Current Trends in Biotechnology and Pharmacy
Vol. 18 (Supplementry Issue 3s) 177 - 190, July-Sept 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2024.3s.13

Extraction and evaluation of Hemiparasitic shrub of *Scurrula ferruginea* leaves: Phytochemical Analysis, Antioxidant, Antimicrobial and Cytotoxic Activities

Ya Chee Lim[1]*, Natasha Keasberry[2], Rajan Rajabalaya[1], Shirley Huan Fang Lee[1], Ihsan Nazurah Zulkipli[1], Anne Catherine Cunningham[1], Farah Abbas[2], Meng Wee Lim[1], Seow Chin Chong[1], Nur Atiqah Mohammad Sahrul Masdi[1], Muhammad Saifullah Bin Sufri[1], Muhammad Norhazim bin Hashim[1] and Sheba R David[1,3]

[1]PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, BE 1410 Gadong, Brunei Darussalam

[2]Faculty of Science, Universiti Brunei Darussalam, Jalan Tungku Link, BE 1410 Gadong, Brunei Darussalam

[3]School of Pharmacy, University of Wyoming, Laramie, Wyoming, 82071, USA

*Corresponding author: yachee.lim@ubd.edu.bn

Abstract

Scurrula ferruginea (SF), a tropical obligate hemiparasitic shrub found on branches of dicotyledonous trees in Southeast Asia, has been used by indigenous people as a medicinal herb. This study aimed to evaluate the antioxidant, antibacterial, and lymphocyte proliferation of *SF* extracts and characterize their bioactive compounds.Leaves of *Scurrula ferruginea* were pulverized into powder followed by extraction method. Total phenolic content and total antioxidant activities were measured. Disc diffusion and well diffusion assays to investigate the antibacterial properties of the extracts against *Vibrio parahaemolyticus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa.* The crude extract was shown to contain phenolic compounds and possessed antioxidant abilities. Preliminary investigations revealed the presence of long-chain alcohols, phytol (diterpene alcohol and a constituent of chlorophyll); squalene, (a triterpene and precursor to steroids); and lupeol (a triterpene) which may contribute to the biological activities of this plant. Aqueous extracts of *SF* (2.5 mg/disc) showed the highest inhibition for *Vibrio parahaemolyticus* and *Staphylococcus epidermidis*, but not *Pseudomonas aeruginosa* by disk diffusion. The extracts (particularly organic solvents) were cytotoxic to primary human lymphocytes above 100 µg/ml. There were no effects on lymphocyte proliferation at lower concentrations.

This study evaluates the leaves of ethyl acetate and aqueous fraction of SF showed the properties of potential antibacterial and antioxidative properties against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus*.

Keywords: Antibacterial; Antimicrobial; Cytotoxic; Antioxidants, *Loranthus*

Introduction

Scurrula ferruginea (SF) is a mistletoe within the *Loranthaceae*family. *SF* is a tropical obligatehemiparasitic shrub that grows on branches of a host plant, usually a dicotyledonous tree, such as *Tabebuia pallida*[1]. The rusty appearance of the leaves has earned the plant local names, such as *dalu-dalu*, *dedalu-api,*or *benalu* in Southeast Asian countries [1,2]. Figure 1 shows the flowers and leaves of *SF,* taken offa host plant, *Tabebuia pallida*. This mistletoe without functional roots acquires water and nutrients from the host plant through a highly modified haustorium[3].

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Figure 1: Flowers and leaves of *Scurrula ferruginea* on its stalk

Mistletoes have long been used as medicinal sources to cure many diseases and were found to be useful against cancers, microbes, oxidation and hypertension[4]. An advantage of using plants to treat diseases is its lowered toxicity to the patient with its potential for combating drug-resistant disease [5]. Antibiotic resistance is rampant globally, and there is a need to for new antibiotics that can fight microbes [6]. *SF* has been used as traditional medicine, exerting antimicrobial effects, and antihypertensive effects in rats[1,4]. The crude acetone extract of the leaves, stems and flowers of *SF* contains various phenolic compounds known to have antibacterial effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas putida*[1,4].

Phytochemical constituents of plants are responsible for their pharmacological activities. Major classes of antimicrobial compounds found in plants include phenols, quinones, flavonoids, and flavanols. A number of these compounds have been shown to be present in *SF* and are thought to be responsible for its antimicrobial effects [7]. The antioxidant activity of various extracts of *SF* have also been studied by several groups. Puneetha *et al.* and Mohsen M *et al*. showed that the plant had a remarkable amount of antioxidant activity[8,9]. *SF* has recently been reported to exert anti-inflammatory effects via nitric oxide generation and certain cytokines such as IL-6, IL-10, IL-1β and TNF-α [10,11]. Additionally, antibacterial studies on *SF* showed that the plant extract moderately inhibited the growth of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas putida* [8]. *SF* also exerted apoptotic effect on human breast cancer cell MDA-MB-231. The study suggested that the plant's antioxidant activity, high phenolic and flavonoid content may induce cytotoxicity and apoptosis in breast cancer cells[8].

The anticancer effects of *SF* were also observed with structures of the specific phytochemical compounds elucidated (flavanols including quercetin, quercitrin and 4'-O-acetylquercitrin)[12]. Among the flavanols, quercetin exhibited the most lethal cytotoxic activity with IC_{50} of 35 µM while quercitrin and its acetyl derivatives remained inactive with IC_{50} of 200 µM. The majority of studies evaluated the cytotoxicity of *SF* extracts in immortalized breast cancer cell lines (MDA-MB-231 and MCF-7), inferring that normal healthy cells would not be affected[8,13]. However, there are limited studies on the structures of bioactive compounds involved, or correlated known bioactive phytocompounds with bioactivity.

Here, we aim to investigate the antimicrobial effects of the extracts of *SF* against three bacterial strains, namely, *Staphylococcus epidermidis, Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* and further isolate and characterize the bioactive compounds. Total phenolic content and antioxidant activity and other analytical techniques such as infrared spectroscopy and GC-MS were also utilized to identify and characterize the bioactive

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compounds. In addition, we evaluated the cytotoxicity of these crude extracts against primary human lymphocytes and their effect on lymphocyte proliferation.

Materials and Methods

Chemicals

Various solvents such as methanol, ethyl acetate, hexane/dichloromethane were obtained from Sigma-Aldrich (MO, USA). The chemicals for cell line studies are sodium carbonate, Folin-Ciocalteu reagent, Gallic acid, phosphomolybdenum, sodium phosphate, ammonium molybdate, hydrogen peroxide and ascorbic acid were purchased from Sigma-Aldrich (MO, USA). For the cell culture method were used as per ethics committee approved such as peripheral blood mononuclear cells, fetal calf serumpenicillin/streptomycin and phytohemagglutinin were purchased from Sigma-Aldrich (MO, USA).

Collection, identification and processing of plant material

Aerial parts of *SF* were collected from *Tabebuia pallida* host plant grown along the roadat latitude N 4° 54’ 57.2", Longitude E 114° 56’ 54.0" in February 2017. The plant was separated into different parts: leaves, stems and flowers. The plant was identified and authenticated by theInstitute of Biodiversity and Environmental Research, andcatalog identification was assigned to the submitted samples (*Scurrulla ferruginea*; S01083) for reference. Leaves were cleaned of any extraneous materials using tap water followed by distilled water. They were oven-dried at 50°C and pulverized into powder in a blender. The extracts were prepared separately via four different methods yielding four different types of extracts, namely: crude maceration, methanolSoxhlet, ethyl acetate and aqueous extracts[14].

Preparation of crude methanol maceration extract

50 g of powdered plant material was dissolved in 200 ml of methanol (Merck, Frankfurter, Darmstadt, Germany) for three days at room temperature with several agitations. Precipitate was filtered using filter paper (Sigma-Aldrich, Lesquin, France). The extraction was repeated three times with fresh solvent each time to ensure that the biological products from the plant were released. The filtrates were pooled together and evaporated using a rotary evaporator (Yamato, Tokyo, Japan) in a water bath at 50°C. The yield was a dark green paste, weighing 3.2 g, which was later stored at 4°C[15].

Preparation of aqueous extract

50 g of prepared powder was dissolved in 50 ml distilled water. Using homogeniser (IKA, Germany), the mixture was homogenised for 5 minutes, followed by 2 hours of 10 minutes intervals at room temperature with Ultrasonic Bath (Branson, Missouri, USA) to break the plants’ cell walls for release of the active biological components[16,17]. The sonicated mixture was filtered and stored in sterile falcon tubes at -80° C refrigerator overnight. The solid frozen mixture was freeze-dried for 48 hours, yielding 3 g of light sand-brown fine powder. The extract was stored at 4° C.

Preparation of ethyl acetate extract

50 g of the prepared powder was dissolved in 50 ml of ethyl acetate and 50 ml of distilled water. The mixture was stirred thoroughly for 15-20 minutes using magnetic stirrer (Sigma, Schnelldorf, Germany). The mixture was then left to separate into two layers in a separating funnel, where the top layer was ethyl acetate while the bottom was aqueous. The extraction was repeated three times and the respective layers were pooled together. The cumulative ethyl acetate layer was evaporated till dry using a rotary evaporator (Yamato, Tokyo, Japan) in water bath at 50° C to obtain 1.4 g of dark green to black paste and weighed 1.4 g. The extract was stored at 4° C[18].

Preparation of methanol Soxhlet extract

Methanol Soxhlet extract was prepared using Soxhlet apparatus (Fischer

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Scientific, Loughborough, UK); 200 g of powdered *SF*leaves were weighed and transferred into the cellulose thimble. 2 L of methanol was then poured into the round bottomed flask of the apparatus, to which a few glass beads was added to prevent excessive boiling of the extracting solvent. The apparatus was set up carefully and the heating mantle was set up to the boiling point of methanol, 65°C. Sixteen cycles of distillation were required for complete extraction of the leaves, followed by evaporation using a rotary evaporator (Yamato, Tokyo Japan) in water bath at 50°C which produced 3.6 g of dark green paste (extract). The extract was stored at 4° C[18].

Fractionation via column chromatography

The ethyl acetate extract from the methanol maceration product was column chromatographed on silica gel 60 (70 – 230 mesh) using a graduated solvent system of increasing polarity starting with hexane (100%), followed by a mixture of hexane/ dichloromethane (90:10), dichloromethane/ methanol (1 – 10 % methanol), and finally 100 % ethyl acetate. Five fractions were obtained from this initial fractionation process[19].

ATR-IR and GC-MS analysis

Infrared spectra (IR) were recorded on an attenuated total reflection (ATR) plate attachment on the FTIR spectrophotometer, between 800 and 4000 cm^{-1}.Gas chromatography with mass spectrometry (GC-MS) analyses were conducted for the crude extract and all the five fractions. Samples (crude extract or fractions) were dissolved in liquid chromatography (LC)-grade methanol at 1 mg/mL concentration, and filtered using a 0.2 µm syringe filter into a *GC* vial. The sample was introduced by injection at a flow rate of 1.22 mL/min with an injection volume of 7 µL[20,21].

Total phenolic content (TPC) and total antioxidant capacity

Total phenolic content of the crude extract was analysed using the Folin-Ciocalteu colorimetric method [8]. 20 µL of 1 mg/mL crude extract was mixed with 0.75 mL of 20 % sodium carbonate solution and 0.25 mL of Folin-Ciocalteureagent. The reaction mixture was incubated for 1 h in the dark. The absorbance of the mixture was measured at 765 nm. Standard Gallic acid in the range of 0 – 100 µg/mL was prepared in the same manner and results were expressed as mg gallic acid equivalent (GAE) per gram of extract.Total antioxidant capacity of the crude extract was analysed using the phosphomolybdenum method [9], where 0.3 mL of the extract 100 – 1000 µg/mL (PPM) was mixed with 3 mL of phosphomolybdenumreagent solution (1 mL of 0.6 M sulphuric acid, 1 mL of 28 mM of sodium phosphate and 1 mL of 4 mM ammonium molybdate). The reaction mixtures were incubated at 95 °C for 90 min. The absorption of the mixture was measured at 695 nm in UV-Vis spectrometer (*UV-1601 PC*, *Shimadzu*, Kyoto, *Japan*). Positive control, ascorbic acid in the range of 100 – 1000 µg/mL was prepared and analysed in the same manner. The percent hydrogen peroxide scavenging activity which is the percentage inhibition was calculated using the formula below:

$$\left[\frac{(AB - AA)}{AB}\right] \times 100\ \%$$

Where, AB is absorbance of blank sample, AA is absorbance of crude extract (or positive control ascorbic acid). Calibration curve was obtained by plotting % inhibition against the concentration of the sample or positive control.

Peripheral blood mononuclear cells (PBMCs) isolation and culture

This study was approved by the University Research Ethics Committee of Universiti Brunei Darussalam (UBD/DVC/ 32.11). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized blood samples from adult volunteers (n =3) by 1:1 dilution with RPMI 1640 and centrifuged at 400 x g over lymphoprep (Robin Scientific; 1.077g/l). Interfacial cells were recovered, washed and resuspended at

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10^6 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% v/v Fetal Calf Serum (*FCS*), 100 U/mL penicillin/streptomycin, 10^{-2}M HEPES and 2 mM glutamine, referred to as 'complete medium', supplemented with 10 μg/ml phytohemagglutinin(PHA).

Lymphocyte Proliferation assay

Assays were performed in round-profile 96 well microtitre plates; 10^5 PBMCs was mixed with a range of extracts in RPMI 1640 complete media in a total volume of 200 μl and incubated at 37°C with 5 % CO_2 for up to 5 days. For every 1 ml of cell suspension, 1 μl of 5000 μMCellTrace™ Carboxyfluoresceinsuccinimidyl ester (CFSE) (Thermo Fisher Scientific, USA) was added to the suspension to achieve a 1:1000 dilution. The suspension was then incubated in a water bath maintained at 37°C for 20 minutes. Following incubation, 10 ml of RPMI 1640 was added to absorb the unbound CFSE stain and centrifuged at 350 x g for 5 minutes until a pellet was formed. The supernatant was decanted and the cell pellet was resuspended. The cells were washed twice in phosphate-buffered saline (*PBS*) before resuspension in the culture medium at 10^6 cells/ml.

Flow Cytometry

Cultured lymphoblasts were transferred to Fluorescence-activated cell sorting (FACS) tubes and spun down at 350 x g for 5 minutes to form individual pellets. The cells were re-suspended and washed twice in 2ml of PBS then resuspended in 200 μl of PBS. Propidium Iodide (PI) solution (5 μl; 0.5 μg/ml) was added to the cell suspensions and samples were immediately run through a BD Accuri™ C6 dual laser flow cytometer (BD Biosciences, San Jose, CA, USA). Data was analysed using BD Accuri C6 software and PI cell viability data was acquired from Fluorescence 3 (FL-3) plots. Modfit (v4.1.7) was used to evaluate cellular proliferation. Data was analysed by SPSS v21 and Prism (v7.05)[18].

Disc *Diffusion Assay*

The bacterial pathogens used were *Staphylococcus epidermidis, Vibrio* parahaemolyticus and *Pseudomonas aeruginosa* from the microbiology laboratory of Raja Isteri Pengiran Anak Saleha (RIPAS) Hospital. The exact amount of 100 μl of the respective bacterial culture (10^6 CFU/ml) was inoculated onto Mueller-Hinton agar and spread evenly. 5 μL of each *SF* extract was loaded onto sterile Whatman filter paper discs of 6 mm diameter. The final amountof extracts loaded on the discs were 0.5 mg, 1.0 mg and 2.5 mg of each *SF*extract dissolved in *DMSO.* The loaded discs were air-dried and then placed carefully onto respective sections of the agar. 5 μL *DMSO* was used as negative control while standard chloramphenicol or gentamicin (30 μg) was used as a positive control. The petri dishes were incubated for 18 hours at 37°C before the zones of inhibition of growth were measured. Three independent experiments were performed[22].

W*ell Diffusion Assay*

100 μl of the respective bacterial suspension (10^6 CFU/ml) was spread evenly onto Mueller-Hinton agar. Three different amounts of each extract were loaded into the 6 mm perforated well in the agar. The final concentrations of the extracts were 5 mg/well, 10 mg/well and 25 mg/well. Chloramphenicol or Gentamicin (50 μg/well) was used as a positive control and *DMSO* was used as negative control. The plates were allowed to stand for 30 minutes at room temperature prior to 18 hours incubation at 37°C. Zones of inhibition were measured to the nearest millimeter and three independent experiments were performed[23].

Results

Fractionation and characterizationof crude extract of SF

SF leaves were collected, cleaned and extracted after identification of the plant. The extraction was further subjected to fractionation and preliminary chemical

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characterisation.Column chromatography of the crude extract gave an initial separation of five fractions. Infrared spectroscopy and GC-MS were carried out for the crude extract and the obtained five fractions. Infrared spectra of the crude extract (Figure 2a) and the first fraction (Figure 2b) showed peaks characteristic of OH and C=O groups at 3343 cm^{-1} and 1635 cm^{-1} respectively. The carbonyl C=O peak was of much lower intensity in fractions 2-5 (Figure 2b), but still showed the presence of OH groups. The OH and C=O groups correlate well to structures of phenols, flavonols and flavonoids which also contain these functional groups.

Although a complete structure cannot be elucidated by GC-MS, an approximation of possible structures or fragments can be obtained. The mass spectrum of the crude extract is shown in (Figure 2c). The mass spectra at specific retention times (here 8.550, 14.942, 20.667 and 25.875 mins) were analysed and compared to an online database, as they were the largest intensity peaks that correlated to non-solvent peaks. GC-MS of the fractions (data not shown) gave similar plots to the methanol maceration crude extract which suggest that multiple fractionation steps or different solvent systems are required for the fractionation.

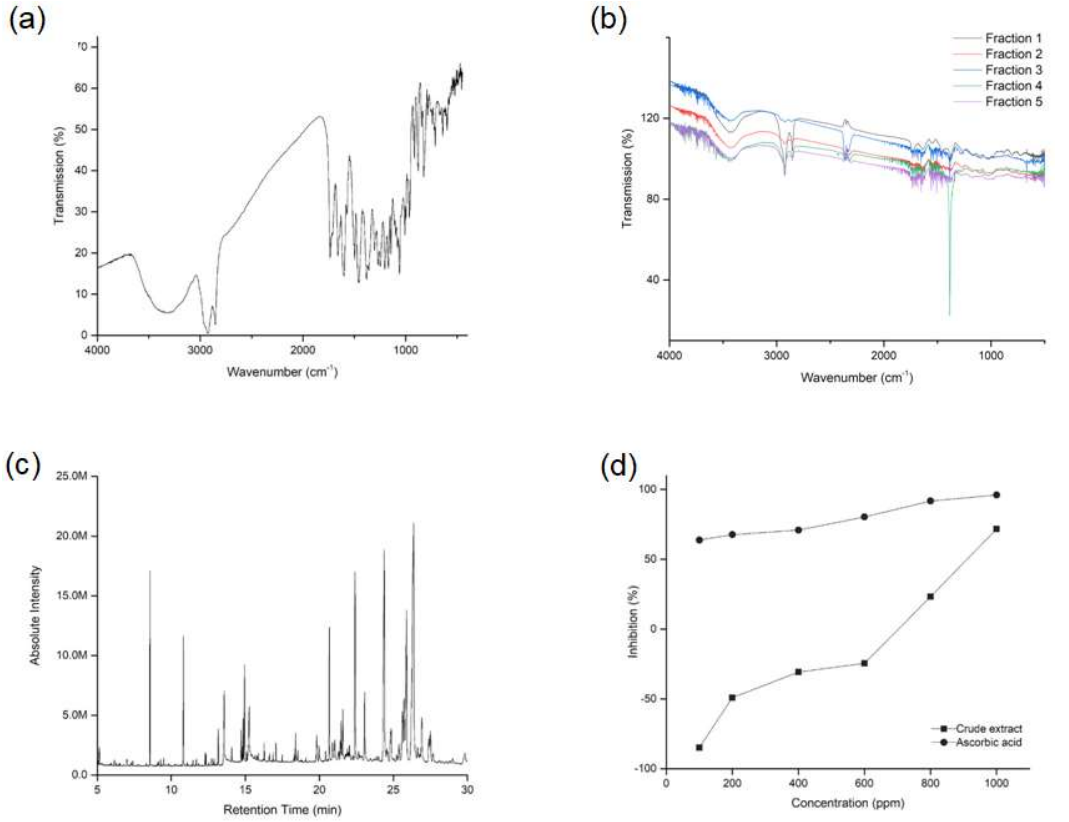

Figure 2: (a) Infrared spectrum of the crude methanol maceration extract. (b) Infrared spectrum of the fractions 1 – 5 obtained from column chromatography (c) *Gas chromatography with mass spectrometry (GC-MS)* of *Scurrula ferruginea* crude extract from methanol maceration. (d) Percent hydrogen peroxide scavenging activity (percentage inhibition) by the crude extract of *Scurrula ferruginea* of concentrations varying from 100 to 100 ppm

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In the analysis and comparison to the database, the focus was to search for compounds that contained phenols, phenyl ethers, and alkyl alcohols as these functional groups are the most likely to be found in the compounds from the plant. Compounds with functional groups similar to those we expect, with a similar mass spectrum fragmentation distribution to the database, and had the highest similarity in molecular mass with those from the GC-MS were picked out. From our findings, some of the compounds that were found to be in our extract were long chain alcohols, phytol (a diterpene alcohol and a constituent of chlorophyll), squalene (a triterpene and precursor to steroids, a component found in many plants), and lupeol (a triterpene which has been shown to have biological activities) [24–26]. The compounds can be separated by additional chromatography of the already separated fractions, for example using prep *TLC*, or *HPLC*. At a much later stage, characterization with *NMR* can be done to aid in the structural determination of the specific compounds.

Total *phenolic content and total antioxidant capacity of crude extract of SF*

The crude extracts were analysed for their total phenolic content and total antioxidant activity as the total antioxidant activity of many medicinal plants depends on the presence of polyphenols and flavonoids of plants. The total phenolic content is used to evaluate the quantity of phenolic compounds present in the crude extract which was found to be157.85 mg GAE/g. The potential of crude extract to reduce Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V) in thephosphomolybdenum assay showed an increase in percentage inhibition with increase in extracts concentration, which correlates to an increase in antioxidant activity (Figure 2d).

Antibacterial properties of SF extracts

The antibacterial activity of the leaf extracts of *SF*was evaluated in terms of zones of inhibition in disc diffusion assays against *Staphylococcus epidermidis*, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa.* For *Staphylococcus epidermidis,* the highest concentration at 2.5 mg/disc showed inhibition for all four extracts, while the two lower concentrations (0.5 mg/disc and 1 mg/disc) did not produce any zone of inhibition (Table 1). 2.5 mg/disc of aqueous extract presented the widest zone of inhibition (8.3 ± 0.0) mm against *Staphylococcus epidermidis* amongst the extracts.No inhibition was observed at 1 mg/disc or below from all the four extracts.As for *Vibrio parahaemolyticus,* the aqueous extract at 0.5 mg/disc showed a zone of inhibition (8.3 mm). Methanol macerated and methanol Soxhlet extracts of *SF*exhibited antibacterial activity at 2.5 mg against *Vibrio parahaemolyticus* with similar zone of inhibitions (9.0 mm each) for both the extracts. No zone of inhibition was observed against *Pseudomonas aeruginosa* with all four *SF* extracts at all tested concentrations (0.5 mg/disc, 1.0 mg/disc and 2.5 mg/disc) (Table 1).

The results obtained from disc diffusion tests were corroborated further with well diffusion assays. Table 2 illustrates the average zones of inhibition of *SF* extracts in a well-diffusion assay against *Staphylococcus epidermidis*, *Vibrio parahaemolyticus*at various concentrations. The three leaf extracts of *SF*(methanol macerated, aqueous maceration and methanol Soxhlet) extracts showed similar zones of inhibition at about 12 mmat concentrations of 5 mg/well against *Staphylococcus epidermidis* (Table 2). Increment in the extracts corresponds with increment in the zones of inhibition, illustrating the different rates of effectiveness of the extracts towards *Staphylococcus epidermidis.* Ethyl acetate extract gave the least inhibition amongst the extracts against *Staphylococcus epidermidis*.

Cytotoxicity *of SF extracts*

All extracts were cytotoxic to primary human PHA stimulated PBMCs (lymphoblasts) as evidenced by Propidium Iodide (PI) uptake.

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Table 1: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* discs

	Staphylococcus epidermidis			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	8.3 ± 0.6	7.3 ± 0.6	7 ± 0.0	7.3 ± 0.6
30 µg/disc Chloramphenicol (positive control)	25 ± 0.0			
	Vibrio parahaemolyticus			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	8.3 ± 1.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	8.6 ± 1.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	9 ± 0.6	9 ± 0.6	0 ± 0.0	8.3 ± 0.6
30 µg/disc Chloramphenicol (positive control)	18.4 ± 1.9			
	Pseudomonas aeruginosa			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
50 µg/disc Gentamicin (positive control)	13 ± 0.0			

Table 2: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* wells

	Staphylococcus epidermidis			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	12 ± 2.0	12.3 ± 0.6	0 ± 0.0	12 ± 0.0
10	13.7 ± 1.5	14.3 ± 0.0	8.3 ± 0.6	14 ± 0.0
25	16 ± 0.6	16 ± 1.0	10.3 ± 0.6	15.7 ± 0.6
50 µg/well Chloramphenicol (positive control)	25 ± 0.0			
	Vibrio parahaemolyticus			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	11 ± 1.0	8.3 ± 0.6	0 ± 0.0	0 ± 0.0
10	13 ± 1.0	8.6 ± 0.6	0 ± 0.0	8.3 ± 0.6
25	15.3 ± 0.6	14 ± 1.0	0 ± 0.0	14.6 ± 0.6
50 µg/well Chloramphenicol (positive control)	21.4 ± 1.4			

(*Contd.*)

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Table 2: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* wells (*Contd.*)				
	Pseudomonas aeruginosa			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
10	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
25	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
100 µg/disc Gentamicin (positive control)	13 ± 0.0			

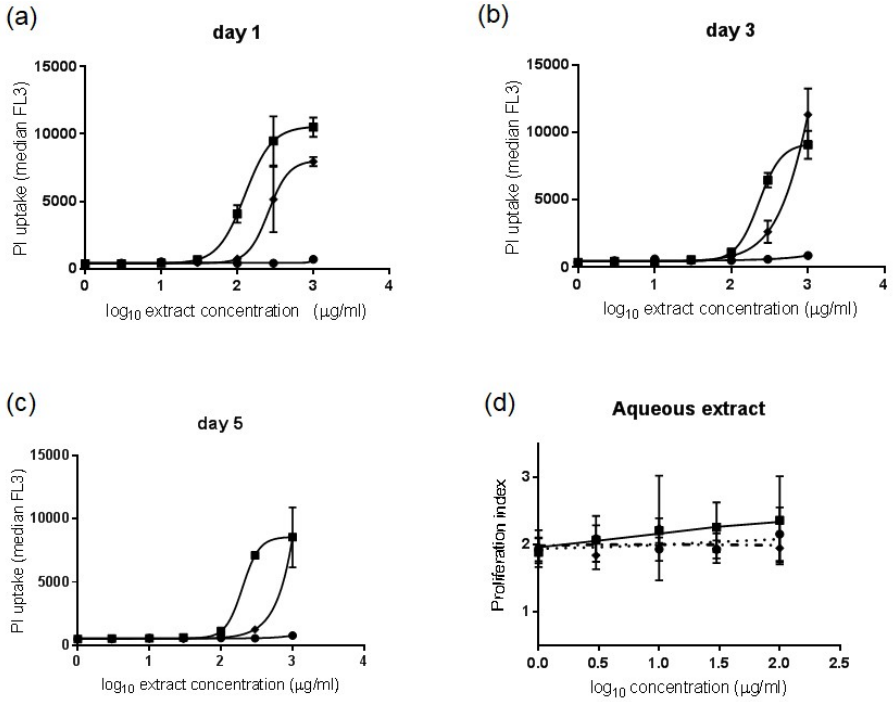

Figure 3: A representative dose response curve illustrating the effect of increasing concentrations of *Scurrula ferruginea* aqueous extract (circle), maceration extract (square), and Soxhlet extract (diamond) on phytohemagglutinin (PHA) stimulated PBMCs over time with (a) at day 1 (b) at day 2 (c) at day 5. Cell death is indicated by propidium iodide (PI) uptake by lymphoblasts. Data represent the mean of median FL3 values ±SD of triplicates. (d) A representative dose response curve of cell trace experiments indicating the effects of the aqueous extract of *Scurrula ferruginea* on lymphoblast proliferation over time (day 1 indicated by circles / dotted line; day 3 indicated by squares/solid line; day 5 indicated by diamonds/hashed line. Data represent the mean proliferation index ± SD of triplicates

This was observed in three different human samples. There was a significant increase in median fluorescence in the presence of each extract as low as 3 µg/ml ($p<0.05$; Figure 3). Solvent extracts of *SF* induced significant cell death, particularly at or above concentrations of 100 µg/ml. The aqueous extract caused less cell death than the methanol extracts

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(maceration and Soxhlet), but it was still evident.Cell trace experiments were performed in order to evaluate the effect of *SF* on lymphocyte proliferation, but the analyses assume there is no significant cell death. When the lower concentrations of the aqueous extract were incubated with human lymphoblasts, there was no significant effect on their proliferation ($p<0.05$; Figure 3).

Discussion

Both infrared spectrum and gas chromatography analyses have shown consistent contents of the extraction and fractionation. The characteristic functional group peaks observed in by infrared and gas chromatography mass spectrometry (GCMS) analyses corroborates each other. High intensity peaks of GCMS illustrated long chain alcohols squalenes, lupeols, and the other antioxidant substances which are usually found in the parasitic mistletoes [4,8]. Phosphomolybdenum assay indicated antioxidant activity by compounds extracted and fractionated from *SF* sample (Figure 2d) and interestingly, the compounds detected by both IR and GCMS have antioxidative properties.

The presence of antioxidative properties prompted the investigation of antibacterial properties of these extracts. Many plants, including *SF*, produce phenolic compounds to defend themselves against certain bacteria, exerting bacteriostatic or bactericidal effects [27,28]. In this study, the antibacterial results against the three bacteria (*Vibrio parahaemolyticus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa)* yielded different results. From the data, extracts of *SF* showed antibacterial effects against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus,* but not *Pseudomonas aeruginosa*. Data from both disc diffusion assay and the well assay correlate well against *Staphylococcus epidermidis*, indicating that all fractions inhibit *Staphylococcus epidermis,* with the ethyl acetate fraction having the least amount of zone of inhibition in both assays (Tables 1 and 2). The dipolar aprotic nature of ethyl acetate may have extracted less hydrophilic phytochemicals than the other solvents[29].

Vibrio parahaemolyticus, a pathogenic *Vibrios*strain, is a major causal organism of gastroenteritis and septicemia characterized by watery diarrhea, vomiting, fever and chills, sourcing from raw or undercooked, contaminated seafood such as raw oysters and mussels[30]. Table 1 illustrated that only *Vibrios parahaemolyticus* was susceptible to aqueous extracts of *SF* at 0.5 mg per disc. Congruent to the disc diffusion data for *Vibrios parahaemolyticus*, the largest zone of inhibition was found with the aqueous extracts of *SF*. Thus, the active compounds that act against *Vibrios parahaemolyticus* are of hydrophilic nature found within the aqueous extracts of *SF*. The strain of *Pseudomonas aeruginosa* used was obtained from a clinical setting. No inhibition of *Pseudomonas aeruginosa* by any extract of *SF*in either disc-diffusion assay or and well-diffusion assay. Thus, the compounds found within all the four *SF* extracts were not able to inhibit this bacterial strain, possibly due to either presence of suitable active compounds or adequate amount. This result differs from the Tunisian *Ruta chalepensis*, which inhibit *Pseudomonas aeruginosa* with diameter of inhibition of 17.7 mm,close to that of the standard antibiotic gentamycin[31]. A comparative analysis between the compounds of these two ethnopharmacological plants (*Ruta chalepensis*and *SF)* will shed light on the compounds that exert antibacterial effects against *Pseudomonas aeruginosa*.

Also, our study indicates that *SF* extracts are cytotoxic to primary human lymphoblasts in a time- and dose-dependent manner at concentrations between 3 μg/ml and 1000 μg/ml. This is in line with the findings of Marvibaigi et al [32]. Solvent extracts were more cytotoxic than aqueous extract of *SF*. Primary human lymphocytes was able to proliferate normally in the presence of lower doses of the aqueous extract of *SF* (3 μg/ml - 100μg/ml) with no apparent detrimental effects. This indicates

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the possibility of using *SF* extracts against a few cancer types.

However, there were a few limitations to our study. First, phytochemical screening of active constituents of plant such as flavonoids, alkaloids, tannins and phenols is required to authenticate our findings. Isolation and identification of these potential antibacterial compounds using methods such as Thin Layer Chromatography (TLC) and High-Pressure Liquid Chromatography (*HPLC*) will be useful for future work. Comparison of the mass spectrum at specific retention times with the mass spectra of known compounds from an online database resulted in an approximation of plausible compounds within the extract only. This study infers through the preliminary chemical characterisation and corroborated by various researchers, the presence of compounds including long chain alcohols, phytol (a diterpene alcohol and a constituent of chlorophyll), squalene (a triterpene and precursor to steroids, a component found in many plants), and lupeol (a triterpene which has been shown to have biological activities) from *SF* extracts. Further analysis with characterisation would be needed to confirm the identity of these compounds, and their specific biological functions [24,25,33]. Secondly, there is a lack of literature on the chemical profiles and GC-MS data on extracts of *SF*, A significant limitation of our study is the inability to pinpoint the specific molecules in the extracts of *SF*. The retention times mentioned were based on the relative higher intensity detected. The molecules postulated were based on mass spectrometry database and there may be other molecules of the same sizes, isomers of long chain molecules (alcohols) or metabolites not mentioned as most plants would have common secondary metabolites. Identification of the compounds responsible for different biological activities and the mechanism of action will be beneficial. Thirdly, the utilisation ofdisc diffusion and well diffusion assays for antibacterial work means that the minimum inhibition concentration of the potent extract cannot be determined. Therefore, future work to determine the minimum inhibition extract of the aqueous and methanol macerated extract of *SF* against both *Staphylococcus epidermidis* and *Vibrio parahaemolyticus* and broth dilution tests (such as the tube-dilution method to generate quantitative results) will shed better insights of the antibacterial effects of the extracts. In addition, these *in vitro* assays are unable to demonstrate any side-effects when these extracts are metabolized physiologically. Therefore, animal models are needed to investigate the therapeutic effects of the *SF*as well toxicity studies. Also, in most studies involving *SF*, the host plant of the mistletoe was not mentioned. This should be noted as few studies have shown that mistletoe growing on different host plant may have different chemical composition. A study of the chemical composition of the European mistletoe, *Viscumalbum* on three host plants has shown significant differences in their chemical composition which may, result in variation in their cytotoxic or antibacterial properties [34].

Therefore, the anticancer effects of *SF* remain a broad area of research to be done. Here, we have explored the phytochemistry, antioxidant, antimicrobial and cytotoxic potential of *SF* extracts. Although our results are still preliminary, we demonstrated that *SF* is a hemiparasitic plant harboring antioxidants with antimicrobial effects towards *Vibrios parahaemolyticus* and *Staphylococcus epidermidis*and cytotoxic effects towards PBMCs.

Conclusion

This study evaluates the properties of this interesting medicinal herb and highlights the potential anti-bacterial properties of *SF*. This work illustrates the selective antibacterial effects against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus,* but not on*Pseudomonas aeruginosa.* In addition, this work sheds light on the antioxidative properties of various *SF* extracts and consequently, portrays the importance of future work on the potential toxicity of *SF*.

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10.5530/ctbp.2024.3s.13

Acknowledgement

The authors would like to acknowledge PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam for the laboratory facilities and materials, Dr Siti Hanna Muharram for the bacterial strains, and Institute for Biodiversity and Environmental Research for assistance with identification and authentication of plant materials.

Competing interests

The authors declare that they have no competing interests.

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