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Anti-acanthamoebic activity of methanolic extract of *Piper sarmentosum* leaves

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ABSTRACT

Aims: *Piper sarmentosum* or locally known as Kaduk, is a tropical herb plant that was investigated for its phenolic content by previous researchers. The present study aimed at the analysis of crude methanolic extract of *P. sarmentosum* leaves for phenolic compounds identification and its anti-amoebic properties against pathogenic *Acanthamoeba castellanii.*

Methodology and results: Folin-Ciocalteu assay was used to determine *P. sarmentosum* leaves methanolic extract (PSLME)'s total phenolic content (TPC). The extract was further characterized by using gas chromatography-mass spectrometry (GC-MS), reverse phase-high performance liquid chromatography (RP-HPLC) and liquid chromatography-mass spectrometry (LC-MS) analyses to determine the chemical constituents in methanolic PSLME extract. The cytotoxicity of the extract was evaluated through the determination of inhibition concentration for half of cell population (IC_{50}) of pathogenic *A. castellanii* followed by cell morphological analysis using inverted light and scanning electron microscopies. Acridine-orange/Propidium iodide (AOPI) staining was also conducted to determine the integrity of cell membrane for quantitative analysis. The results demonstrated that the TPC from PSLME was 142.72 mg [GAE]/g with a total of 33 phenolic compounds identified. The IC_{50} value obtained for *A. castellanii* was low (74.64 µg/mL) which indicates promising anti-acanthamoebic activity. Microscopy analyses showed that the plant extract caused cells encystment, in which exhibited by distinctive morphological changes on the cells shape and organelle, as well as shortening of acanthopodia. The dual staining and its quantitative analysis prove compromised membrane integrity in the treated amoeba.

Conclusion, significance and impact of study: This finding provides the evidence that PSLME contains active phenolic compounds contributing to the anti-acanthamoebic activity on pathogenic *Acanthamoeba* species.

Keywords: Piper sarmentosum, phenolic compound, chromatography, *Acanthamoeba castellanii*, anti-amoeba

INTRODUCTION

Acanthamoeba castellanii is a free-living protozoan amoeba that lives as bacterial consumer in natural environment such as seawater, river, lakes and contaminated water sources (Tsvetkova *et al.*, 2004; Lorenzo-Morales *et al.*, 2005). This amoeba can easily infect human through lower respiratory tract or ulcerated and broken skins which could produce toxins that can be lethal (Siddiqui and Khan, 2012). These infections could progress to chronic diseases such as granulomatous amoebic encephalitis, serious infection of the brain and spinal cord, as well as *Acanthamoeba* keratitis, which is a vision threatening keratitis in human involving *A. castellanii* (Kamel *et al*., 2005).

Phenolic compounds constitute major secondary metabolites in plants as they play important roles for the plant protection against physical pressures such as microbial pathogens, insects and harsh environment condition (Khoddami *et al.*, 2013). Plant phenolics comprise of many subclasses such as simple phenols, coumarins, lignins, lignans, tannins, benzoic acid, cinnamic acid, phenolic acids and flavonoids (Soto-Vaca *et al.*, 2012; Khoddami *et al*., 2013). Over decades, phenolic compounds are discovered to provide pharmacological properties including anti-inflammatory, anti-allergenic, anti-thrombotic, anti-artherogenic and anti-microbial against bacteria, fungi and microalgae (Dykes and Rooney, 2007; Zakaria *et al.*, 2010; Hussain *et al.*, 2012).

Piper sarmentosum or locally known as Kaduk, is a terrestrial herb that has been used as traditional food and for many traditional medicinal purposes. The plant leaves are commonly used as raw vegetable whilst the whole

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plant is effective as remedy to treat minor ailments such as coughs, headache, toothache, influenza, asthma and feet fungoid dermatitis (Atiax *et al*., 2011). It is widely distributed in Southeast Asia countries including Thailand, Indonesia, Philippines and Malaysia. Recent researches proved that some of the plant solvents-based extracts showed antimicrobial properties against wide ranges of microorganisms especially bacteria, microalgae, protozoa and fungi (Rahman *et al.*, 2014a; 2016). These findings reported that the demonstrated antimicrobial properties of the plant associated with high amount of phenolic content. However, the specific potential of phenolic compounds from *P. sarmentosum* against *A. castellanii* is yet to be investigated. Hence, this research aimed to identify the phenolic substances present in *P. sarmentosum* leaves crude methanolic extract and to evaluate the cytotoxicity effects toward pathogenic *A. castellanii.*

MATERIALS AND METHODS

Plant material

Fresh leaves of *P. sarmentosum* were collected from the botanical garden of Glasshouse and Nursery Complex, International Islamic University Malaysia (IIUM) Kuantan Campus, Pahang. The taxonomic identification was performed by Dr. Shamsul Khamis from Universiti Kebangsaan Malaysia, and the voucher specimen (No: PIIUM 0239-3) was deposited in the Herbarium at Kulliyyah of Pharmacy, IIUM. Samples were frozen at –80 °C for 24 h and freeze dried for three days.

Extraction of phenolic compounds

Extraction of phenolic compounds from *P. sarmentosum* leaves was carried out using improved maceration technique based on Rahman *et al.* (2014a). Powdered leaves (350 mg) were dissolved in 25 mL of pure methanol. The solution was sonicated in a water bath sonicator at 35 °C for 40 min. A 10 mL of 0.01 M hydrochloric acid was then added slowly to the solution over 5 min. The solution was transferred to 100 mL round bottomed flask and stirred using a magnetic stirrer under nitrogen condition at 35 °C for 16 h. After cooling and filtering through Whatman No. 1, the methanol solvent was evaporated to dryness using a rotary evaporator at vacuum pressure of 337 mb in a water bath at 60 °C.

Determination of total phenolic content

Total phenolic content (TPC) in the plant extract was determined according to Folin-Ciocalteu method (Pieroni *et al.*, 2011). Samples were evaluated at final concentration of 0.1 mg/mL after dilution with methanol. A total volume of 0.5 mL of extract aliquot was mixed with 2.5 mL Folin-Ciocalteu reagent (previously diluted with distilled water 1:10, v/v) and 7.5 mL aqueous sodium carbonate solution. The tube was incubated at room temperature for 120 min for color development. Absorbance was measured spectrometrically at 760 nm. TPC was determined by interpolation of the absorbance of the samples against a linear calibration curve made with gallic acid standard ranged from 1 to 500 µg/mL. TPC was expressed as mg gallic acid equivalents (GAE) per g of crude extract. The samples were made in triplicates.

Gas chromatography mass spectrometry (GC-MS) analysis

The characterization of phenolic compounds in *P. sarmentosum* leaves methanolic extract (PSLME) was performed through gas chromatography mass spectrometry (GC-MS) analysis based on the modified method from Rahman *et al.* (2014a). The plant crude extract was diluted in liquid chromatography (LC) grade methanol to obtain the final concentration of 5 mg/mL. The diluted solution was filtered through a sterile nylon membrane (pore size of 0.45 µm and diameter of 13 mm). GC-MS analysis of the plant extract was conducted on Clarus^{TM} SQ 8 GC/MS instrument equipped with Elite 5MS capillary column (30 m length, 0.25 µm film thickness, 0.25 mm inner diameter, coupled with a Clarus 600C mass spectrometer. 99.9% purified helium gas was used as the carrier gas at 68.8 kPa pressure maintained at the flow rate of 1.0 mL/min. The ionizing energy was set at 70 eV and the mass range at m/z 25-700. The oven temperature condition was programmed from 70 °C (hold for 5 min) to 280 °C at a rate of 7 °C/min. The total program run time was 46 min. Phenolic compositions were identified by comparing the mass spectrums and retention time of authentic compounds in the National Institute of Standards and Technology (NIST) and Wiley libraries. The composition of each detected phenolic compounds was expressed as the percentage of the compound peak area in the total extract.

Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

PSLME was diluted in LC grade methanol to produce the working concentration of 2.5 mg/mL. The standards used for reverse phase-high performance liquid chromatography (RP-HPLC) analysis of phenolic compounds were gallic acid, tannic acid, quercetin and naringenin. Quantification of phenolic compounds was performed by using an external standard method, which was achieved via calibration curves generated from the serial dilutions of standard solution with concentration sequences from 1 to 200 µg/g. All standards were filtered through 0.45 µm pore size sterile nylon membrane filter preceding the analysis. The linear calibration charts that were comprised of the area under the peak versus standard concentrations were subsequently used for phenolic compounds quantification according to their corresponding standards.

Qualitative and quantitative analysis for phenolic compounds in *P. sarmentosum* through RP-HPLC was performed using FX-15 HPLC system. The instrument was equipped with FX-15 UHPLC pump, FX-UV/vis

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UHPLC detector, an auto sampler and vacuum degasser. Separation of the phenolic compounds was achieved using LiChosorb C-18 HPLC analytical column (5 µm diameter, 3.0 µm inner diameter, 250 mm length). The analysis was programmed based on Rahman *et al.* (2014b) with minor optimization. Separation of individual phenolic compounds was performed by isocratic elution of the mobile phase in binary mode and the column temperature was maintained at 28 °C throughout analysis. Prior to analysis, the column was washed with HPLC grade acetonitrile for 30 min and was re-conditioned with HPLC grade methanol for 15 min between runs. The details of the isocratic condition with respect to each targeted phenolic compound are described in Table 1. Each solvent was filtered through 0.45 µm membrane filter and degassed in a water bath sonicator for 15 min before used. The four standards were pre-analyzed to determine the compound retention times, in order to compare to the sample extracts under the same conditions. All of the standards and samples were injected into the system with injection volume of 10 µL. Chromatograms, linear calibration curve of standard and quantitative measurements of phenolic compounds peak area were recorded and processed by Chromera and TotalChrom software (version 6.3).

LC-MS analysis

The identification of phenolic compounds through liquid chromatography mass spectrometry (LC-MS) was performed using Ultra Performance Liquid Chromatograhy (UPLC) coupled to Vion IMS QToF Mass Spectrometer. The system featured XS Ion Optics and Quadrupole Time-of-Flight (QToF) as the detection system. The phenolic compounds chromatographic separation was achieved by using ACQUITY UPHLC® HSS T3 column. The low collision energy was set at 4 eV and the high collision ramp started at 10 eV and ended at 45 eV. The interface used in the analysis was Electron Spray Ionization (ESI) ionization source type with the source temperature of 120 °C. Pure nitrogen (99.9%) was used as desolvation gas at the temperature of 550 °C, with the flow rate of 800 L/h. The mobile phase used to move the solute through the column was LC grade water (Solvent A) and LC grade acetonitrile (Solvent B) and the flow rate was maintained at 0.5 mL/min. The program was set in gradient mode with the following conditions as described in Table 2. The column temperature was maintained at 40 °C throughout analysis. The spectra were recorded in negative mode within the mass range of 100 to 1000 m/z. The identification of individual compounds detected was based on the retention times and theoretical exact mass driven by UNIFI scientific information system (version 1.8).

Table 2: The gradient condition of LC-MS analysis.

Time (min)	Composition of mobile phase solvent (%)	
	Water (Solvent A)	Acetonitrile (Solvent B)
0.0-5.0	99	1
5.0-16.0	65	35
16.0-18.0	0	100
18.0-20.0	99	1

Determination of inhibition concentration value (IC_{50}) for half of cell population against *A. castellanii*

Acanthamoeba castellanii strain 1501/2A of Culture Collection of Algae and Protozoa (CCAP), UK was used throughout this study. Approximately 10^4 cell/mL was seeded in each well of sterile 96-well plate filled with peptone-yeast-glucose (PYG) culture medium. Cells were incubated at 30 °C for 24 h. The cells were treated with *P. sarmentosum* leaves methanolic extract (PSLME) with different concentrations ranging from 3.91 to 1000 µg/mL from two-fold dilution series. The determination of half-maximal inhibitory concentration (IC_{50}) was conducted using eosin dye method founded by Wright *et al.* (1988). The absorbance of final was measured at 492 nm using Multiskan™ GO Microplate Spectrophotometer. The percentage of cell growth inhibition was plotted against the logarithm of the extract sample concentrations in GraphPad Prism software to obtain non-linear sigmoidal dose-response curve, where the extract concentration

Table 1: The isocratic condition of RP-HPLC analysis with respect to target compounds.

Target compounds	Mobile phase				Ratio (v/v)	Flow rate (mL/min)	Wavelength (nm)
	Solvent A	Solvent B	Solvent C	Solvent D			
Gallic acid	Pure LC grade methanol	1% trifluoroacetic acid in ultrapure water			A:B 80:20	0.8	280
Tannic acid	Pure LC grade methanol	1% trifluoroacetic acid in ultrapure water			A:B 70:30	0.7	270
Naringenin	Pure LC grade methanol	1% trifluoroacetic acid in ultrapure water			A:B 90:10	0.8	280
Quercetin	Pure LC grade methanol		LC grade acetonitrile	Ultrapure water	A:C:D 5:50:45	0.8	370

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that inhibited 50% (IC_{50}) of *Acanthamoeba* population was determined. Positive control, chlorhexidine (0.25%) used the same protocol as above.

Microscopy observation of *Acanthamoeba* cell morphology

Cells concentration of 1 × 10^5 cells/mL was treated with PSLME at their IC_{50} values in T-25 culture flask and incubated in 30 °C for 72 h. After incubation, the cell morphology was observed directly under Olympus IX51 inverted light microscope. The cell images were acquired and processed using Analysis LS Research software (version 2.6) connected to the microscope. The image of the extract-treated *A. castellanii* was compared to the positive (chlorhexidine) and negative (cells without treatment) controls. Each treatment was prepared in triplicates.

Measurement of cell confluence in axenic culture

Extract-treated *A. castellanii* was subjected to visual examination of adherent cells confluence. The technique was based on Busschots *et al.* (2015). A cardboard cover was designed to fit the bottom surface of T-25 flask having two windows located close to the top and bottom of the flask surface with the area measurement of 1 cm^2. The cardboard was glued underneath the culture flask. Upon viewing, 4× objective lens was placed over the cardboard window and was adjusted to focus on the cell nuclei. Photographs were taken from each window, representing an approximate confluence value of the whole flask. Measurement of adherent cells confluence was conducted using Image J software (version 10.2) (Fiji, USA) producing an area fraction (AF) output.

Scanning electron microscopy (SEM)

Cells density of 1 × 10^4 cells/mL *A. castellanii* which treated with PSLME at IC_{50} value in a 30 °C incubator for 72 h was observed by SEM. The *A. castellanii* cells were subjected to dry fixation based on Nakisah *et al.* (2012). After incubation, the culture medium was carefully replaced with 2.5% (v/v) gluteraldehyde in phosphate buffered saline (PBS) solution for 1 h at 30 °C. The cover slips of which contained the amoeba cells were then gently washed with sterile PBS three times. The treatment was followed with post-fixation using 1% osmium tetraoxide that was previously diluted in PBS solution for 1 h at 30 °C in dark condition. The cover slips were then rinsed with PBS for three times. Following that, the cell specimens were dehydrated in graded series of ethanol (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) for 5 min in each soaking. Final dehydration in 100% ethanol was repeated twice. Later, dehydrated *Acanthamoeba* specimens were subjected to critical point drying by adding few drops of hexamethyldisilazane. The cover slips were carefully mounted on a 13 mm aluminium stub by using the double-sided adhesive conductive carbon sticky tape to maintain the position of the cover slips on the SEM stub. Prior to visual examination under SEM, the specimen stub was coated with gold by JEOL Smart Coater. Similar protocol was applied for the negative and positive controls. Scanning Electron Microscope (JEOL JSM-6360 LV) was operated at 5 to 20 kV accelerating voltage.

Determination of cell membrane integrity

The cell suspension in PBS was centrifuged at 800 rpm for 3 min using Minispin microcentrifuge (Eppendorf, Germany). The supernatant was then discarded, and the pellets were resuspended in 50 µL of Acridine-orange/Propidium iodide (AOPI)(1:1 v/v). The cell suspensions were observed under an upright binocular microscope (Leica Dmire LB2, Germany). Then, 100 *Acanthamoeba* cells were randomly calculated on each slide of three replicates and were classified based on their membrane integrity. Green cells were classified as intact cells while the cells with red or orange precipitates were classified as cells with non-intact membrane. Statistical analysis of t-test (SPSS version 17.0) (IBM, USA) was carried out to determine whether there was significant difference in the effects of PSLME on the membrane integrity between the treated and untreated cells. $p<0.05$ was considered as statistically significant.

RESULTS

Total phenolic content

Calculation of the total phenolic content was achieved from a linear calibration curve of gallic acid standard with coefficient of determination (R^2) equals to 0.998. By using the linear regression equation, high total phenolic content was measured which was 142.72 mg [GAE]/g of crude extract.

Gas chromatography mass spectrometry (GC-MS) analysis

Gas chromatography mass spectrometry (GC-MS) analysis of PSLME showed eight peaks in its total chromatogram as shown in Figure 1. The compounds mass spectrums and retentions were compared with the National Institute of Standards and Technology (NIST) and Wiley libraries and revealed that six peaks represented six phenolic compounds, while the other two peaks were unidentified. The identified phenolic compounds are listed in Table 3. The relative amount of the compound detected was expressed as percentage of peak area (total area normalization). α-Asarone, a phenylpropanoid was the major chemical that constituted 59% of the total compound content while, its isomer, ß-asarone only constituted 1.9% of the extract. Besides, PLSME also contains 1.5% of another phenylpropanoid called benzenepropanoic acid, methyl ester. Meanwhile, the compositions of phenylpropenes detected were elemicine (33.3%), myristicine (2.8%) and methyleugenol (1.5%).

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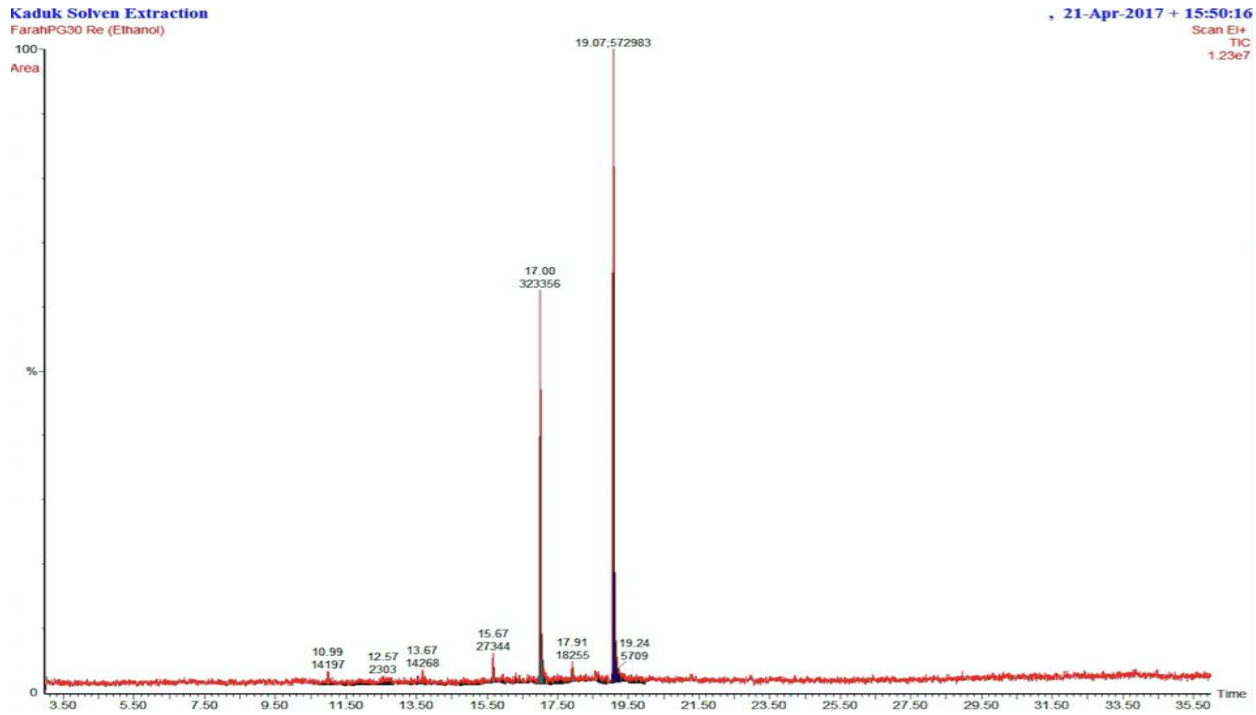

Figure 1: GC-MS chromatogram of phenolic compounds detected in methanolic extract of *P. sarmentosum* leaves. Retention time 19.07: α-Asarone, 17.91: ß-Asarone, 17.00: Elemicine, 15.67: Myristicine, 13.67: Methyleugenol, 10.99: Benzenepropanoic acid, methyl ester.

Table 3: Chemical constituents detected in methanolic extract of *P. sarmentosum* leaves from GC-MS analysis.

No.	Name of compound	Chemical formula	Class of phenolic compound	Retention time (min)	Exact mass (g/mol)	Peak area (%)
1	α-Asarone	$C_{12}H_{16}O_3$	Phenylpropanoid	19.07	208.1099	59.046
2	ß-Asarone	$C_{12}H_{16}O_3$	Phenylpropanoid	17.91	208.1099	1.881
3	Elemicine	$C_{12}H_{16}O_3$	Phenylpropene	17.00	208.1099	33.322
4	Myristicine	$C_{11}H_{12}O_3$	Phenylpropene	15.67	192.0786	2.818
5	Methyleugenol	$C_{11}H_{14}O_2$	Phenylpropene	13.67	178.0994	1.470
6	Benzenepropanoic acid, methyl ester	$C_{10}H_{12}O_2$	Phenylpropanoid	10.99	164.0837	1.463

Table 4: Composition of phenolic compounds in methanolic extract of *P. sarmentosum* leaves detected from RP-HPLC analysis.

No.	Name of compound	Chemical formula	Class of phenolic compound	Retention time (min)	Exact mass (g/mol)	Amount of compound (µg/g)
1	Gallic acid	$C_7H_6O_5$	Phenolic acid	3.32	170.12	115.50
2	Tannic acid	$C_{76}H_{52}O_{46}$	Phenolic acid	3.70	1701.21	62.50
3	Naringenin	$C_{15}H_{12}O_5$	Flavonoid	3.89	272.26	3.56
4	Quercetin	$C_{15}H_{10}O_7$	Flavonoid	4.38	302.24	0.29

Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

The RP-HPLC analysis of PSLME revealed that gallic acid was the major phenolic compound constituent (115.50 µg/g), followed by tannic acid (62.5 µg/g), naringenin (3.56 µg/g) and quercetin (0.29 µg/g) as presented in Table 4. Figure 2 shows the representative chromatograms of compounds detection in the plant extract with respect to the standard retention times.

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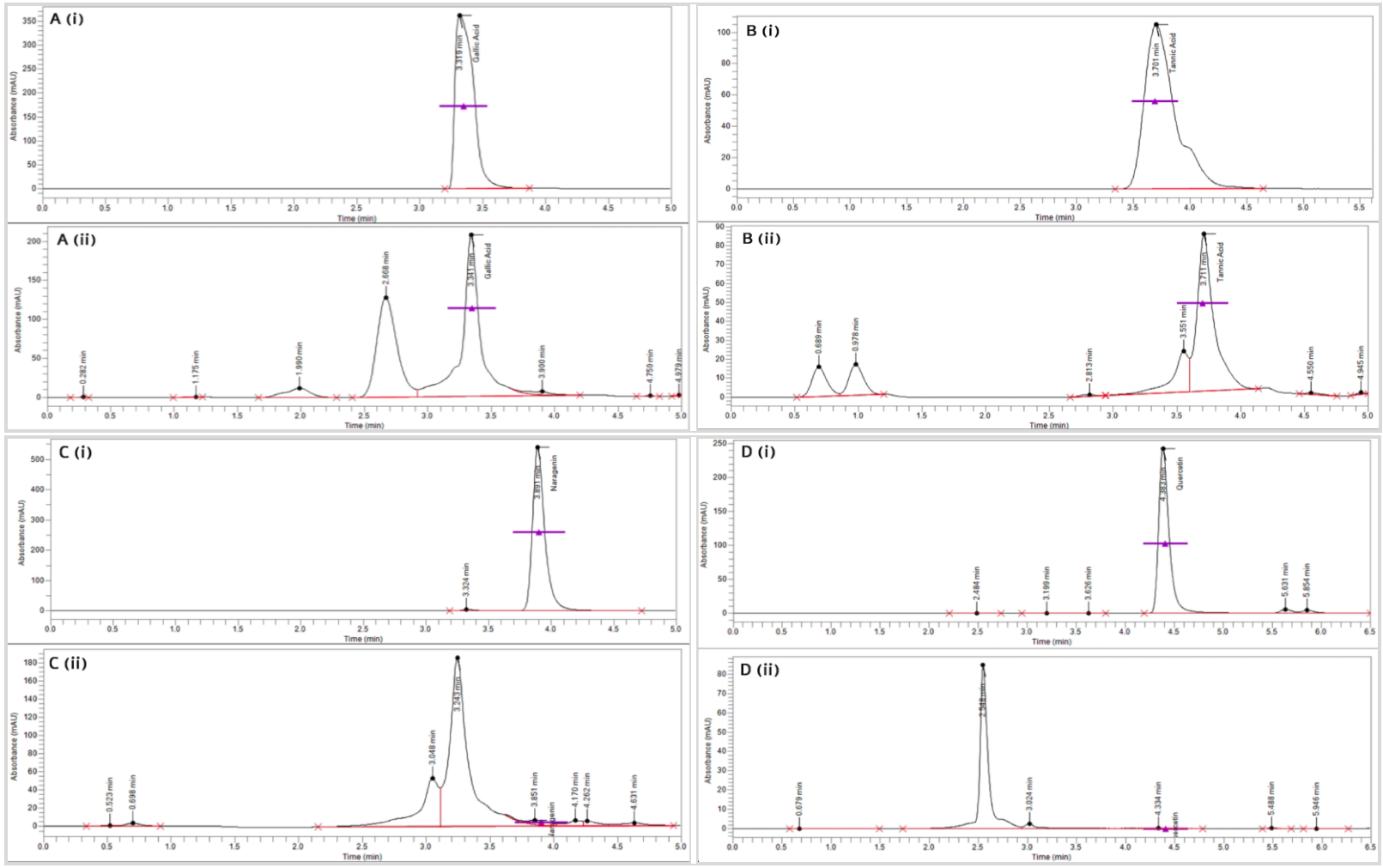

Figure 2: Representative chromatograms of (i) standard solution and (ii) *P. sarmentosum* leaves methanolic extract from RP-HPLC analysis. (A) Gallic acid, (B) Tannic acid, (C) Naringenin and (D) Quercetin.

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LC-MS analysis

Through LC-MS analysis, a total of 23 phenolic compounds were identified in *P. sarmentosum* leaves methanolic extract. The list of identified compounds is presented in Table 5. Based on the calculation of compound peak area, kuzubutenolide A was the largest constituent in the total extract (30%) while the least constituent was aspidinol of 0.619% of the crude extract. The total chromatogram of phenolic compounds detected in *P. sarmentosum* leaves methanolic extract (PSLME) is shown in Figure 3.

Table 5: The list of phenolic compounds identified in *P. sarmentosum* leaves methanolic extract from LC-MS analysis.

No.	Name of compound	Chemical formula	Class of phenolic compound	Retention time (min)	Exact mass (g/mol)	Peak area (%)
1	Isoduartin	$C_{18}H_{20}O_6$	Isoflavan	13.1	377.124	0.962
2	1-Galloyl-glucose	$C_{13}H_{16}O_{10}$	Tannin	3.728	331.067	6.678
3	Aspidinol	$C_{12}H_{16}O_4$	Aenicide phloroglucinol derivatives	8.835	269.102	0.619
4	Renifolin	$C_{18}H_{24}O_7$	Prenylated chalcone (flavonoid)	13.597	397.149	0.661
5	Tribulusamide A	$C_{36}H_{36}N_2O_8$	Lignanamides	12.665	669.245	2.102
6	Tribulusamide B	$C_{36}H_{34}N_2O_9$	Lignanamides	0.681	683.225	1.01
7	Forsythoside A	$C_{29}H_{36}O_{15}$	Phenylpropanoid	12.242	623.198	0.761
8	N-trans-Coumaroyltaramine	$C_{17}H_{17}NO_3$	Phenylpropenic acid	9.66	282.114	0.735
9	2,3,5,4'-Tetrahydroxystilbene-2-O-(6''-O-α-D-glucopyranosyl)-β-D-glucopyranoside	$C_{26}H_{32}O_{14}$	Stilbene (phenolic glycoside)	10.386	567.172	3.572
10	2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucopyranoside	$C_{20}H_{22}O_9$	Stilbene (phenolic glycoside)	10.47	405.119	1.1
11	2,4,6-Trihydroxyacetophenone-2,4-di-O-β-D-glucopyranoside	$C_{20}H_{28}O_{14}$	Phloroglucinol (phenolic glycoside)	4.976	491.141	3.943
12	Cimidahurine	$C_{14}H_{20}O_8$	Amorphous phenolic glycosides	3.632	315.108	13.136
13	2′-Acetylacteoside	$C_{31}H_{38}O_{16}$	Phenylethanoid glycosides	10.941	665.21	4.737
14	Cistanoside A	$C_{36}H_{48}O_{20}$	Phenylethanoid glycosides	0.815	845.272	2.085
15	Cistanoside H	$C_{22}H_{32}O_{13}$	Phenylethanoid glycosides	8.047	503.177	1.273
16	Decaffeoylacteoside	$C_{20}H_{30}O_{12}$	Phenylethanoid glycosides	9.284	461.166	0.641
17	Tubuloside E	$C_{31}H_{38}O_{15}$	Phenylethanoid glycosides	13.408	649.215	0.594
18	Meliadanoside B	$C_{15}H_{20}O_8$	Phenylpropanetriol glycosides	11.975	327.111	1.045
19	Kuzubutenolide A	$C_{23}H_{24}O_{10}$	Phenolic glucoside	7.62	505.135	30.612
20	Caesalpins P	$C_{16}H_{12}O_6$	Phenolic cassane diterpenoids (flavonoid)	15.097	299.056	2.678
21	Coniferol	$C_{10}H_{12}O_3$	Phenolic cinnamic alcohol	10.24	179.071	1.414
22	Darendoside A	$C_{19}H_{28}O_{11}$	Phenethyl alcohol glycosides	5.565	431.156	0.834
23	Feroxin A	$C_{17}H_{24}O_8$	Anthocyanin	6.12	401.145	10.192

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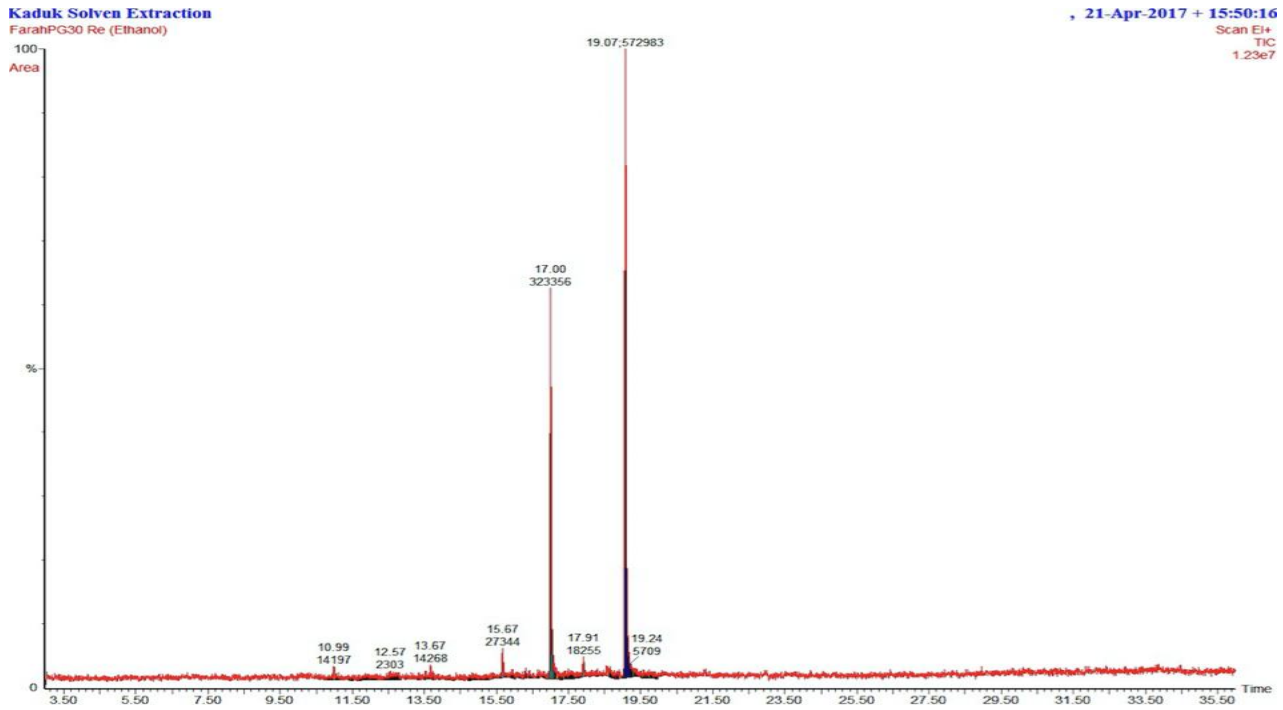

Figure 3: LC-MS chromatogram of phenolic compounds detected in methanolic extract of *P. sarmentosum* leaves.

Determination of inhibition concentration value (IC_{50}) for half of cell population against *A. castellanii*

The inhibition concentration of PSLME for 50% of *A. castellanii* cell population (IC_{50} values) was obtained from the non-linear sigmoidal dose-response curve of percentage of cell population inhibition against the logarithm of PSLME concentration (Figure 4). The result showed that the plant leaves methanolic extract has good anti-amoebic properties against *A. castellanii* with the IC_{50} value of 74.62 µg/mL.

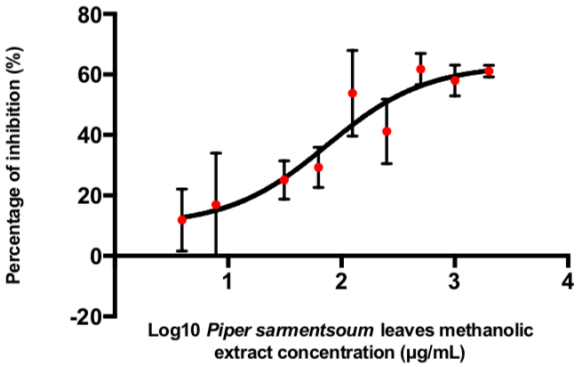

Figure 4: Non-linear dose response curve of *P. sarmentosum* leaves methanolic extract on *A. castellanii.* Error bars based on standard error of mean (SEM).

Microscopy observation of *A. castellanii* cell morphology

Distinctive alterations were exhibited in the PSLME-treated *A. castellanii* cell morphology as shown in Figure 5. The healthy untreated cells or trophozoites had irregular shapes with distinct acanthopodia (Figure 5A). The presence of vacuoles and nucleus were easily detected in the trophozoites. Meanwhile, the extract-treated *A. castellanii* were reduced in size (Figure 5B). The cells were observed to be detached from the flat surface due to the shortening and loss of acanthopodia and became rounded. The features of the nucleus and contractile vacuoles were non-distinguishable. The chlorhexidine-treated cells were observed to cause more severe damages by significant reduction in the cell size and become spherical (Figure 5C). The formation of double cell membrane was also clearly detected.

Measurement of cell confluence in axenic culture

The aim of the analysis was to gain an overview image of the cell population and to analyse the cell confluence using ImageJ. It was noted that the cell confluence changes after 72 h treatment with the plant extract at IC_{50} values (Figure 6). Under the microscopy observation, the bright-orange dots represented the cells colonies while the dark space represented the space between the cells colonies. The higher the confluency of the cells observed, the lesser the dark space observed (Figure 6A). Based on the calculation of area fraction (AF), healthy untreated *A. castellanii* is classified as a confluent population as it

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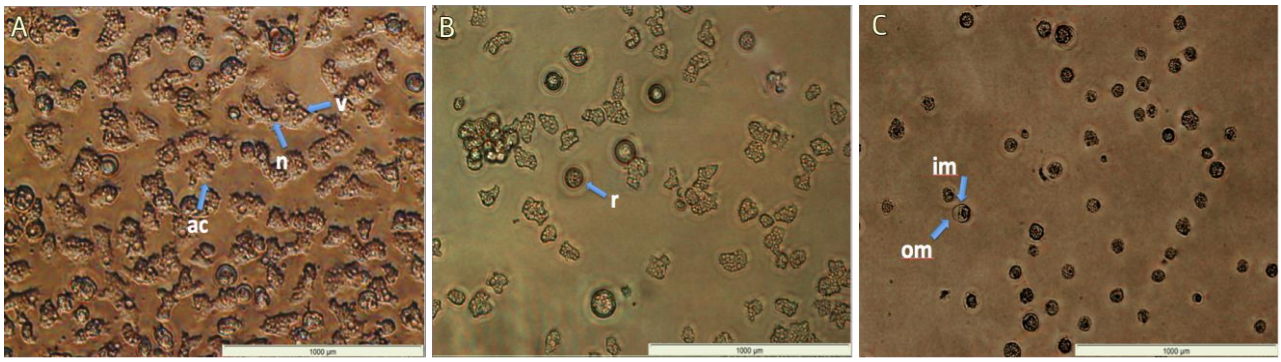

Figure 5: Inverted light microscopy images of *A. castellanii* (CCAP 1501/2A). (A) Untreated cells, (B) *P. sarmentosum* leaves extract-treated cells and (C) Chlorhexidine-treated cells (positive control). Note: nucleus (n), vacuoles (v), acanthopodia (ac), rounded cell (r), inner membrane (im), outer membrane (om). Scale bars 1000 µm; magnification 20×.

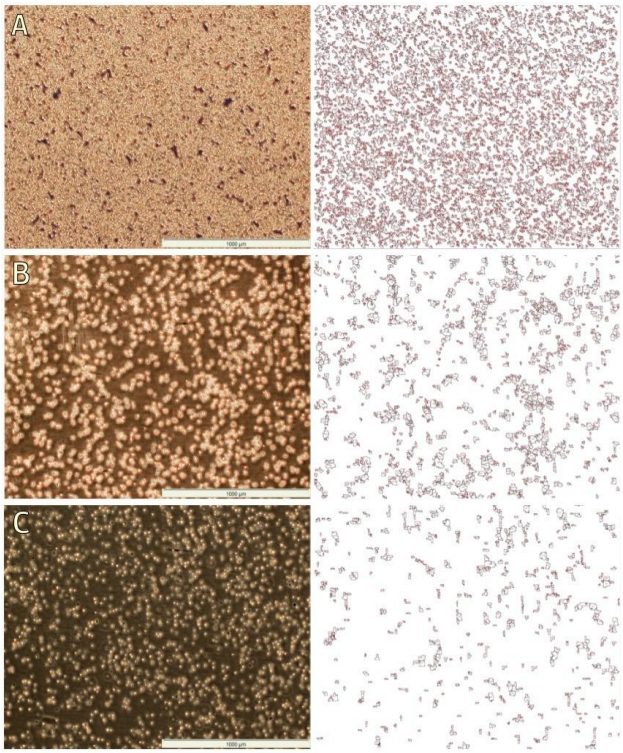

Figure 6: Measurement of *A. castellanii* (CCAP 1501/2A) cell confluency. Left image: Cells image under inverted light microscope (Scale bars 1000 µm; magnification 4×). Right image: Drawing of Area Fraction (AF) output generated by ImageJ software. (A) Untreated cells, (B) *P. sarmentosum* leaves extract-treated cells and (C) Chlorhexidine-treated cells (positive control).

achieved AF value of above 30 (31.99). However, the cell confluence was reduced when treated in PSLME with the AF value of 19.06. The positive controls showed the lowest AF values of 5.76.

Scanning electron microscopy (SEM)

Under SEM, healthy untreated *A. castellanii* cells displayed oval or triangular cell shape with the presence of many slender protrusions throughout the cells surface. Acanthopodia features were distinguishable indicated by the spine-like projections around the cell membrane. The presence of distinct vacuoles and nucleus embedded on the cell cytoplasm demonstrated that the amoeba lived in a favourable growth condition (Figure 7A). The PSLME-treated *Acanthamoeba* was shrunken as the cell shape started to change from irregular into rounded form with smoother cell surface (Figure 7B). The vacuoles and nucleus were not visible. Meanwhile, the chlorhexidine-treated cell exhibited a complete cyst form with massive reduction in cell size, total loss of acanthopodia, vacuoles and nucleus (Figure 7C).

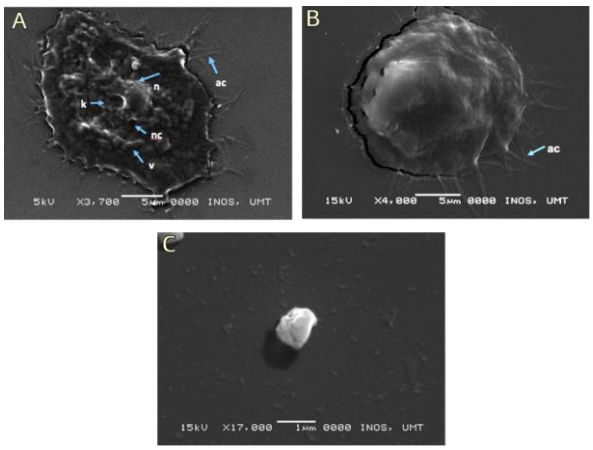

Figure 7: Scanning electron micrograph of *A. castellanii* (CCAP 1501/2A) single cell. (A) Healthy untreated cell (magnification 3700×), (B) PSLME-treated cell (magnification 4000×) and (C) Chlorhexidine-treated cell (positive control) (magnification 17000×). Note: acanthopodia (ac), nucleus (n), nucleolus (nc), karyosome (k), vacuoles (v).

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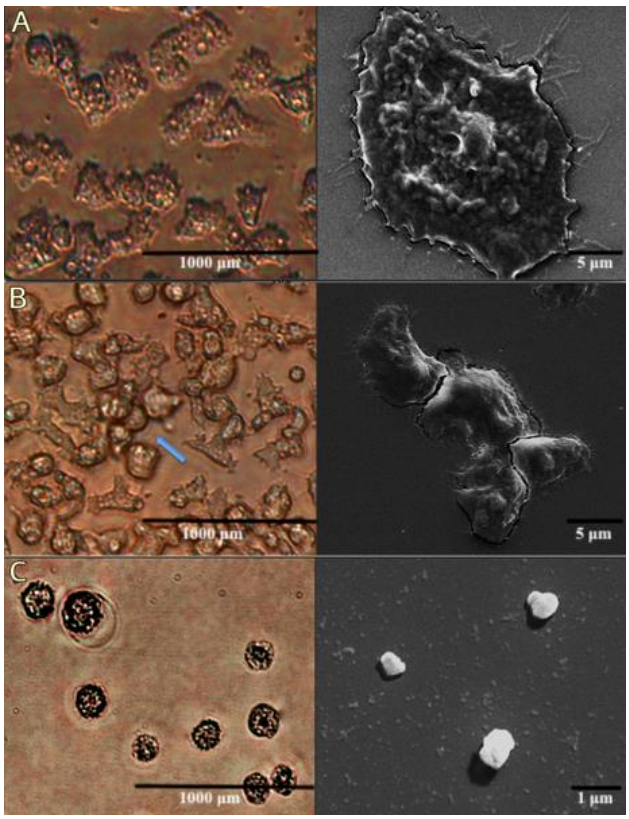

Figure 8: Zoomed-in microscopy image of *A. castellanii* (CCAP 1501/2A) morphology in axenic culture. Left image: Inverted light microscopy. Right image: Scanning electron microscopy. (A) Untreated cell, (B) *P. sarmentosum* leaves extract-treated cell and (C) Chlorhexidine-treated cell (positive control). Note: Arrow indicates clumping cells.

Changes of *Acanthamoeba* morphology in axenic culture

It was also observed that PSLME had altered the morphology of *A. castellanii* cells as the growth environment became unfavourable. Figure 8 shows the microscopic images of *A. castellanii* morphology in axenic culture. In the untreated condition, healthy *A. castellanii* grew as individual trophozoite cells (Figure 8A). Meanwhile, extract-treated cells were observed to grow in small clumps with their neighbouring cells. Chlorhexidine-treated *Acanthamoeba* displayed individual cystic-appearance form of cell (Figure 8C).

Determination of membrane integrity

The cells that were stained green to indicate that their membrane was intact, whereas cells that were stained orange or stained green with the granules in the cells stained red or orange indicated that the cell membranes' integrity had been compromised by the extract and therefore, they were classified as non-intact. In *A. castellanii,* the extract had caused membrane blebbing as indicated in Figure 9B. However, the PSLME-treated cells still maintain their membrane integrity. The positive control of chlorhexidine had caused more significant damage to cell membrane integrity, which was exhibited by the red fluorescence (Figure 9C).

Quantitative evaluation of membrane intactness was conducted. Figure 10 shows the bar charts of the percentage of membrane intactness. The results showed that without any treatment, almost all counted cells had intact membrane that gave the percentage of 94%. The treatment of PSLME extract led to reduction in the percentage of the cells with intact membrane, which was 57%. Chlorhexidine caused more severe effect than the extract with only 8% *A. castellanii* left with intact cell membrane. T-test analysis showed that there was significant difference between cells with non-intact from intact membrane (p<0.05) when treated in PSLME.

DISCUSSION

The selection of methanol as the solvent to extract phenolic compounds was based on its characteristic as a high polar solvent that could increase the solubility of phenolic components, therefore was capable of yielding high amount of phenolic compounds (Babbar *et al.*,

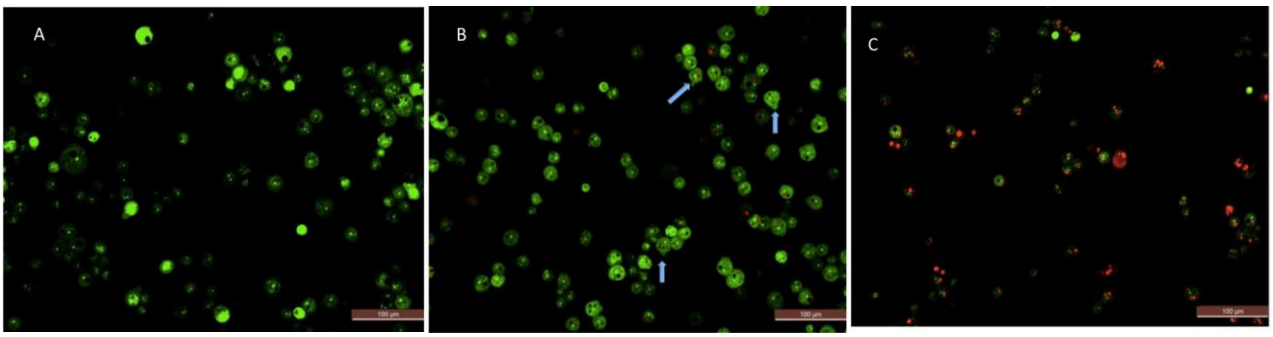

Figure 9: Fluorescence micrographs of *A. castellanii* (CCAP 1501/2A) stained with AO/PI dyes attached with zoomed-in cell image. (A) Untreated cells, (B) Extract treated-cells and (C) Chlorhexidine-treated cells (positive control). Note: Arrows indicate membrane blebbing. Scale bars: 100 µm; magnification 20×.

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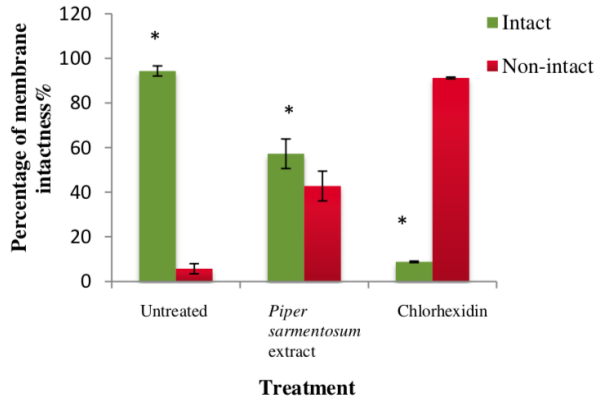

Figure 10: Percentage of membrane intactness of *A. castellanii* (CCAP 1501/2A). Data represent mean ± standard error of mean from three replicates.

2012). According to Tsao (2010), most of phenolic compounds are classified as hydrophilic where the interaction between the polar sites of hydrophilic phenolic compounds and the solvent would occur, resulting in better solvation of the *P. sarmentosum* leaves bioactive phenolics in methanol.

The total phenolic compound (TPC) value in this study surpassed the yield from *P. sarmentosum* methanolic extract reported by Mustafa *et al.* (2010) with the values of 90.86 mg [GAE]/g. Ugusman *et al.* (2012) also showed lower value of TPC yield of 91.02 mg [GAE]/g obtained from *P. sarmentosum* aqueous extract. The reduced TPC observed in these previous studies emphasized the evidence that different extraction method and solvent could influence the yield of phenolic compounds (Babbar *et al*., 2012). In a study reported by Sárosi *et al.* (2011), plants grown in higher temperature and solar irradiance could accumulate higher amounts of phenolic compounds. As the sample in this study was cultivated under direct sun with periodically irrigation, this explains the highest amount of TPC in *P. sarmentosum* compared to the aforementioned previous studies.

Upon chromatography analyses, it was noted that there was no similar compounds detected between GC-MS, RP-HPLC and LC-MS results. According to Dai and Mumper (2010), due to the complexity of the plant phenolics, different compounds may have different reactivity towards the assay solvents or reagents. The present GC-MS result was in accordance with the report by Rahman *et al.* (2014a), which found similar content of benzenepropanoic acid and asarone. The presence of asarone in the leaves of *P. sarmentosum* extract was also reported by many authors (Sim *et al.*, 2009; Bactiar and Fahami, 2019; Zainol Abidin *et al.*, 2020). Meanwhile, Tuntiwachwuttikul *et al.* (2006) had detected significant amount of benzenepropanoic acid in *P. sarmentosum* ethanolic extract. It is an intermediate in plant phenylpropanoid biosynthesis pathway. Detection of myristicine in *P. sarmentosum* was revealed in the study conducted by Rameshkumar *et al.* (2017) and Chaprapai and Chavasiri (2017). In other works, a study reported by Chieng *et al.* (2008) proved that the myristicine isolated from *P. sarmentosum* leaves oil has significant inhibitory activity against subterranean termite namely *Coptotermes* sp. Additionally, the finding of methyleugenol in this study was validated by the reviews done by Sanusi *et al.* (2017) and Sun *et al.* (2020).

The presence of gallic acid, tannic acid, quercetin and naringenin in this study was supported the previous report by Rahman *et al.* (2014b) and Rahman *et al.* (2016) through RP-HPLC analysis. In addition, the reviews by Azelan *et al.* (2020) and Sun *et al.* (2020) also cited the presence of naringenin and quercetin in *P. sarmentosum* extract. According to several authors, *P. sarmentosum* is reported to contain the isolation of a number of amides and phenylpropanoids (Likhitwitayawuid *et al*., 1987; Masuda *et al*., 1991; Parmar *et al.*, 1997; Stöhr *et al*., 1999; Rahman *et al.*, 2016). These reports are in line with the findings from the LC-MS result, which also reported the same class of phenolic compounds. Other than that, the presence of tannin also corresponded to several findings by some authors in *P. sarmentosum* research (Chanwitheesuk *et al.*, 2005; Hussain *et al.*, 2009; Sanusi *et al.*, 2017).

The anti-acanthamoebic activity of PSLME was classified as moderately active based on the cytotoxicity assays reported by Calzada *et al.* (2005). The antiprotozoal activities of medicinal plants are significantly active when the IC_{50} values are less than 38 µg/mL and moderately active when the IC_{50} values ranging from 41.7 to 100 µg/mL. The presented IC_{50} values results were relatively low when compared to previous works that studied the anti-amoebic activities of varieties of medicinal plants extracts on *A. castellanii.* For example, the methanolic extracts of *Passiflora caerulea*, *P. incarnate*, and *P. alata* Curtis leaves and calluses gave the IC_{50} values ranges from 4 to 15 mg/mL (Hadaś *et al.*, 2017), while the methanolic extract of *Rubus ulmifolius* showed IC_{50} value of 61.79 mg/mL (Hajaji *et al.*, 2017).

Naringenin and quercetin that were detected in the PSLME extract had been highlighted to possess strong antimicrobial, anti-inflammatory and antiprotozoal properties (Tasdemir *et al.*, 2006; Jananie *et al.*, 2011; Sre *et al.*, 2012). Naringenin, which was isolated from *Lippia graveolens* Kunth had been shown to have higher antiprotozoal activity against *Entamoeba histolytica*, compared to other flavonoids of sakuranetin and cirsimaritin (Quintanilla-Licea *et al.*, 2014). Meanwhile, *in vivo* test conducted by Wong *et al.* (2014) demonstrated that quercetin was the most potent compounds possessing leishmanicidal potential to combat the infection caused by *Leishmania donovani* in mouse models.

Maddox *et al.* (2009) emphasized that plant phenolic compounds exhibit significant antimicrobial activity against wide ranges of pathogenic microorganisms. According to Sanchez-Mata and Tardío (2016), the number of hydroxyl groups and its specific locations in the benzene ring are closely associated with the level of toxicity of a phenolic compound to microorganism, noting

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that the higher the hydroxylation process involved, the higher the inhibitory effect. The effect of hydroxylation can be seen for simple phenolics such as aspidinol. The presence of one hydroxyl group at C4' that is attached to one methoxyl group at C7' in a phenolic compound is essential for anti-parasitic properties (Grecco *et al.*, 2012).

Through microscopic morphological observation, PSLME caused distinctive alterations in the amoeba morphology with notable cells rounding as a result of shortening of acanthopodia. Acanthopodia is important in the cells movement for food and prey capturing, adhesion to surfaces and communication between cells (Bowers, 1969). The acanthopodia were observed to be thickened and elongated as an effort for cells to remain tightly attached to the surface substratum. SEM analysis revealed that PSLME-treated cells had altered acanthopodia. Acanthopodia is crucial in signal transmissions to induce the encystment process as the cells survival strategy by entering the dormant and resistance stages through diverse molecular and cellular modifications (Aguilar-Díaz *et al.*, 2011).

In the presence of PSLME, the cells organelles especially nucleus and vacuoles were also shrunken and undistinguishable. At this stage, *A. castellanii* cells are metabolically inactive as they are undergoing encystment process. Shrunken nucleus causes the process of DNA replication and genetic information translation to cease. Additionally, the microscopy images of healthy *A. castellanii* also showed that numerous contractile vacuoles were embedded in the cell cytoplasm playing the key role in maintaining the cell osmotic regulation. In that process, the vacuoles contract to expel excess water from the inner part of the cells, simultaneously facilitates the cells to move and capture food (Bowers and Korn, 1974). The treatment with PSLME led to massive loss of vacuoles in *A. castellanii,* hence, causing disruption in the cell homeostasis, at the same time, diminishing the phagocytosis process for food and prey (Kusrini *et al.*, 2016). Collectively, the morphology alterations in extract-treated *A. castellanii* lead to reduction in the cell population size indicated by low measurement of cell confluence.

The emission of bright green fluorescence is corresponding to the AO dye property that is readily permeable into the plasma membrane of *Acanthamoeba* cells (Hashim *et al.*, 2015). Meanwhile, cells with non-intact membrane would allow the exclusion dye of PI to penetrate into the inner part of cells through the leakages and pores on the cell membrane (Schlicher *et al.*, 2010). Therefore, *A. castellanii* emitted precipitates of orange or red fluorescence dispersed in the green cytoplasm due to the cytotoxic effect of PSLME. The previous study conducted by Nakisah *et al.* (2012) also showed similar result when *A. castelanii* was treated with *Aaptos* sp. extracts. The cells also became shrunken and smaller in size, which indicated an apoptosis has taken place.

CONCLUSION

By employing GC-MS, RP-HPLC and LC-MS, 33 phenolic compounds were identified in PSLME. The plant extract was found to be active in inhibiting the cells proliferation of *A. castellanii in vitro* indicated by low IC_{50} value of 74.62 µg/mL. The cytotoxic effects are consistent in the subsequent microscopy analyses that demonstrated that the plant extract induces encystment process, hence making the cells to become inactive. The study provides the first preliminary data that describes the potential of phenolic content in PSLME of having potent inhibitory effects against *A. castellanii*. This fundamental research could provide new knowledge to benefit pharmacological field to treat *Acanthamoeba*-related diseases by utilizing phenolic compounds from *P. sarmentosum*.

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