-90.**

Pasqualotto, A. and Denning, D. (2006). Post-operative aspergillosis. *Clinical Microbiology and Infection* **12(11), 1060-1076.**

Pilmis, B., Garcia-Hermoso, D., Alanio, A., Catherinot, E., Scemla, A., Jullien, V., Bretagne, S. and Lortholary, O. (2018). Failure of voriconazole therapy due to acquired azole resistance in *Aspergillus fumigatus* in a kidney transplant recipient with chronic necrotizing aspergillosis. *American Journal of Transplantation* **18(9), 2352-2355.**

Prasanna, S., Jayakumar, K. and Jayashree, V. (2016). Primary cutaneous aspergillosis-Tinea Pedis caused by *Aspergillus niger* in an immunocompetent adult individual residing in silk city of Kancheepuram district. *International Journal of Advanced Research* **4(9), 443-446.**

Punturee, K., Wild, C. P. and Vinitketkumneun, U. (2004). Thai medicinal plants modulate nitric oxide and tumor necrosis factor-α in J774.2 mouse macrophages. *Journal of Ethnopharmacology* **95, 183-189.**

Raju, M., Varakumar, S., Lakshminarayana, R., Krishnakantha, T. P. and Baskaran, V. (2007). Carotenoid composition and vitamin A activity of medicinally important green leafy vegetables. *Food Chemistry* **101(4), 1598-1605.**

Rattanakom, S. and Yasurin, P. (2015). Chemical profiling of *Centella asiatica* under different extraction solvents and its antibacterial activity, antioxidant activity. *Oriental Journal of Chemistry* **31(4), 2453-2459.**

Restuati, M. and Diningrat, D. S. (2018). Antimicrobial profile of *Premna pubescens*. Blume and *Centella asiatica* extracts against bacteria and fungi pathogens. *International Journal of Pharmacology* **14(2), 271-275.**

Ring, L. C., Yenn, T. W., Wahidin, S., Tan, W. N., Abdullah, S. Z. B., Jamil, N. A. M. and Rahman, M. S. A. (2019). Antimicrobial wound dressing film utilizing nano-cellulose and gelatin as drug delivery system for *Garcinia mangostana* L. extract. *Malaysian Journal of Microbiology* **15(5), 373-378.**

Rosa, L. H., Almeida Vieira, M. d. L., Santiago, I. F. and Rosa, C. A. (2010). Endophytic fungi community associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) in Antarctica. *FEMS Microbiology Ecology* **73(1), 178-189.**

Sakthi, S. S., Geetha, M. and Saranraj, P. (2011). Pharmacological screening of *Datura metel* and *Acalypha indica* for its antifungal activity against pathogenic fungi. *International Journal of Pharmaceutical Science and Health Care* **2(1), 15-30.**

Santos, M. C., Farias, L. S., Merlugo, L., de Oliveira, T. V., Barbosa, F. S., Fuentefria, A. M., Henriques, A. T., Garcia, C. V. and Mendez, A. S. L. (2018). UPLC-MS for identification of quercetin derivatives in *Cuphea glutinosa* Cham. & Schltdl (Lythraceae) and evaluation of antifungal potential. *Current Pharmaceutical Analysis* **14(6), 586-594.**

Schairer, D. O., Chouake, J. S., Nosanchuk, J. D. and Friedman, A. J. (2012). The potential of nitric oxide releasing therapies as antimicrobial agents. *Virulence* **3(3), 271-279.**

Schelz, Z., Molnar, J. and Hohmann, J. (2006). Antimicrobial and antiplasmid activities of essential oils. *Fitoterapia* **77(4), 279-285.**

Schrijvers, R. and Debyser, Z. (2012). Combinational therapies for HIV: A focus on EVG/COBI/FTC/TDF. *Expert Opinion on Pharmacotherapy* **13(13), 1969-1983.**

Sivaraj, R., Rahman, P. K., Rajiv, P., Narendhran, S. and Venckatesh, R. (2014). Biosynthesis and characterization of *Acalypha indica* mediated copper oxide nanoparticles and evaluation of its antimicrobial and anticancer activity. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **129, 255-258.**

Solomon, R. D. J., Kallidass, S. and Vimalan, J. (2005). Isolation, identification and study of antimicrobial property of a bioactive compound in an indian medicinal plant *Acalypha indica* (Indian-nettle). *World Journal of Microbiology and Biotechnology* **21(6-7), 1231-1236.**

Sreelatha, S., Padma, P. R. and Umasankari, E. (2011). Evaluation of anticancer activity of ethanol extract of *Sesbania grandiflora* (Agati Sesban) against Ehrlich ascites carcinoma in Swiss albino mice. *Journal of Ethnopharmacology* **134(3), 984-987.**

Sulaiman, S. F., Sajak, A. A. B., Ooi, K. L. and Seow, E. M. (2011). Effect of solvents in extracting polyphenols and antioxidants of selected raw vegetables. *Journal of Food Composition and Analysis* **24(4-5), 506-515.**

Supramani, S., Rahayu Ahmad, Z. I., Annuar, M. S. M., Klaus, A. and Wan, W. A. A. Q. I. (2019). Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified *Ganoderma lucidum* strain QRS 5120 using response surface methodology. *AIMS Microbiology* **5(1), 1-19.**

Supritha, P. and Radha, K. V. (2018). Estimation of phenolic compounds present in the plant extracts using high pressure liquid chromatography, antioxidant properties and its antibacterial activity. *Indian Journal of Pharmaceutical Education and Research* **52(2), 321-326.**

Taemchuay, D., Rukkwamsuk, T., Sakpuaram, T. and Ruangwises, N. (2009). Antibacterial activity of crude extracts of *Centella asiatica* against *Staphylococcus aureus* in bovine mastitis. *Journal Veterinary Medicine of Kasetsart University* **19(3), 119-128.**

Tapsell, L. C., Hemphill, I., Cobiac, L., Patch, C. S., Sullivan, D. R., Fenech, M., Roodenrys, S., Keogh, J. B., Clifton, P. M., Williams, P. G., Fazio, V. A. and Inge, K. E. (2006). Health benefits of herbs and spices: The past, the present, the future. *Medical Journal of Australia* **185(4), 4-24.**

Thouri, A., Chahdoura, H., El Arem, A., Hichri, A. O., Ben Hassin, R. and Achour, L. (2017). Effect of solvents extraction on phytochemical components and biological activities of Tunisian date seeds (var. Korkobbi and Arechti). *BMC Complementary and Alternative Medicine* **17, 1-10.**

Varma, S. R., Sivaprakasam, T. O., Mishra, A., Kumar, L. M., Prakash, N. S., Prabhu, S. and Ramakrishnan, S. (2016). Protective effects of triphala on dermal fibroblasts and human keratinocytes. *PLoS ONE* **11(1), e0145921.**

Vunduk, J., Wan-Mohtar, W. A. A. Q. I., Mohamad, S. A., Abd Halim, N. H., Mohd Dzomir, A. Z., Žižak, Ž. and Klaus, A. (2019). Polysaccharides of *Pleurotus flabellatus* strain Mynuk produced by submerged fermentation as a promising novel tool against adhesion and biofilm formation of foodborne pathogens. *LWT,* **112, 108221.**

Wan-Mohtar, W. A. A. Q. I., Mahmud, N., Supramani, S., Ahmad, R., Zain, N. A. M., Hassan, N. A., Peryasamy, J. and Halim-Lim, S. A. (2018). Fruiting-body-base flour from an oyster mushroom - a waste source of antioxidative flour for developing potential functional cookies and steamed-bun. *AIMS Agriculture and Food* **3(4), 481-492.**

Wan-Mohtar, W. A. A. Q. I., Viegelmann, C., Klaus, A. and Lim, S. A. H. (2017). Antifungal-demelanizing properties and RAW264.7 macrophages stimulation of glucan sulfate from the mycelium of the mushroom *Ganoderma lucidum. Food Science and Biotechnology* **26(1), 159-165.**

Wan-Mohtar, W. A. A. Q. I., Young, L., Abbott, G. M., Clements, C., Harvey, L. M. and McNeil, B. (2016). Antimicrobial properties and cytotoxicity of sulfated (1,3)-beta-D-glucan from the mycelium of the mushroom *Ganoderma lucidum*. *Journal of Microbiology and Biotechnology* **26(6), 999-1010.**

Wilson, G. and Garthwaite, J. (2009). Nitric oxide. *Encyclopedia of Neuroscience*, **1151-1156.**

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64(1), 3-19.**

Yoo, N., Lee, H. R., Son, J. M., Kang, H. B., Lee, H. G., Yoon, S. R., Yoon, S. Y. and Kim, J. W. (2016). Genkwadaphnin promotes leukocyte migration by increasing CD44 expression via PKDI/NF-kappa B signaling pathway. *Immunology Letters* **173, 69-76.**

Zahidin, N. S., Saidin, S., Zulkifli, R. M., Muhamad, I. I., Ya'akob, H. and Nur, H. (2017). A review of *Acalypha indica* L. (Euphorbiaceae) as traditional medicinal plant and its therapeutic potential. *Journal of Ethnopharmacology* **207, 146-173.**

Zhang, Q. H., Fan, D., Xiong, B. J., Kong, L. B. and Zhu, X. D. (2013). Isolation of new flavan-3-ol and lignan glucoside from *Loropetalum chinense* and their antimicrobial activities. *Fitoterapia,* **90, 228-232.**