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Autophagic cell death induction in a clinical isolate of *Acanthamoeba* sp. treated with methanolic extracts of *Pereskia bleo* Kuhn

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ABSTRACT

Aims: This study was aimed to investigate the effects of methanol extracts from various parts of the *Pereskia bleo* Kuhn plant on *Acanthamoeba* sp. The antioxidant levels of each extract from different plant parts were measured after the extraction process. These extracts were then exposed to *Acanthamoeba* sp. to assess dose-response, IC_{50} values, changes in cell morphology, internal cell activity and apoptosis based on alterations in phospholipids.
Methodology and results: The total phenolic content, carotenoid estimation and antioxidant activity of the leaves, flowers and fruits of *P. bleo* were measured based on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) assay. Its anti-amoebic properties were tested using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay for the IC_{50} determination. The morphological and biochemical changes in the *Acanthamoeba* sp. were observed under light and fluorescence microscopy using the acridine orange and propidium iodide double staining (AO/PI). The IC_{50} values of *P. bleo* leaves, flowers and fruits methanolic extracts were 5.884%, 0.1646% and 20.69%, respectively. Morphological observation displayed shortened acanthapodia with darkened cytoplasms. AO/PI-stained *Acanthamoeba* sp. cells appear with orange-fluorescent organelles in their green cytoplasm, indicating autophagic cell deaths. Apoptotic and necrotic *Acanthamoeba* sp. cells were absent based on Annexin V labelling.
Conclusion, significance and impact of study: This study confirmed that the methanolic crude extracts of *P. bleo* exhibit high cytotoxic potential towards *Acanthamoeba* sp. trophozoites by inducing an autophagic mode of cell death.

Keywords: Acanthamoeba keratitis, Annexin V, antioxidant, autophagy, β-carotene

INTRODUCTION

Acanthamoeba sp. is a pathogenic protozoan of the rhizopodan order, Amoebida. These free-living amoebae are the causative agents of granulomatous amoebic encephalitis (GAE), a fatal disease of the central nervous system (CNS) and *Acanthamoeba* keratitis (AK), a painful sight-threatening disease of the eyes. Over the years, an increase in the cases of AK has been reported correlating with the increased use of contact lenses. Improper care, such as unclean lens practice, can cause keratitis infection initiated by pathogens like amoeba, bacteria, and fungi. This study was conducted on *Acanthamoeba* sp. samples from keratitis patients in Kuala Lumpur Hospital (HKL). *Acanthamoeba* keratitis is one of the most challenging eye diseases to treat and the recurrence is very high due to the amoeba's interchangeability from active trophozoites to dormant cyst stages.

Treatment of the *Acanthamoeba* sp. cysts can be very challenging as they become resistant to various chemotherapy due to their newly formed cell wall structures, which are absent in trophozoites. Tragically, eye infection allows the cells to be in both trophozoite and cyst phases. During the trophozoite phase, the *Acanthamoeba* sp. is much more vulnerable and sensitive to treatments. This study shows the presence of anti-amoebic properties in local plant methanolic extracts, namely *P. bleo*, commonly known as Kunth or rose cactus, collected from around the Universiti Malaysia Terengganu (UMT) Campus area. *P. bleo*, belonging to the Cactaceae family, is widely used in traditional medicine by the locals in Malaysia to treat various health

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issues. Recently, *P. bleo* has received increased attention in the therapeutic and medicinal fields to develop traditional or modern treatments against an array of diseases. Antioxidant analyses were performed to identify the total phenolic and carotenoid content (beta-carotene). DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was used to determine the antioxidant properties of *P. bleo* flowers, fruits and leaves. The cytotoxicity potential of flowers, fruits and leaves methanolic crude extracts of this medicinal plant towards *Acanthamoeba* sp. (a corneal scrapping isolate from a keratitis patient, HKL) was evaluated based on the mode of cell death shown by the cells.

The present study investigated the individual cytotoxicity potential of *P. bleo* flowers, fruits and leaves on *Acanthamoeba* sp. Several studies have investigated the potential pharmacological activities of *P. bleo* methanolic crude extracts, including antioxidant, anti-inflammatory and anticancer properties (Wahab *et al.*, 2009). In this study, each part of this plant was successfully extracted with methanol and these methanolic crude extracts were assessed for their cytotoxicity effects on *Acanthamoeba* sp. by cell viability assay (MTT) for the establishment of IC_{50} values and through light and fluorescence microscopy using AO/PI staining. The methanolic crude extracts of *P. bleo* flowers, fruits and leaves revealed a toxicity range of less than 10% in their IC_{50} values and through microscopy, strongly suggest the possession of efficient cytotoxic effects towards *Acanthamoeba* sp. cells. The morphological alterations in *Acanthamoeba* sp. cells and autophagic cell death supported the cytotoxic properties of this plant. The confirmation of the mode of cell death in *Acanthamoeba* sp. induced by the methanolic crude extracts of *P. bleo* flowers, fruits and leaves were based on the absence of apoptosis and necrotic cell death by flow cytometry with Annexin V-FITC and PI staining technique. Annexin V-FITC kit allows fluorescent detection of annexin V bound to apoptotic cells and quantitative determination by flow cytometry. The Annexin V-FITC kit uses annexin V conjugated with fluorescein isothiocyanate (FITC) to label phosphatidylserine sites on the membrane surface. Hence, the detection and confirmation of the preferable mode of cell death (apoptosis) in *Acanthamoeba* sp. post-treatment with the crude extracts provided information on the properties of this plant in treating eye-related diseases. In addition, the results obtained from this study also indicated whether the different methanolic crude extraction from each part of the *P. bleo* would exhibit different effects on *Acanthamoeba* sp. cells.

MATERIALS AND METHODS

Sample preparations

The fresh flowers, fruits and leaves of *P. bleo* were collected from around the UMT Campus area. Methanolic crude extracts from different parts of *P. bleo* were obtained using different techniques due to the varying water content, juice and drying interval. The details on the methods used for all three parts of the *P. bleo* are as follows:

The collected flower samples were washed and dried in the oven at 44.5 °C for 24 h and were ground into fine powder 10 g of dried flower samples were extracted using 125 mL of 80% methanol and kept in a rotary shaker at 190–220 rpm for 24 h. Then, the *P. bleo* flower extract was filtered using filter paper in a vacuum pump, followed by centrifugation at 10000 rpm for 5 min. The supernatant was collected while the solvent was let to evaporate in the fume chamber. Finally, the *P. bleo* flower extract was stored in a blue cap bottle at 4 °C (Ruban and Gajalakshmi, 2012).

To prepare the methanolic crude extracts of *P. bleo* fruits, the collected fruits were washed and dried in the oven at 44.5 °C for 24 h. Then, the dried fruits were ground into fine powder. At room temperature, approximately 20 g of the ground dried fruits were extracted using 250 mL of 80% methanol. The macerate was evaporated using a rotary evaporator at 50 °C for 24 h. Finally, the *P. bleo* fruit extract was stored at 4 °C in a blue cap bottle (Sutomo *et al.*, 2014).

To prepare *P. bleo* leaves methanolic crude extracts, leaves were washed and dried in the oven at 44.5 °C for 24 h. The dried leaves were ground into fine powder. Then, 20 g of the powdered dried leaves were extracted using 250 mL of 80% methanol using a shaking water bath for 2 h at 40 °C. Next, the aliquot was filtered with filter paper using a vacuum pump. Lastly, the *P. bleo* leaves extract was concentrated using a rotary evaporator at 40 °C before being stored in a blue cap bottle at 4 °C (Hassanbaglou *et al*., 2012).

Antioxidant analyses

Total phenolic content (TPC) was conducted by collecting the sample by centrifugation at 1000 rpm for 15 min before being analyzed. An aliquot of 200 µL of the supernatant was mixed with 1.5 mL of Folin-Ciocalteu reagent (previously diluted tenfold with distilled water) and was allowed to stand at room temperature for 5 min. Following 90 min incubation, 1.5 mL of 6% (w/v) sodium bicarbonate solution was added to the mixture. After the incubation period, the absorbance was read spectrophotometrically at 725 nm. The standard calibration (0.01–0.05 mg/mL) curve of gallic acid in the 80% methanol curve was plotted. Results were expressed as gallic acid equivalents (GAE) in mg per 100 g sample extracts (Maisarah *et al*., 2013).

For total carotenoid estimation (β-carotene), 20 mL petroleum ether was added into a separating funnel. Next, 15 mL of acetone extraction was added and allowed to stand for 15 min. Then, 150 mL of distilled water was added until the formation of two phases was observed, where the aqueous phase was removed. The petroleum ether phase was washed four times with 100 mL of distilled water to remove residual acetone. Next, the petroleum ether was collected and mixed with 7.5 g of anhydrous sodium sulfate to remove residual water. Lastly, it was filtered using filter paper and β-carotene

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content was determined based on molar absorptivity β-carotene $E^{\%}$ = 2590 at λ_{max} = 450 nm derived from the standard plots (Sahabi *et al.*, 2012).

DPPH free radical scavenging assay was performed using the method Maisarah *et al*. (2013) described; 200 µL of the sample extracts (0.62–4.96 mg/mL) was added to 1 mL of 0.1 mM DPPH in 80% methanol. The mixture was shaken vigorously and left to stand in a dark room for 30 min at room temperature. The absorbance of the solution was then measured spectrophotometrically at 517 nm with 80% methanol as blank. The assay is based on the measurement of the scavenging capacity of antioxidants. The capability of the samples to scavenge the DPPH radical was calculated using the equation as follows:

Scavenging effect (%)
= 1 – (Absorbance of the sample at 517 nm/Absorbance of control at 517 nm) × 100

Acanthamoeba sp. culture media preparation

Protease yeast glucose (PYG) media was prepared by adding 3.75 g of protease, 3.75 g of yeast and 7.5 g of D+ glucose in the Page's amoeba saline (PAS) solution that was earlier prepared by mixing 5 mL of Stock 1 and 5 mL of Stock 2 in 990 mL distilled water. Stock 1 is a 12 g NaCl and 0.4 g $MgSO_4$ mixture in 500 mL distilled water. Stock 2 is a mixture of 14 g Na_2HPO_4 and 13.6 g KH_2PO_4 in 500 mL distilled water.

Acanthamoeba sp. culture and maintenance

The *Acanthamoeb*a sp. cells (HKL isolate) were cultured in T-25 tissue culture flasks with 5 mL of PYG media. The flasks were then incubated at 30 °C and were observed every day. *Acanthamoeb*a sp. cells were subcultured every three to four days or when they reached confluency.

IC_{50} value of *P. bleo* methanolic crude extract on *Acanthamoeba* sp.

Acanthamoeba sp. cells (~1 × 10^5 cells per well) were seeded in 96-well plates containing 100 µL of PYG media. After 8 h of incubation, PYG media containing different concentrations of extracts from the flowers, fruits and leaves of *P. bleo* ranging between 30% to 0.45% were added into the wells with three replicates for each extraction. This method was conducted with a negative control, the untreated cells. For IC_{50} determination, the stock solution of MTT (5 mg MTT/mL PBS) was added to all 96-well plates and then incubated at 30 °C for another 4 h. Following the incubation, 150 µL of DMSO was added, and cells were resuspended to dissolve all the formazan blue crystals. The plates were then read using a Dynatech MR580 MicroElisa reader at a wavelength of 570 nm. This method was adapted from Mossman (1983).

Morphological assessment of *Acanthamoeba* sp. cells

The effects of the extracts on the morphology of *Acanthamoeba* sp. were assessed and compared with the control cultures after 24 h. The treatments with *P. bleo* extract (leaves, fruits, flowers extract) were carried out in 6-well plates containing 3 mL of culture medium with 10^4 cells/mL at their IC_{50} concentration. The morphological changes of the cells were observed directly on the 6-well plates using an inverted microscope (Leica Dmire Microscope, Germany). The particular alteration in the acanthapodia structure, the cells' shape and reduced cell sizes indicate the effects of the extracts on the *Acanthamoeba* sp. cells (Nakisah *et al.*, 2012).

For internal biochemical changes observation, the untreated and *P. bleo* extracts-treated *Acanthamoeba* sp. cells were harvested by centrifugation at 3000 rpm for 15 min after a 24 h incubation time. Then, the supernatant was decanted, and the pellet was washed once with PBS and centrifuged at 1000 rpm for 5 min. The pellets were resuspended with 100 µL AO/PI solutions that were prepared by adding 2 µL of acridine orange (1 mg/mL) and 2 µL propidium iodide (1 mg/mL) in 996 µL PBS. The cell suspensions were then incubated in the dark for 10 min since both dyes are light-sensitive. Finally, the *Acanthamoeba* sp. cell suspensions were observed directly under the inverted fluorescence microscope (Nakisah *et al.*, 2012).

Apoptosis confirmation

To determine and confirm the apoptosis events in *Acanthamoeba* sp. post-treatment with the flowers, fruits and leaves extracts of *P. bleo*, annexin V-FITC was used to detect the membrane alterations based on phosphatidylserine by flow cytometry. Firstly, the cells were spun down for 1 min at 3000 rpm and the supernatant was decanted. The cells were then washed in ice-cold PBS at pH 7.4 and gently resuspended. Following resuspension, the cells were spun down again for another 1 min at 3000 rpm, where the supernatant was decanted. The excess PBS was removed using a pipette, and the cells were resuspended in 1× binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, filtered through 0.2 µm pore filter) followed by the adjustment of the cell density to 2–5 × 10^5 cells/mL. Next, 10 µL of Annexin V-FITC was added to 190 µL of cell suspension and mixed gently. The cells were then washed with 1x binding buffer after an incubation period of 10 min at room temperature. Following that, the cell resuspension with 190 µL binding buffer was repeated. Finally, 10 µL of 20 µg/mL propidium iodide stock solution was added before analysis using flow cytometry (Life Technologies-Novex).

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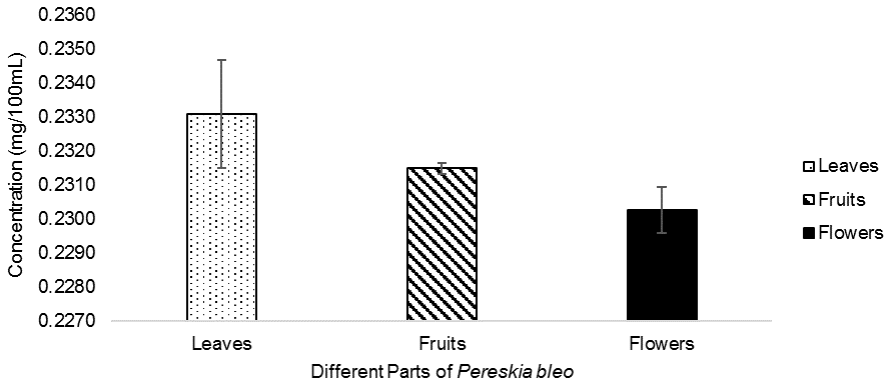

Figure 1: Total phenolic content in leaves, fruits and flowers of the *Pereskia bleo.* Error bars represent standard error.

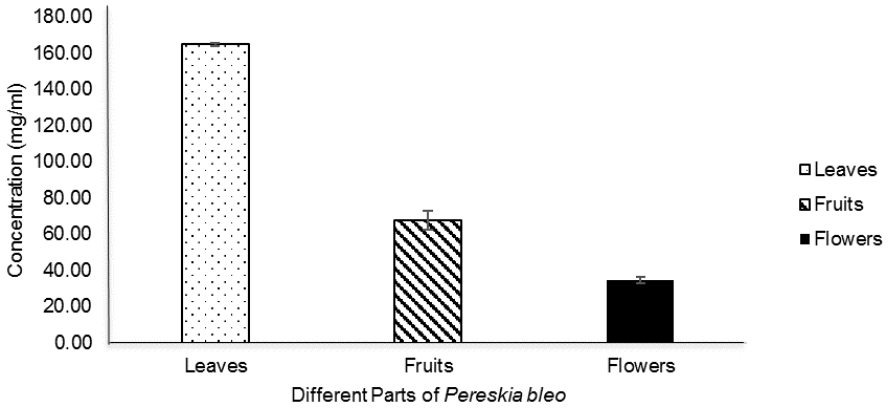

Figure 2: Total carotenoid content in leaves, fruits and flowers of *Pereskia bleo.* Error bars represent standard error.

RESULTS

Antioxidants analyses

The total phenolic content (TPC) for each sample was estimated in comparison with the standard gallic acid solution (mg/100 mL). Based on Figure 1, TPC was highest in the leaves. However, there was no significant variation between different parts of *P. bleo* at the $p>0.05$ [$F(2, 6) = 2.014$, $p = 0.214$]. The results displayed that the total phenolic content contained in leaves, fruits and flowers are 0.2331 ± 0.0028 mg/100 mL, 0.2303 ± 0.003 mg/100 mL and 0.2296 ± 0.0012 mg/100 mL, respectively.

Total carotenoid estimation was measured at OD=475 for the different parts of the plant. They ranged from 20–160 mg/mL. Based on the results presented in Figure 2, the leaves of *P. bleo* contain the highest total carotenoid content (165.04 ± 1.63 mg/100 mL), compared to the fruits (67.71 ± 9.31 mg/100 mL) and flowers (36.53 ± 2.72 mg/100 mL) of the plant. There was a significant variation between the three parts of *P. bleo* at the $p<0.05$ [$F(2, 6) = 427.428$, $p = 0.000$]. Post hoc comparison using the Tukey HSD test indicated that the mean score for the leaves (M = 1.65, SD = 0.016) was significantly different from fruits (M = 0.35, SD = 0.027) and flowers (M = 0.68, SD = 0.093).

The antioxidant activity was determined using the DPPH method. The estimated values of the antioxidant activities by DPPH ranged between 70–74% scavenging effects against the samples. Based on Figure 3, the flower extract of this plant exhibited better antioxidant potential compared to standard ascorbic acid by the DPPH scavenging assay method. However, no significant difference between the subjects was detected using one-way ANOVA at $p>0.05$ [$F(2, 6) = 1.728$, $p = 0.255$]. The scavenging activity in leaves, fruits, and flowers is estimated at 74.44 ± 0.86%, 71.79 ± 1.18% and 70.38 ± 4.46%, respectively.

Determination of *Acanthamoeba* sp. cell viability and IC_{50} value

Cell viability assay (MTT) is an appropriate method for the fast screening of new substances. The cytotoxicity study was conducted to analyze the *in vitro* anti-amoebic activity of flowers, fruits, and leaves methanolic crude extracts of *P. bleo* on the pathogenic species of *Acanthamoeba* (HKL isolate). After incubating the *P. bleo* methanolic crude extracts-treated cells with tetrazolium

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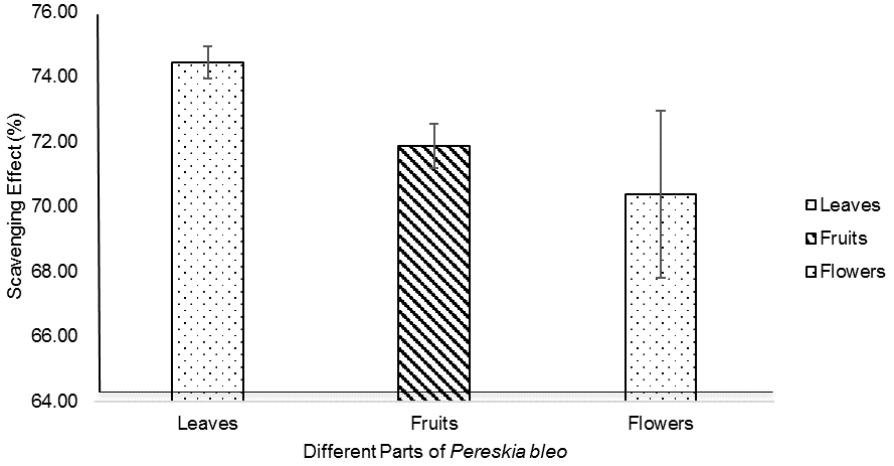

Figure 3: The scavenging activity in different parts of the *Pereskia bleo*. Error bars represent standard error.

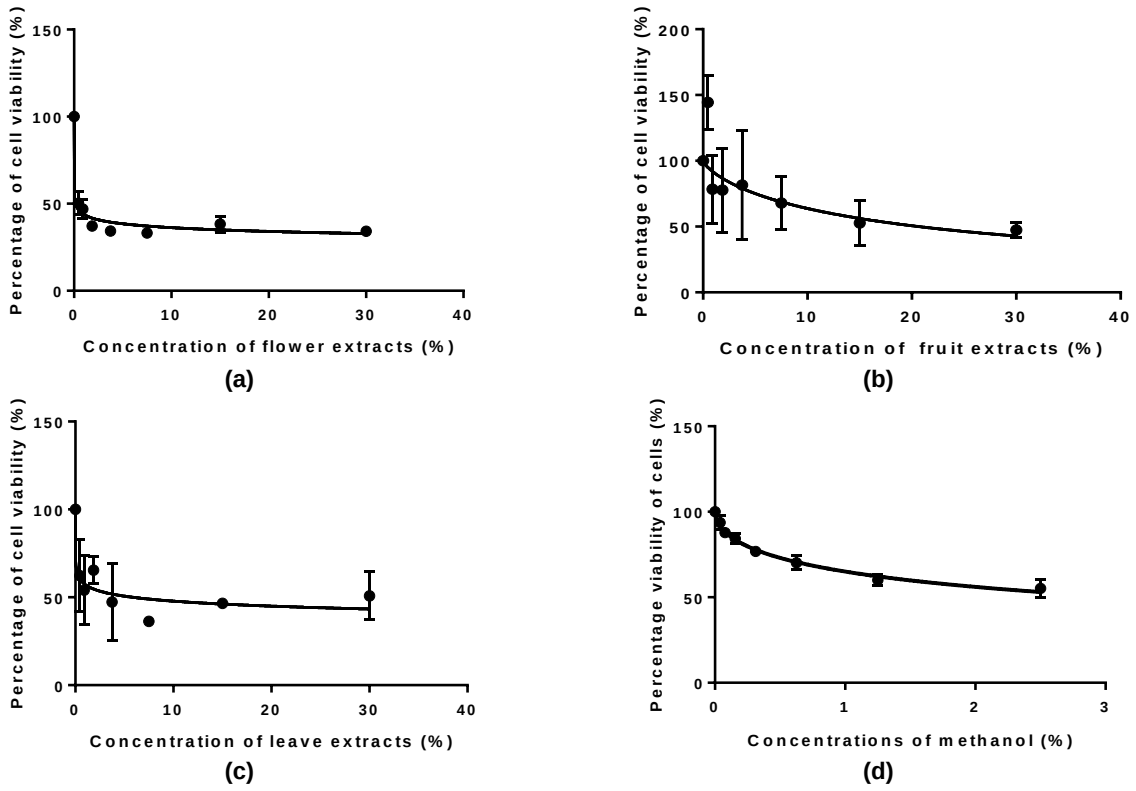

Figure 4: Percentage of *Acanthamoeba* sp. cell viability post-treatment with (a) Methanolic crude extract of *Pereskia bleo* flower (IC_{50} value = 0.1646%), (b) Methanolic crude extract of *P. bleo* fruit (IC_{50} value = 20.69%), (c) Methanolic crude extract of *P. bleo* leave (IC_{50} value = 5.884%) and (d) Methanol as our negative control (no IC_{50} value).

salt for several hours, the dark blue crystal of the formazan product was formed. DMSO was used to dissolve it. The half-maximal inhibitory concentration (IC_{50} value) was obtained based on the absorbance reading using a Micro Elisa reader with a wavelength of 570 nm. The curves derived from Graph Prism software are presented in Figure 4.

Referring to Figure 4, the concentration of *P. bleo* flowers, fruits, and leaves methanolic crude extracts that can inhibit the cells' biological process by half percent (IC_{50}) were 0.1646%, 20.69% and 5.884%, respectively. To ensure significant results, various dilutions of methanol were used to study the effects of the solvent for extracting *P. bleo* crude extracts towards *Acanthamoeba*. Based on

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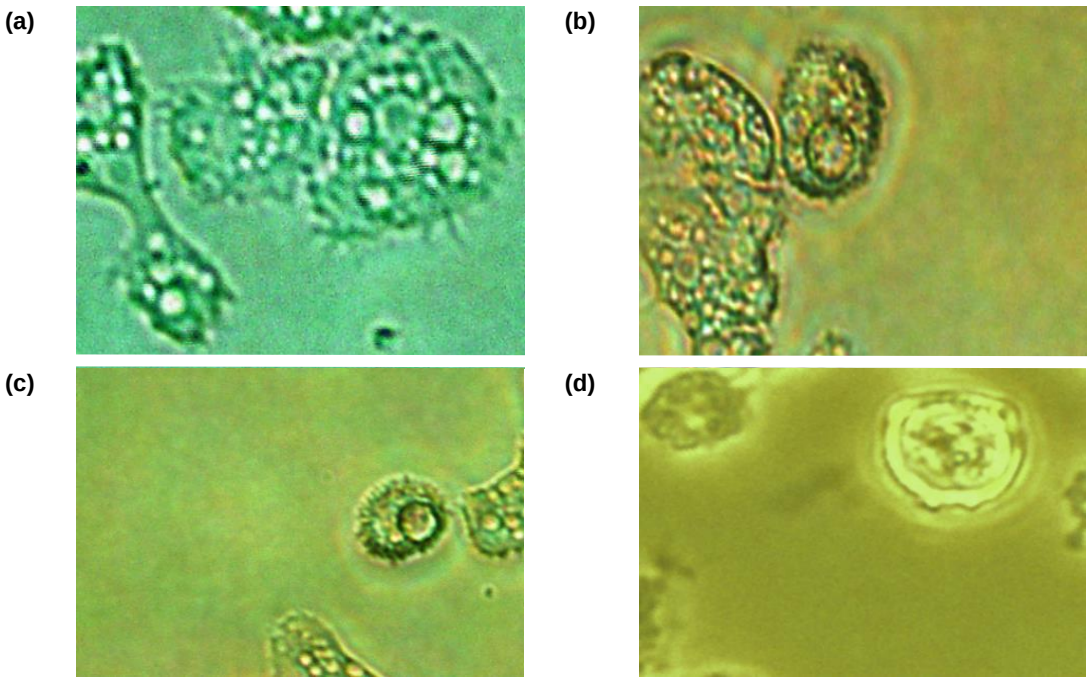

Figure 5: Images of *Acanthamoeba* sp. cells under light microscopy. (a) Untreated as negative control, (b) Post-treatment with methanolic crude extract of *Pereskia bleo* flowers, (c) Post-treatment with methanolic crude extract of *P. bleo* fruits and (d) Post-treatment with methanolic crude extract of *P. bleo* leaves (Magnification ×200).

the results obtained, *Acanthamoeba* sp. cells showed a reduction in viability upon exposure to the treatments.

Morphological changes on *Acanthamoeba* cells

Morphological observation in Figure 5 showed the difference in the morphological structure of untreated *Acanthamoeba* sp. cells and *P. bleo* methanolic crude extracts-treated *Acanthamoeba* sp. cells were identified using light microscopy. The untreated *Acanthamoeba* sp. cells exhibited the distinct structure of acanthapodia; a spiny surface projection (black arrow), vacuoles (orange arrows) and nuclei are shown in Figure 5a. However, post-treatment with the flowers, fruits, and leaves with methanolic crude extracts of *P. bleo* (Figure 5b–5d), the cell's nucleus was not prominent and the *Acanthamoeba* sp. cells decreased in size. Besides the shortened acanthapodia, the large-sized vacuoles were also visible. The shape of the cells became rounded and displayed a cystic appearance, suggesting that the *P. bleo* methanolic crude extracts can induce the encystment of *Acanthamoeba* sp. cells.

The mode of cell death induced by methanolic crude extracts is indicated by the biochemical changes observed under fluorescent microscopy with acridine orange and propidium iodide as dyeing agents. Figure 6 displays the fluorescence microscopy observation of *Acanthamoeba* sp. cells. Upon exposure to AO/PI staining solution, untreated *Acanthamoeba* sp. cells appeared as green fluorescence cells. The light green colour of the nucleus and the dark-coloured vacuoles were observed (Figure 6a). Furthermore, post-treatment with flowers, fruits and leaves with methanolic crude extracts of *P. bleo* (Figure 6b–6d), the *Acanthamoeba* sp. cells were stained green with orange fluorescence organelles in their green cytoplasm, indicating high lysosomal activity compared to the untreated *Acanthamoeba* sp. cells which were stained green entirely showing healthy viability in the untreated cells.

Apoptosis detection

As indicated in AO/PI observation using fluorescence microscopy, no apoptotic events were observed in methanolic extracts-treated *Acanthamoeba* sp. cells. Annexin V also confirmed this result with the absence of apoptosis cell death in *Acanthamoeba* sp. cells. Apoptosis in *Acanthamoeba* sp. cells as early as 24 h post-treatment was confirmed by flow cytometry stained with annexin V-FITC and propidium iodide (PI) dyes. This method was chosen due to its sensitivity in detecting apoptotic and necrotic cell death in eukaryotic cells, where the binding ability of annexin V-FITC to phosphatidylserine on the cell's membrane and also the binding of the PI dye to the cellular DNA in cells can produce significant differences in the apoptotic and necrotic cells. Figure 7 shows the results of the flow cytometry analysis of *Acanthamoeba* sp. cells.

The flow cytometry results indicated typical quadrant analysis of fluorescence intensity of gated *Acanthamoeba* sp. cells in annexin V-FITC and PI channels post-treatment with *P. bleo* flowers, fruits, and leaves

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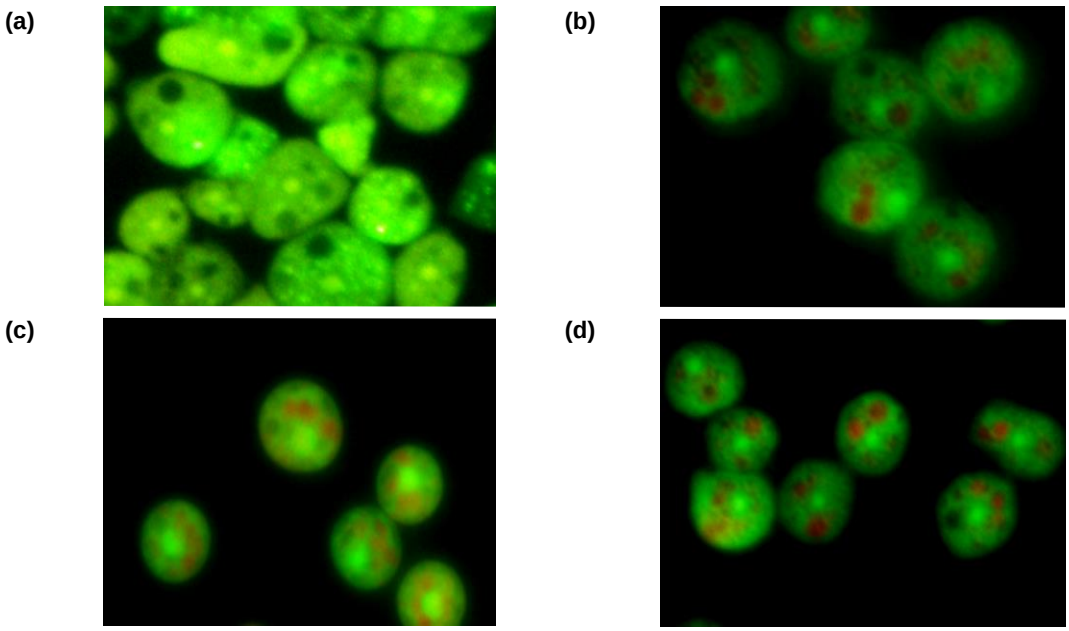

Figure 6: Images of *Acanthamoeba* sp. cells under fluorescence microscopy observation. (a) Untreated cells as negative control, (b) Post-treatment with methanolic crude extract of *Pereskia bleo* flowers, (c) Post-treatment with methanolic crude extract of *P.bleo* fruits and (d) Post-treatment with methanolic crude extract of *P. bleo* leaves (Magnification ×200).

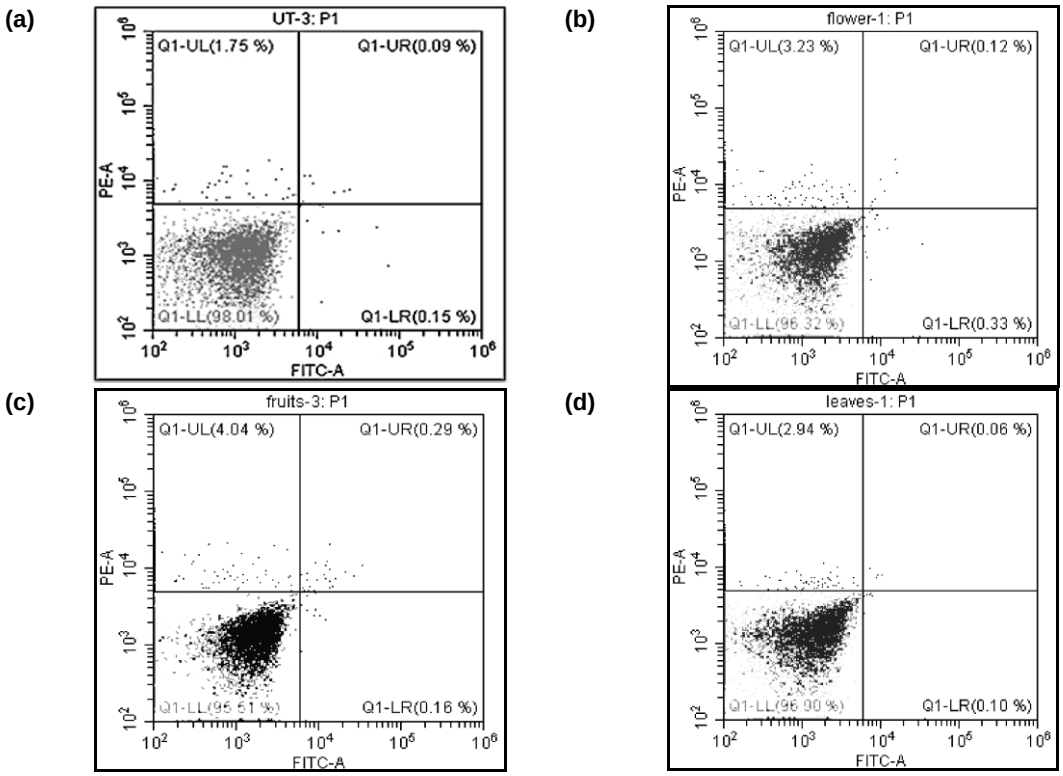

Figure 7: Results of flow cytometry analysis of *Acanthamoeba* sp. cells. a) Untreated cells as negative control, (b) Post-treatment with methanolic crude extract of *Pereskia bleo* flowers, (c) Post-treatment with methanolic crude extract of *P. bleo* fruits and (d) Post-treatment with methanolic crude extract of *P. bleo* leaves.

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methanolic crude extracts. In each figure, the lower left quadrant represents viable cells, the lower right quadrant represents early apoptotic cells, the upper right quadrant represents late apoptotic cells that lead to necrosis, and the upper left quadrant represents cells that underwent necrotic cell death. Based on the graphs, no apoptotic event was detected in all *Acanthamoeba* sp. cells treated with *P. bleo* flowers, fruits and leaves methanolic crude extracts with less than 1% percentage of apoptotic cells detected.

DISCUSSION

The phenolic content was highest in *P. bleo* leaves, followed by fruits and flowers. The phenolic content was determined based on the synthetic antioxidant gallic acid standard curve. Crude extracts of herbs, spices, and other plant materials high in phenols have been suggested to have therapeutic characteristics and excellent effects on human health and they are gaining popularity since they have been demonstrated to be very efficient oxidant scavengers and lipid peroxidation inhibitors (Alu'datt *et al.*, 2017). The methanolic extraction method was used to obtain the phenolic content in different parts of the plant to maximize the extraction volume of each part (Maisarah *et al*., 2013). Polar solvents like methanol, ethanol and water produced a high yield of phenolic compounds more efficiently than non-polar solvents (Huda-Faujan *et al.*, 2015). Due to breakdown and polymerization processes, these variables may influence the quantity and quality of antioxidants in the extracts (Bimakr *et al.*, 2011). Besides that, phenolic phytochemicals contained in plants act as promising anti-inflammatory, anti-thrombotic, anti-allergenic and antimicrobial compounds (Huda-Faujan *et al.*, 2015). Phenolic compounds protect against nontransmissible chronic diseases (NTCDs) such as cardiovascular disease, cancer, obesity and diabetes not only through their antioxidant activity but also through their ability to regulate a variety of cellular processes at various levels, such as enzyme inhibition, gene expression modification, protein phosphorylation and so on (de la Rosa *et al*., 2018). The phenolic compounds believed to possess redox properties act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelation action (Huda-Faujan *et al*., 2015). Based on the findings of this study, phenolic compounds in leaves of *P. bleo* are the highest antioxidant contained among other plant parts. Research on gooseberry revealed a similar pattern, with total phenolic content changing by the portion of the plant, with leaves having the most significant value (Medina-Medrano *et al*., 2015; Ertürk *et al*., 2017; Nurhaslina *et al*., 2019).

Carotenoids are a family of pigmented compounds found only in plants, such as yellow-orange fruits and dark green, leafy vegetables (Olson and Krinsky, 1995). Carotenoids are lipophilic secondary metabolites produced by the isoprenoid pathway and found in most plant organs (Howitt and Pogson, 2006). The plant's capacity influences the carotenoid concentration of plant tissues to synthesise carotenoids in the tissue, which is one strategy for managing carotenoid accumulation (Yamamizo *et al*., 2009). Over 90% of the carotenoids exhibited in the human diet include β-carotene, α-carotene, lycopene, lutein and cryptoxanthin (Gerster, 1997). In this study, β-carotene in different parts of the *P. bleo* was determined using the synthetic antioxidant β-carotene standard curve. In addition, the acetone extraction method was used to extract β-carotene contents from different parts of the plant to maximize each part's extraction volume: the leaves, flowers and fruits (Hassanbaglou *et al*., 2012).

Based on the β-carotene determination, leaves contained the highest β-carotene content compared to fruits, followed by flowers. The lowest β-carotene content in flowers might be due to the extraction method that distinguished the yellow-orange color from the flowers, compared to the leaves and fruits, which maintained their color during the extraction process. β-carotene, lutein and alpha-carotene were found in abundance in the petals of yellowish-white cultivars, whereas β-carotene and α-carotene were found in abundance in the petals of orange-red cultivars (Wang *et al*., 2018).

To determine the antioxidant activity, the DPPH free radical scavenging assay was used based on the ability of antioxidant properties in the extracts to scavenge the stable radical DPPH (Olajuyigbe and Afolayan, 2011). The scavenging activity of extracts might be due to the phenolic content and total carotenoid estimation (β-carotene) in the *P. bleo* plant. For this assay, methanolic extraction was used because a previous study provided evidence that higher antioxidant activity of extracts can be found in alcoholic extracts such as methanol or ethanol (Huda-Faujan *et al.*, 2015). The correlation between antioxidant content and activity was proven in this study, where the leaves of *P. bleo* contained the highest phenolic and carotenoid (β-carotene) contents, exhibiting the best results for DPPH free radical scavenging assay, followed by its fruits and flowers. Bleaching of β-carotene is a free-radical-mediated process occurring from the hydroperoxides generated from linoleic acid by air oxidation. In the absence of antioxidants, β-carotene molecules oxidize and lose their double bonds and distinctive orange color, which may be measured using spectrophotometry (Sim *et al*., 2010). Since this plant has been used to treat vision problems, its potential to treat microbial keratitis, *Acanthamoeba*, was also evaluated. An *in vitro* assay was done to determine the dose-response and the mode of cell death in *Acanthamoeba* cells post-treatment with the leaves, fruits and flowers' methanolic crude extracts of *P. bleo*.

In this study, MTT colorimetric assay was used to measure and quantitate the formation of dark-purple water-insoluble formazan dye of the tetrazolium salt to assess the cytotoxicity effects of *P. bleo* flowers, fruits and leaves methanolic crude extracts against *Acanthamoeba* sp. Furthermore, the ELISA reading obtained for the treated *Acanthamoeba* sp. cells was directly proportional to the number of viable cells in the culture plate after 24 h of incubation. Based on the results

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obtained, the methanolic crude extract of the *P. bleo* flower had the lowest IC_{50} value, which required 0.01646% of the extract to inhibit half of the *Acanthamoeba* cells population, followed by the leaves and fruits extracts, which needed 0.58840% and 2.069%, respectively, to inhibit half of the *Acanthamoeba* sp. cell population. Methanol was also used in this study as a negative control.

It is postulated that the varying IC_{50} values between the three parts of *P. bleo* were due to the differing phytoconstituents in the different parts of the plant. To date, among the 20 plant-derived phytoconstituents, phytol, alkaloids, fatty acids, glycosides, lactones, phenolic, sterol, terpenoid and carotenoid compounds have been reported in the leaves and two other components in the fruits are responsible for the biological activities of the *P. bleo* extracts (Zareisedehizadeh *et al.*, 2014). However, there was a lack of reports on the phytoconstituents isolated from the *P. bleo* flowers because leaves and fruits of *P. bleo* plant are most studied as this plant has been used mostly in traditional medicine. On the other hand, Daniels *et al*. (2011) stated that flavonoids of polyphenolic contents are the major contributor to the antioxidant properties in *Gethyllis multifolia* L. Bolus flowers. Thus, it is also believed that these constituents may be present in the *P. bleo* flowers, which could contribute to the cytotoxic potential of these *P. bleo* flowers against *Acanthamoeba* sp. cells. Hence, more research is recommended to develop further studies on the phytoconstituents in the different parts of *P. bleo*. The difference in toxicity between the flowers, leaves, and fruits of *P. bleo* in this study is assumably due to the presence of phytoconstituents.

In addition, *Acanthamoeba* sp. cells observed under light microscopy provided strong evidence of cellular changes within *Acanthamoeba* sp. cells when exposed to *P. bleo* flowers, leaves and fruits methanolic crude extracts for an incubation period of 24 h. Based on the light microscopy observation, the *P. bleo* methanolic crude extracts affected the *Acanthamoeba* sp. cell viability and induced morphological changes in the *Acanthamoeba* sp. cells. This event is a typical morphological change when *Acanthamoeba* is under a changing environment due to exposure to foreign substances. Similar observations were also recorded in other studies (Kusrini *et al*., 2018; Kusrini *et al*., 2020; Zamli *et al*., 2020). The observations demonstrated irregular cell shapes with numerous acanthapodia structures and a high number of vacuoles. Moreover, the *P. bleo* flowers, leaves and fruits methanolic crude extract-treated *Acanthamoeba* sp. cells exhibited a decrease in cell size and a reduction in the number of vacuoles, indicating the encystment event in *Acanthamoeba* sp. cells (Ibrahim *et al*., 2014). Encystment is a process where *Acanthamoeba* sp. cells change their life cycle due to the unfavorable conditions faced by the cells, such as starvation, low temperature, and exposure to biocides. This process leads to the formation of resilient cysts from vegetative trophozoites (Fatimah *et al*., 2011). Hence, the results obtained in this study indicate that the methanolic crude extract of *P. bleo* flowers, leaves and fruits could induce encystment events by creating stressful conditions for *Acanthamoeba* sp. cells.

On the other hand, through the morphological assessment, one of the most apparent changes detected in the treated *Acanthamoeba* sp. cells is the cell membrane disruption that resulted in the loss of tiny projection acanthapodia in the cells. According to Bowers and Korn (1968), acanthapodia is a spike-like projection of *Acanthamoeba* sp. cells that help in the cells' locomotion (to move within a substrate and catch prey). The treated *Acanthamoeba* sp. cells lost their acanthapodia, reducing pathogenicity. Based on the results, all of the methanolic crude extracts of *P. bleo* modified the structure of acanthapodia on the surface of *Acanthamoeba* sp. cells. This structure plays a crucial role in the pathogenesis of *Acanthamoeba* infections, such as *Acanthamoeba* keratitis. Besides, acanthapodia promotes the binding of the pathogenic *Acanthamoeba* sp. cells and initiates contact with the invaded host cells. This structure can also bind to corneal epithelial cells during *Acanthamoeba* infection (Siddiqui and Khan, 2012). Moreover, based on the morphological observation of the treated *Acanthamoeba* sp. cells using inverted light microscopy in this study, floated cells were observed in the culture medium, indicating the loss of the acanthapodia structures, causing the cells to be detached from the culture plate, which lead to the low pathogenicity effect of the *P. bleo* flowers, leaves and fruits methanolic crude extract-treated *Acanthamoeba* sp. cells. Thus, the alteration of acanthapodia structures between the untreated and treated *Acanthamoeba* sp. cells in this study revealed the cytotoxic potential of *P. bleo* flowers, leaves, and fruits methanolic crude extracts towards *Acanthamoeba* sp. cells.

The distinction of the cell death type is very important and relevant in the determination of the cytotoxic effects of *P. bleo* flowers, fruits and leaves methanolic crude extracts on *Acanthamoeba* sp. cells in this study. The mode of cell death determination reveals whether the *Acanthamoeba* sp. cells undergo three major types of cell death: apoptosis, necrosis, or autophagic cell death after being treated with the *P. bleo* flowers, fruits, and leaves methanolic crude extracts at their IC_{50} value for 24 h of incubation.

Membrane integrity plays a vital role in maintaining the inner compartment to ensure its survival. In the present study, the effects of the methanolic crude extracts on *Acanthamoeba* sp. cells were also evaluated using fluorescence dyes (acridine orange and propidium iodide (AO/PI)). The application of AO/PI staining performed in many previous studies helped differentiate the intact and non-intact membranes of the cells. Moreover, Ibrahim *et al*. (2014) stated that the AO/PI staining was based on membrane integrity. In contrast, Darzynkiewicz *et al.* (1997) noted that the cell death mode could be verified based on the cells' uptake of AO or PI after being employed by these dyes.

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The AO/PI staining observations by fluorescence microscopy obtained for both the untreated and treated *Acanthamoeba* sp. cells supported the previous findings of the light microscopy that the *P. bleo* flowers, fruits and leaves methanolic crude extracts altered the morphology of *Acanthamoeba* sp. cells due to pressure. However, Kusrini *et al.* (2018) reported that the internal morphological changes of treated *Acanthamoeba* sp. cells could not be confirmed and supported based on light microscopy observations alone; hence, the use of fluorescence microscopy with two fluorescence dyes was included in this study. The acridine orange and propidium iodide dyes were used to discriminate the mode of cell death induced by all compounds. Based on the AO/PI staining results, the untreated *Acanthamoeba* sp. cells appeared as green fluorescence cells. The cells were fluoresced with green cytoplasm and green nuclei, as seen in Figure 6a. Furthermore, the green fluoresced cells with an intact nucleus and cell membrane indicate that the cells are viable and healthy (Kusrini *et al.*, 2015). According to Mascotti *et al*. (2000), AO is a membrane-permeable, nucleic acid selective dye that binds to viable cells at low concentrations to generate green fluorescence.

Meanwhile, the *P. bleo* flowers, fruits and leaves methanolic crude extracts-treated *Acanthamoeba* sp. cells were stained green with an abundance of orange fluorescence organelles in their green cytoplasm, indicating the autophagic cell death compared to the untreated cells. Autophagic cell death involves a massive accumulation of a double membrane containing a vacuole known as an autophagosome that fuses with a lysosome, forming an autolysosome. The degradation of the autophagosomal content causes the cells to die upon exposure to cytotoxic drugs (Tsujimoto and Shimizu, 2005). In addition, Levine and Yuan (2005) reported that the excessively and continuously self-digestion of cell materials, including organelles, will eventually lead to autophagic cell death. From the results obtained, it is strongly believed that the orange fluorescence precipitates observed were the active lysosomes that were stained by AO dye due to the high content of hydrolytic enzyme indicated by the green to orange changes of the AO dye. This phenomenon was also reported in the mammalian cell culture system, as seen in MCF-7 cells (Kwan *et al.*, 2016) and HT-29 cells (Hajiaghaalipour *et al*., 2017).

AO, a cationic dye, enters an acidic compartment such as lysosomes, which in low pH conditions produces orange fluorescence precipitates that can be observed by fluorescence microscopy. The lysosome is an organelle within *Acanthamoeba* sp. cells that contains digestive enzymes that help in the degradation of any pathogenic materials (Scheid, 2015). In this study, the reaction between the lysosomes and the treatment given is believed to have caused changes in the pH within lysosomes to occur (low pH) due to the high activity of the hydrolytic enzyme. The AO uptake resulted from the active lysosomes proton pump where the high concentration of protons (low pH) caused the AO dye to enter the lysosomes in an uncharged form, become protonated and later be trapped in the organelle to emit orange fluorescence precipitates (Kusrini *et al*., 2015).

It is also noted that the orange-red color of both nuclei and cytoplasm of *Acanthamoeba* sp. cells, which were observed in Figure 6b, 6c and 6d, indicating the necrotic cells as PI staining dye can only enter the compromised-*Acanthamoeba* sp. cells membrane to emit red fluorescence (Kusrini *et al*., 2015). Therefore, results obtained in the present study confirmed that the *P. bleo* flowers, fruits and leaves methanolic crude extracts are capable of inducing an autophagic type of cell death in *Acanthamoeba* sp. cells after being exposed to the treatment at their IC_{50} value with 24 h incubation. In addition, the flow cytometry method was also used and labelled with annexin V-FITC and PI dyes to detect the presence of phosphatidylserine (PS) externalization event in *Acanthamoeba* sp. cells and membrane integrity. The externalization of PS to the outer layer of the cell's plasma membrane normally occurs during the early stages of apoptosis (Homburg *et al.*, 1995). In normal cells, PS is predominantly located on the inner leaflet of the cell membrane (Hingorani *et al*., 2011). In addition, Koopman *et al*. (1994) reported that a healthy blood cell displays a significant membrane phospholipid asymmetry in which phosphatidylcholine and sphingomyelin are predominantly located on the outer leaflet of the membrane, and most of the membrane's phosphatidylethanolamine and PS are located in the cytosolic side of the blood cells' plasma membrane. Thus, this study's detection and confirmation of apoptosis are based on the externalization of PS in the *Acanthamoeba* sp. cell membranes. Andree *et al.* (1990) stated that annexin V is a protein with a high affinity towards phospholipids, especially PS and this method has also been conducted with both annexin V-FITC and propidium iodide (PI) staining that can totally differentiate the apoptosis or necrosis type of cell death when it is performed together. Besides, Vermes *et al.* (1995) stated that the detection procedure of this method involves the binding of Annexin V-FITC to PS in the cell membrane at the beginning of the apoptotic process and the insertion of propidium iodide (PI) to the cellular DNA, which indicates the occurrence necrosis.

Based on the results obtained from this study, *P. bleo* flowers, fruits, and leaves methanolic crude extract treatments on *Acanthamoeba* sp. cells indicate no apoptosis indication at the membrane layer of *Acanthamoeba* sp. cells with a very low percentage (lower than 1%) of apoptosis detected by flow cytometry (Figure 7b–7e). The cell membrane of *Acanthamoeba* is unlike mammalian cells, which indicates the apoptosis event by PS exposure to the outer layer of the phospholipid bilayer. Upon the stress condition, *Acanthamoeba* rapidly turns its cellular metabolic activity into a secure condition, the cyst stage. At the beginning of the encystment process, trophozoites of *Acanthamoeba* synthesize glycogen molecules from glucose, followed by cellulose production to form ecto- and endocyst layer of the pre-cyst stage the pseudocyst of the *Acanthamoeba*

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*(*Moon and Kong, 2012). We also suggest other mechanisms of apoptosis detection in *Acanthamoeba* cells, as the PS component is absent in this eukaryotic microorganism.

On the contrary, plant extracts-treated *Acanthamoeba* sp. cells exhibited no signs of apoptosis in *Acanthamoeba* sp. cells through flow cytometry. This is due to the occurrence of different reactions produced by *Acanthamoeba* sp. cells treated with the plant extracts. In this case, the methanolic crude extracts of *P. bleo* might form separate particles and precipitation that lead to phagocytosis or pinocytosis, in which autophagosomes will subsequently fuse with lysosomes. The cytotoxic particles from the methanolic crude extracts enter the cell directly through phagocytosis or pinocytosis mechanisms and interact with the *Acanthamoeba* sp. internal cytosolic environment. Hence, based on the results obtained, it is highly suggestive that there was no PS externalization of the phospholipid bilayer event occurring in the methanolic crude extract-treated *Acanthamoeba* sp. cells. The particles within the plant-extract solutions interact directly with lysosomes after being engulfed or absorbed through phagocytosis or pinocytosis activities in *Acanthamoeba* sp. cells, leading to high activity of lysosomal (Műller, 1969; Hong *et al*., 2018) and proceeding to autophagic cell death.

Induction of cell death based on autophagosomal activity is another novel cell death mechanism in eukaryotic microorganisms *viz*. *Acanthamoeba* sp. has different phenotypes in its life cycle. Biochemical observations in *Acanthamoeba* cell death revealed that this death pathway is the primary mechanism for non-viable stage induction in *Acanthamoeba* and thus, elimination of this microorganism from the host is, in theory, can be achieved. Recurrence of infection can occur as excystment of the cyst would happen if the cyst of the *Acanthamoeba* environment is suitable for the phenotypic changes. Further investigation is needed to confirm the amoebicidal status in every anti-amoebic test.

CONCLUSION

In conclusion, it can be deduced that methanol extracts exhibit high antioxidant properties. Furthermore, they demonstrate promising antiamoebic qualities, as evidenced by the IC_{50} values derived from dose-response studies and significant changes observed in cell morphology and biochemical activities upon exposure to *P. bleo* methanol extracts. Therefore, there is a need for further exploration of this plant, not only for traditional uses but also for its potential in medicine against pathogens causing emerging diseases.

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