Original article

An evaluation of the phytochemical composition, antioxidant and cytotoxicity of the leaves of *Litsea elliptica* Blume – An ethnomedicinal plant from Brunei Darussalam

May Poh Yik Goh [a], Ajmal Faiz Kamaluddin [a], Terence Jit Loong Tan [b], Hartini Yasin [b], Hussein Taha [a], Abdalla Jama [a], Norhayati Ahmad [a,*]

[a] Environmental and Life Sciences, Faculty of Science, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE1410, Brunei Darussalam
[b] Chemical Sciences, Faculty of Science, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE1410, Brunei Darussalam

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ABSTRACT

Litsea elliptica is traditionally believed to prevent and treat stomach ulcers, cancer, fever and headaches. This study investigates the phytochemical composition, antioxidant and cytotoxic effects of *L. elliptica* leaf extracts. The phytochemical content was determined via GCMS analysis and total phenolic content (TPC) and total flavonoid content (TFC) were analysed using the Folin-Ciocalteu and aluminium-chloride assays. Antioxidant activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging and ferric-ion reducing antioxidant power (FRAP) assays, whereas cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and calcein/ethidium viability assays. The mechanism of cytotoxicity was investigated using Annexin V/propidium iodide. Modifications in the mitochondria were investigated using MitoTracker Red CMXRos. Ten and twenty-six compounds were characterized in the young-leaf and mixed-leaves extracts, respectively. The young-leaf methanolic extract demonstrated the highest antioxidant capacity of at least four-folds greater than the mixed-leaves and ethanolic extracts. The methanolic extract also had higher TPC and TFC values compared to the ethanolic extract. Although the mixed *L. elliptica* leaves had lower antioxidant capacities compared to the young leaves, the mixed leaves extract has demonstrated greater cytotoxicity against the A549 cancer cell line. Further investigation revealed that the *L. elliptica* leaves-induced cytotoxicity on A549 cells was possibly via the non-inflammatory mitochondria-mediated apoptotic pathway. Overall, our results showed the potential of the *L. elliptica* leaves possessing cytotoxic activities against carcinoma cells where the compounds present can be further investigated for its therapeutic application.
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1. Introduction

Knowledge of the biological properties and phytochemical content of known medicinal plants used traditionally is required prior to further exploration of its potential use in different applications such as drug development, biotechnology and the food industry.

In Brunei Darussalam, the use of herbal and traditional remedies has been practiced for generations. Being geographically located in an area with highly diverse plants and natural resources, access to these herbs and medicinal plants is within easy reach and availability.

Litsea elliptica Blume is a species within the Lauraceae family that can be widely found in the South East Asian forests (Wong et al., 2014; Taib et al., 2009; Goh et al., 2017). The leaf of this tropical tree has been remarked for its ability to treat and prevent ailments such as stomach ulcers, cancer, fever and headaches by the local communities of Brunei Darussalam, Indonesia and Thailand (Taib et al., 2009; Goh et al., 2017; Grosvenor et al., 1995; Suksamerkun et al., 2013; Bhamarapravati et al., 2002). In these regions, locals respectively have referred to the plant as "Pawas", "Madang Perawas" and "Tham-mung". There have been previous

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reports on the antioxidant activities of *L. elliptica* where its roots were shown to have high antioxidant activity compared to the synthetic antioxidant butylated hydroxytoluene (BHT) (Wong et al., 2014). However, further investigation on the potential pharmaceutical benefits of the *L. elliptica* leaves are still lacking.

In addition to *L. elliptica*, antioxidant activities have also been reported for the extracts of other *Litsea* species including the essential oil of *Litsea cubeba* (Wang et al., 2012), methanolic extracts of the leaves and bark of *Litsea monopetala, Litsea glutinosa, Litsea assamica and Litsea laeta* (Choudhury et al., 2013), methanolic extracts of various parts of *Litsea garciae* (Hassan et al., 2013) as well as the fermented beverage of *Litsea glaucescens* (Gamboa-Gómez et al., 2016). Other than antioxidant activities (Wang et al., 2012), two fractions of the methanolic extract of the *L. cubeba* fruit residue showed inhibitory effects against the proliferation of HeLa cells, a human cervical cancer cell line, *via* cell cycle arrest at the G1/S phase (Piyapat et al., 2013).

Other plants from the Lauraceae family including the leaves of *Litsea salicifolia* and the roots of *Litsea acuminate* have also shown cytotoxicity against brine shrimp nauplii in brine shrimp lethality bioassay (BSLA) and the HeLa cell line respectively whereas the *L. glutinosa* roots were potent against human colon and skin cancer cell lines (Roy et al., 2016; Tanaka et al., 2015; Ndi et al., 2016). This suggests that plants of the Lauraceae family have high cytotoxic potential. However, the cytotoxicity and chemo-preventive properties of *L. elliptica* against cancer cell lines are yet to be explored. Evaluation of the inhibitory effects of *L. elliptica* against cancer cell lines is necessary to understand the medicinal potential of the plant and its underlying mechanism of action. In light of the current interests of finding new potential source of compounds for further development of effective anticancer agents, this plant can be a candidate for further exploration. The present study reports the antioxidant activities of the ethanolic and methanolic extracts of the young and mixed leaves of *L. elliptica*, and the phytochemical composition and cytotoxicities of the methanolic extracts of the young and mixed *L. elliptica* leaves against A549, a human lung cancer cell line.

2. Materials and method

2.1. Plant material

Samples of the mixed leaves (consisting of approximately 20% young and 80% matured leaves) of *L. elliptica* were collected from Kampung Batu Ampar, Brunei Darussalam whereas the young leaves were purchased from the Tutong Market (Kompleks Pasarneka dan Tamu Tutong). Species identification was kindly confirmed by the taxonomist from Faculty of Science, Universiti Brunei Darussalam. A voucher specimen of the plant (Record No. S/00755; Collector No.: MGPY001) was deposited to the Universiti Brunei Darussalam Herbarium (UBDH).

2.2. Sample preparation

The collected samples were washed and rinsed to remove visible debris. Leaves were left to air dry in the shade at room temperature for about 14 days. The dried samples were pulverised using a domestic blender and stored in airtight containers at room temperature until further use.

2.3. Extract preparation of Litsea elliptica

2.3.1. Crude methanol and ethanol extraction by soaking and shaking

20 mg/mL stock concentrations of the extracts were made by soaking the powdered samples in either methanol or ethanol. The mixtures were then placed in a 40 °C water bath and shaken at 250 rpm for 1 h. Subsequently, the mixtures were centrifuged at 4500 rpm for 5 min followed by filtration through a Whatman No. 1 filter paper. The filtrates were then collected as the extract solutions and stored at 4 °C until further analysis.

2.3.2. Crude methanol extraction using the Soxhlet extraction method

Approximately 200 g of the pulverised samples were extracted with 1L of methanol using the standard Soxhlet extractor. The extraction cycle was repeated several times until the methanol collected in the reservoir of the Soxhlet extractor ran clear. The mixtures were then filtered by vacuum filtration and the methanol was evaporated from the filtrate using a rotary evaporator. The remaining crude extracts were then collected and stored at 4 °C until further use.

2.4. Identification of compounds via Gas Chromatography–Mass Spectrometry (GC-MS)

The methanolic extracts of the *L. elliptica* leaves obtained via Soxhlet extraction were analysed using GC-MS. The carrier gas used was helium. The pressure was set at 100.1 kPA, while the column and total flow rate were 1.69 mL/min and 50.0 mL/min respectively. The GC oven temperature was first held at 50 °C for 1 min. It was then increased to 140 °C at a rate of 20 °C/min and again to 300 °C at a rate of 10 °C/min and held for 10 min. Splitless injection was used to inject the sample at 250 °C. The interface temperature was 250 °C whereas the ion source temperature was 200 °C. The total time taken to run the analysis of each sample was 31.50 min. The detector scanned masses ranging between 2 and 500 *m/z*. The mass spectra of the compounds in the extracts were obtained *via* electron ionization at 70 eV. The mass spectra and retention indices of the volatile compounds in the extracts were compared to the compounds in the literature and National Institute Standard and Technology (NIST) library database.

2.5. Antioxidant capacity determination

2.5.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

The method described by Delgado-Andrade et al. was adopted to determine the radical scavenging activity of the plant samples with slight modifications (Delgado-Andrade et al., 2005). A 40 μg/mL DPPH solution in methanol was freshly prepared for the analysis. 0.2 mL of the serially diluted extract or quercetin was added to 1.0 mL of the methanolic DPPH solution and kept in the dark for 30 min. The absorbance was then recorded at 517 nm using a UV–visible spectrophotometer against methanol as the negative control. A formula was used to determine the percentage of radical scavenging activity for each of the concentrations:

$$\% \text{ Radical Scavenging Activity} = (A_c - A_s)/A_c \times 100$$

$$A_c = \text{Absorbance of DPPH control without sample}, A_s$$
$$= \text{Absorbance of sample}.$$

The IC_{50} was obtained from the curve of % radical scavenging activity against the concentrations of each plant extract. The quercetin equivalent antioxidant capacities (QEAC) of the extracts were also determined based on the formula:

$$QEAC = (IC_{50} \text{ quercetin}/IC_{50} \text{ extract}) \times 1000$$

2.5.2. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities of the extracts were deter-

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mined based on the protocol reported by Thaipong et al. with slight modifications (Thaipong et al., 2006). A stock solution of ABTS$^{\bullet+}$ radical cation was prepared by mixing 7.4 mM of aqueous ABTS and 2.6 mM of aqueous potassium persulfate in a ratio of 1:1 followed by a 12- to 20-hour incubation in the dark at room temperature. 3 mL of the ABTS$^{\bullet+}$ solution was then diluted in approximately 100 mL of methanol to obtain a working ABTS$^{\bullet+}$ solution with an absorbance of 1.1 ± 0.05 at 734 nm. 50 μL of the serially diluted extract or trolox was then mixed with 950 μL of the working ABTS$^{\bullet+}$ solution and allowed to react in the dark for 30 min. The absorbance of the reacted mixture was recorded at 734 nm using a UV–visible spectrophotometer, which was blanked with distilled water. The IC$_{50}$ values of the extracts or standard were then determined from the linear graph of % ABTS inhibition against extract or standard concentrations. The trolox equivalent antioxidant capacities (TEAC) of the extracts were then calculated based on the IC$_{50}$ values of the standard and the extracts such as shown in Section 2.5.1.

2.5.3. Ferric-ion reducing antioxidant power (FRAP) assay

The antioxidant capacities of the extracts were further evaluated using the ferric-ion reducing antioxidant power (FRAP) assay described by Gan et al. with slight modifications (Gan et al., 2010). 10 mM acidified 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 20 mM FeCl$_3$·6H$_2$O were prepared in 40 mM of hydrochloric acid and distilled water respectively. Fresh FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), TPTZ and FeCl$_3$·6H$_2$O solutions (10:1:1) and kept warm at 37 °C until use. Briefly, 50 μL of the serially diluted trolox or 1000 mg/L extracts was allowed to react with 950 μL of the FRAP reagent for 30 min in the dark. The absorbance was recorded at 593 nm using a UV–visible spectrophotometer and the FRAP result of the extract was estimated based on the linear regression of the standard trolox using the following equation:

$$[FRAP] = ([T] \times V \times D \times 100 \text{ mg})/m,$$

where [FRAP] = ferric-ion reducing antioxidant power in mg trolox equivalent (TE)/100 mg dry extract, [T] = trolox concentration determined from the calibration curve in mg/L, V = volume of extract in L, D = dilution factor of extracts, m = weight of dry plant sample extract in mg.

2.6. Total phenolic content (TPC) determination

The total phenolic contents of the extracts were quantified in gallic acid equivalents using the Folin-Ciocalteu's reagent method as described by Othman et al. with slight modifications (Othman et al., 2014). The extract stock solutions were diluted to 1,000 μg/mL. 0.5 mL of the diluted extract was added to 2.5 mL of ten-fold diluted Folin-Ciocalteu's reagent and 2.0 mL of 75 g/L of Na$_2$CO$_3$ and kept in the dark for 30 min. The absorbance was then measured at 760 nm against distilled water as blank. The total phenolic content of each extract was estimated from the standard calibration curve of gallic acid using the equation:

$$[TPC] = ([GA] \times V \times D)/m,$$

where [TPC] = total content of phenolic compounds in mg gallic acid equivalent (GAE)/g dry sample, [GA] = gallic acid concentration determined from the calibration curve in mg/L, V = volume of extract in L, D = dilution factor of extracts, m = weight of dry plant sample extract in g.

2.7. Total flavonoid content (TFC) determination

The method described by Madaan et al. was used to determine the total flavonoid contents of the extracts with slight modifications (Madaan et al., 2011). 0.5 mL of the 20,000 μg/mL extract

was added to 1.5 mL of 95% methanol, 0.1 mL of 10% AlCl$_3$, 0.1 mL of 1 M CH$_3$COOK and 2.8 mL of distilled water and kept in the dark for 30 min. The absorbance was then recorded at 415 nm using a spectrophotometer against a blank made up of 3.4 mL distilled water, 1.5 mL of 95% methanol and 0.1 mL of 1 M CH$_3$COOK. The total flavonoid contents of the extracts were estimated from the standard calibration curve of rutin using the equation:

$$[TFC] = ([R] \times V \times D)/m,$$

where [TFC] = total content of flavonoid compounds in mg rutin equivalents (RE)/g dry sample, [R] = rutin concentration determined from the calibration curve in mg/L, V = volume of extract in L, D = dilution factor of extracts, m = weight of dry plant sample extract in g.

2.8. Cytotoxic effect of L. elliptica against A549 lung cancer cell line

2.8.1. Cell culturing and harvesting

In the present study, A549 (human lung cancer cell line) were obtained from American Type Culture Collection (ATCC), USA. Cells were maintained in Dulbecco's modified essential medium (DMEM) (Sigma-Aldrich, Germany) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and cultured in a humidified 37 °C, 5% CO$_2$ incubator. The cultures were passaged every 2–3 days.

Once the cells have reached the exponential growth phase, they were harvested by first trypsinising the cells with 1.5 mL of 1× Trypsin-EDTA solution (SIGMA) followed by incubation at 37 °C for 5 min or until the cells were completely detached from the flask surface. The cells were then seeded into 96-well microplates at a concentration of 50,000 cells/mL and allowed to adhere to the bottom of the wells overnight.

2.8.2. Treatment of cells with extract

The crude methanolic extracts of the young and mixed L. elliptica leaves obtained via Soxhlet extraction were tested in the cytotoxicity study. A 40 mg/mL extract:DMSO solution was prepared and serially diluted in the media with 5% DMSO to obtain concentrations ranging from 0.03125 to 2 mg/mL.

80 μL of the media mixture containing 0.5% DMSO was added into each well of the microplate seeded with the cells. The cells were then treated with 20 μL of each of the working extract dilutions and a negative control consisting of media with 0.5% DMSO only. The cells were then incubated for 24, 48 and 72 h before their viability and morphologies were analysed.

2.8.3. Estimation of cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The Vybrant® MTT Cell Proliferation Assay Kit (Life Technologies) was used to determine the cytotoxic potential of the extract against A549 cells. Following 24, 48 and 72 h of treating the cells with the extract, 150 μL was removed from each well followed by the addition of 100 μL of 0.5 mg/mL MTT solution. The plates were then incubated for 2 h at 37 °C in 5% CO$_2$. Subsequently, the medium was removed and an excess of DMSO was added. The absorbances were recorded at 540 nm and 690 nm (background absorbance) with a microplate reader to determine the percentage cell viability in each well.

2.8.4. Analysis of cell morphology

The morphologies of the cells incubated with the extracts for 24, 48 and 72 h were analysed and images were captured using a microscope (Olympus IX73).

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2.8.5. LIVE/DEAD® cell viability assay

The live A549 cells were selectively stained in green fluorescence while the dead cells were stained red with the acetoxymethyl diacetylester of calcein (calcein-AM) and ethidium homodimer-1 (EthD-1) dyes respectively. Both dyes were obtained from the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes). The Hoechst 33342 dye (Molecular Probe) was also incorporated to stain the nucleus of the cells in blue fluorescence. A staining solution of 2 μM calcein-AM, 4 μM EthD-1 and 5 μg/mL Hoechst 33342 was prepared in sterile phosphate buffer saline (PBS) following the manufacturer's instruction.

The A549 lung cancer cells were initially cultured overnight in a 6-well microplate. The seeded cells were subjected to 24, 48 and 72-hour treatment with the extract at the maximum and IC_{50} concentrations that had demonstrated potent cytotoxicities against the cell line. The cells were then washed with sterile PBS and stained with 500 μL of the staining solution followed by a 30-minute incubation at room temperature. The fluorescence of the stained cells were captured using an inverted microscope equipped with camera (Olympus IX73).

2.8.6. Detection of early apoptosis using fluorescein isothiocyanate (FITC) Annexin V

The early and late apoptosis induced by the *L. elliptica* plant extracts was further investigated using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) according to manufacturer's protocol. The cells that had been treated with the extract for 24, 48 and 72 h were rinsed with PBS and stained with 500 μL of the staining solution for 15 min. The resulting fluorescence of the stained cells were observed and captured with the inverted microscope (Olympus IX73).

2.8.7. Mitochondrial analysis

The mitochondria of the A549 cancer cell were analysed using the MitoTracker™ Red CMXRos stain (ThermoFisher Scientific) according to manufacturer's instructions. A staining solution of 0.5 μM MitoTracker™ Red CMXRos was prepared in growth medium. The extract-treated cells were rinsed with PBS and stained with 500 μL of staining solution for 30 min. The fluorescent images of the stained cells were later evaluated and captured using the inverted microscope (Olympus IX73).

2.9. Statistical analysis

The data in this study was analysed using the one-way ANOVA method to determine the differences between the different extracts or the different treatment periods. Student's *t*-test was also used to assess the differences between two individual extracts. A significance level of $p < 0.05$ was set. All data were from triplicates independent tests and expressed in their average ± their standard deviation (SD).

3. Results

3.1. Phytochemical compounds of the young and mixed leaves of Litsea elliptica

The young-leaf extract was identified with ten compounds whereas the mixed-leaves extract was characterised with twenty-six compounds. The biological activities and reported applications of the compounds identified in the young-leaf and mixed-leaves extracts were determined from the literature and listed in Tables 1 and 2 respectively.

Both extracts of the young and mixed leaves were characterised with catechol, palmitic acid, methyl ester, linoleic acid, methyl ester, phytol, mono(2-ethylhexyl) phthalate and *dl*-α-tocopherol. This shows that these six compounds were present throughout the early and late stages of development of the *L. elliptica* leaves. The young-leaf extract possessed the highest level of phytol (**6**), octacosanol (**9**), palmitic acid, methyl ester (**4**), mono(2-ethylhexyl) phthalate (**7**) and oleic acid isopropyl ester (**8**) whereas the mixed-leaves extract had the highest level of pyrogallol (**5, 6**), palmitic acid (**15**), palmitic acid, methyl ester (**14**) phytol (**10, 13**) and mono(2-ethylhexyl) phthalate (**22**). Interestingly, phytol, palmitic acid and mono(2-ethylhexyl) phthalate were commonly present at high levels in both young-leaf and mixed-leaves extracts of *L. elliptica*.

3.2. Antioxidant activities of the young and mixed leaves of Litsea elliptica

The quercetin equivalent antioxidant capacities (QEAC) of the *L. elliptica* leaf extracts extracted using the soaking and shaking method were determined based on their DPPH radical scavenging activities as shown in Fig. 1.

The methanolic extracts of both young and mixed *L. elliptica* leaves have higher antioxidant capacities (QEAC = 55.34 ± 3.81 and 12.06 ± 0.90 mg QEAC/g dry sample respectively) compared to their ethanolic extracts (QEAC = 7.05 ± 0.23 and 3.51 ± 0.02 mg QEAC/g dry sample respectively). These results indicate that the methanolic extracts of the *L. elliptica* leaves possess higher antioxidant capacities than the ethanolic extract regardless of the maturity of the leaves. All of the extracts of the young *L. elliptica* leaves demonstrated significantly higher antioxidant capacities compared to the mixed-leaves extracts.

The young-leaf methanolic extract obtained *via* Soxhlet extraction had a DPPH radical scavenging activity of 273.89 ± 26.09 mg QEAC/g dry extract. The ABTS radical scavenging activity of the Soxhlet extract (IC_{50} = 116.24 ± 3.47 μg/mL) was about 20% lower than that of trolox (IC_{50} = 90.74 ± 0.91 μg/mL), which is equivalent to an ABTS scavenging activity of 781.25 ± 23.49 mg trolox equivalent antioxidant capacities (TEAC)/g dry extract. The Soxhlet extract of the young leaves have also demostrated a ferric-ion reducing antioxidant power (FRAP) of 29.54 ± 1.31 mg trolox equivalent (TE)/100 mg dry extract.

The antioxidant activities of the Soxhlet methanolic extracts of the mixed leaves and stem of *L. elliptica* are as shown in Fig. 2. The IC_{50} values of the extract of the leaf and stem were 326.28 ± 4.72 μg/mL and 1,859.01 ± 69.03 μg/mL respectively. This indicates that the leaves of *L. elliptica* have higher antioxidant activity than its stem. The IC_{50} values of quercetin and trolox were 19.43 ± 2.33 μg/mL and 53.59 ± 0.58 μg/mL respectively.

3.3. Total phenolic and flavonoid content (TPC and TFC) of the young L. elliptica leaves

The TPC and TFC of the methanolic and ethanolic extracts of the young *L. elliptica* leaves are as shown in Fig. 3. Both the methanolic and ethanolic extracts of the young *L. elliptica* leaves produced high TPCs of 134.97 ± 9.97 mg GAE/g dry sample and 108.00 ± 0.17 mg GAE/g dry sample respectively. In contrast, both of the alcoholic extracts have shown low TFCs of 15.90 ± 0.08 mg RE/g dry sample and 12.39 ± 0.09 mg RE/g dry sample.

3.4. Cytotoxicity of the methanolic extract of the Litsea elliptica leaves

As shown in Fig. 4, the IC_{50} values of both young-leaf and mixed-leaves extracts were the highest after 24 h of treatment (IC_{50} (young leaf) > 200 μg/mL; IC_{50} (mixed leaves) = 145.63 ± 17.11 μg/mL) followed by 48 h (IC_{50} (young leaf) = 136.70 ± 23.54 μg/mL; IC_{50} (mixed leaves) = 56.09 ± 23.38 μg/mL) and 72 h (IC_{50} (young leaf) = 44.00 ± 4.35 μg/mL; IC_{50} (mixed leaves) = 39.22 ± 7.

Table 1
Biological activities and nature of the phytochemical compounds identified from GC-MS analysis of the methanolic extract of the *L. elliptica* young leaves.

Peak No.	Retention Time (min)	Relative Peak Area (%)	IUPAC Name	Group	Biological Properties/Reported Applications
1	6.009	0.46	Benzene-1,2-diol Synonym: 1,2-Dihydroxybenzene; Catechol; *o*-Hydroquinone	Aromatic alcohol	Antibacterial, anti-dermatic, fungicidal, dye, antiseptic [1], anticancer (breast), antioxidant and pesticidal [2]
2	6.211	0.44	2,3-Dihydro-1-benzofuran Synonym: Coumaran	Coumaran	Anti-inflammatory, analgesic [3] and antimicrobial [4]
3	6.613	0.63	2-(4-Methylcyclohexyl) propan-2-ol Synonym: *p*-Menthan-8-ol; α-Dihydroterpineol	Cyclic alcohol	*No available information*
4	13.173	3.16	Methyl hexadecanoate Synonym: Hexadecanoic acid, methyl ester; Palmitic acid, methyl ester	Fatty acid ester	Antioxidant, hypercholesterolemic, pesticidal [2], antifungal, antiandrogenic, flavour, haemolytic, 5-alpha reductase inhibitor and potent antimicrobial activity [5]
5	14.788	0.91	Methyl (9Z,12Z)-octadeca-9,12-dienoate Synonym: 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; Linoleic acid, methyl ester	Fatty acid ester	Antibacterial [6] and anticancer [7]
6	14.94	4.66	(E,7R,11R)-3,7,11,15-Tetramethylhexadec-2-en-1-ol Synonym: Phytol; 3,7,11,15-Tetramethylhexadec-2-en-1-ol	Diterpene	Antimicrobial, anti-inflammatory, anticancer, diuretic [4] and cancer preventive [8]
7	18.544	3.06	2-(2-Ethylhexoxycarbonyl) benzoic acid Synonym: Mono(2-ethylhexyl) phthalate; Phthalic acid, mono-(2-ethylhexyl) ester	Phthalic acid monoester	Antifungal, anti-retroviral, anti-tumour, anti-diabetic, anticancer, antioxidant, anti-scabies, anti-inflammatory, potent antimicrobial agent [5], plasticizer for PVC and other resins and used as dielectric fluid [9]
8	21.519	2.59	Propan-2-yl (E)-octadec-9-enoate Synonym: 9-Octadecenoic acid (9Z)-, 1-methylethyl ester; Oleic acid isopropyl ester	Fatty acid ester	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, anticoronary, antiacne, antieczemic, and insectifuge [10]
9	22.832	3.29	Octacosan-1-ol Synonym: Octacosanol	Alcohol	Anticancer, hypocholesterolemic, anticoagulant, increase stamina, improve strength and reaction time for athletes [11]
10	23.05	2.07	2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydrochromen-6-ol Synonym: dl-α-Tocopherol; Vitamin E	Vitamin E	Anti-aging, analgesic, anti-diabetic, antioxidant, anti-inflammatory, antidermatitic, antileukemic, antitumor, anticancer, antibronchitic, vasodilator, anticoronary, antiulcerogenic, antispasmodic, anticoronary, antistroke, hypocholesterolemic and hepatoprotective [12]

[1] Saravanan et al. (2014), [2] Manorenjitha et al. (2013), [3] Hameed et al. (2015), [4] Kishore and Alluraiah (2013), [5] Martins et al. (2010), [6] Yu et al. (2005), [7] Ali et al. (2017), [8] Hema et al. (2011), [9] Waghmare and Kurhade (2014), [10] Ramesh and Dhanaraj (2016), [11] Venkata Raman et al. (2012), [12] Promprom and Chatan (2017).

92 µg/mL) respectively. Due to an inverse relationship between the IC$_{50}$ values and cytotoxic activities, the results showed that the cytotoxicities of both young-leaf and mixed-leaves extracts against the A549 cells increased with treatment time. The 24-hour treatment with the young leaves was regarded ineffective as its IC$_{50}$ above the maximum concentration of 200 µg/mL could not be determined in this study.

3.5. Morphological changes of A549 cells following treatment with methanolic leaf extract of Litsea elliptica

An inverted light microscope was used to analyse the morphological changes in the A549 lung cancer cells that were treated with the methanolic extract of the mixed *L. elliptica* leaves for 24, 48 and 72 h. An evident reduction in number of viable cells was observed with an increase in extract concentration and treatment duration. A closer examination of the treated cells revealed several alterations in the cell morphology that resembled characteristic changes in apoptotic cells such as nuclear compaction, membrane blebbing and apoptotic bodies as shown in Fig. 5. These hallmarks of apoptosis were more significant in the cells treated with 200 µg/mL of the extract following 24 h of treatment, and those receiving 50 to 200 µg/mL doses at 48 and 72 h of treatment.

3.6. Live/dead staining

The LIVE/DEAD® Viability/Cytotoxicity assay was performed to confirm the cytotoxic activity of the methanolic extract of the mixed *L. elliptica* leaves on A549 cells. This assay involved dual staining using calcein-AM and ethD-1. The results showed a reduction in viable cells after being treated with the *L. elliptica* mixed-leaves extracts at 56.09 µg/mL (Fig. 6). A further decline in cell population was observed when the cells were treated with a concentration of 200 µg/mL of the extract. In comparison, the population of dead cells stained in red fluorescence by ethD-1 was increased as the extract concentration increases.

A large proportion of the untreated A549 cells reflected the morphology of healthy viable cells as shown in Fig. 7. Hoechst stained-control cells showed that the nuclei of the untreated cells remained uniform and intact. As the dosage of the extract increased to 56.09 µg/mL and 200 µg/mL, the fluorescence of the stained nuclei intensified suggesting that the nuclei of the treated cells were increasingly deteriorating with the increase in extract concentration (Pieme et al., 2014). As opposed to the untreated A549 cells, the bright blue fluorescence on the treated cells have accentuated various apoptotic hallmarks in the cellular nuclei such as nuclear compaction (labelled "NC") and nuclei/DNA fragmentation (labelled "DF") as the chromatin in the apoptotic nuclei became highly condensed and fragmented indicating that apoptosis occurred in the *L. elliptica* treated A549 cells.

3.7. Induction of apoptosis by Litsea elliptica on A549

Cells that were subjected to 24 h treatment with 145.63 µg/mL of the *L. elliptica* extract demonstrated signs of late apoptosis (Fig. 8). In addition, we also found cells from the treated population which were in the early apoptotic stage displaying only the green fluorescence of Annexin V. A close-up observation revealed green

Table 2
Biological activities and nature of the phytochemical compounds identified from GC-MS analysis of the methanolic extract of the *L. elliptica* mixed leaves.

Peak No.	Retention Time (min)	Relative Peak Area (%)	IUPAC Name	Group	Biological Properties/ Reported Applications
1	5.603	2.10	3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one	Flavonoid	Antimicrobial, anti-inflammatory [1], antioxidant [1-2], anti-proliferation and pro-apoptotic against colon cancer cells through NF-κB inhibition [3]
2	5.931	2.08	Benzene-1,2-diol Synonym: 1,2-Dihydroxybenzene; Catechol; *o*-Hydroquinone	Aromatic alcohol	Antibacterial, anti-dermatic, fungicide, dye antiseptic [4], anticancer (breast), antioxidant and pesticidal [5]
3	6.233	0.66	5-(Hydroxymethyl)furan-2-carbaldehyde Synonym: 5-Hydroxymethylfuralde-hyde	Furaldehyde	Antimicrobial and preservative [6]
4	6.543	0.66	3-Methoxybenzene-1,2-diol Synonym: 3-Methoxycatechol; Methoxy-*o*-hydroquinone	Phenolic	*No available information*
5	7.474	1.80	Benzene-1,2,3-triol Synonym: 1,2,3-Trihydroxybenzene; Pyrogallic acid; Pyrogallol	Aromatic alcohol	Antioxidant, antiseptic, antibacterial, antidermatitic, fungicidal, pesticidal, antimutagenic and dye [7]
6	7.531	20.33			
7	8.489	0.28	(7*aR*)-4,4,7*a*-Trimethyl-6,7-dihydro-5*H*-1-benzofuran-2-one Synonym: Dihydroactinidolide; 2(4*H*)-Benzofuranone, 5,6,7,7*a*-tetrahydro-4,4,7*a*-trimethyl-, (*R*)-	Furanone	Antifungal, antialgal, antioxidant, antibacterial [8], analgesic and anti-diabetic [9]
8	9.276	0.79	Dodecanoic acid Synonym: Dodecylic acid; Lauric acid; Laurostearic acid	Fatty acid	Antioxidant [10], antimicrobial [11] and flavour [12]
9	11.306	0.62	Tetradecanoic acid Synonym: Myristic acid	Fatty acid	Cosmetic, antioxidant, cancer preventive, nematicidal, lubricant, hypocholesterolemic [13], flavour and pesticidal [12]
10 13	12.158 12.644	3.06 1.08	(*E*,7*R*,11*R*)-3,7,11,15-Tetramethylhexadec-2-en-1-ol Synonym: Phytol; 3,7,11,15-Tetramethylhexadec-2-en-1-ol	Terpene alcohol	Antimicrobial, anti-inflammatory, anticancer, diuretic [12] and cancer preventive [13]
11	12.285	0.45	6,10,14-Trimethylpentadecan-2-one	Sesqui-terpenoids	Allelopathic and antibacterial [14]
12	12.338	0.71	Pentadecanoic acid Synonym: Pentadecylic acid; *n*-Pentadecanoic acid	Fatty acid	*No available information*
14	13.117	5.01	Methyl hexadecanoate Synonym: Hexadecanoic acid, methyl ester; Palmitic acid, methyl ester	Fatty acid ester	Antioxidant, hypercholesterolemic, pesticidal [5], antifungal, antiandrogenic, flavour, haemolytic, 5-alpha reductase inhibitor and potent antimicrobial [15]
15	13.171	20.31	Hexadecanoic acid Synonym: *n*-Hexadecoic acid; Palmitic acid	Fatty acid	Antioxidant, hypercholesterolemic [5], nematicidal, pesticidal, antiandrogenic, flavour, haemolytic, 5-alpha-reductase inhibitor [12], cancer-preventive, anti-inflammatory [16], lubricant [17], antifungal, potent antimicrobial and antimalarial [8]
16	13.579	0.75	Heptadecanoic acid Synonym: *n*-Heptadecanoic acid; *n*-Heptadecylic acid; Margaric acid	Fatty acid	Antioxidant [18]
17	14.465	0.29	(3*R*)-5-[(1*S*,4*aS*,8*aS*)-5,5,8*a*-Trimethyl-2-methylidene-3,4,4*a*,6,7,8-hexahydro-1*H*-naphthalen-1-yl]-3-methylpent-1-en-3-ol Synonym: Manool; Labda-8(20),14-dien-13-ol, (13*R*))	Terpene alcohol	*No available information*
18	14.544	1.28	Methyl (9Z,12Z)-octadeca-9,12-dienoate Synonym: 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; Linoleic acid, methyl ester	Fatty acid ester	Antibacterial [19] and anticancer [20]
19	14.939	1.59	Methyl octadecanoate Synonym: Methyl stearate; Stearic acid, methyl ester; Octadecanoic acid, methyl ester	Fatty acid ester	Potent antifungal, antimicrobial, antibacterial and at low pH 3, 5-alpha-reductase inhibitor, cosmetic, flavour and hypocholesterolemic [21]
20	15.089	1.78	(9Z,12Z)-Octadeca-9,12-dienoic acid Synonym: Linoleic acid; Telfairic acid; 9,12-Octadecadienoic acid (*Z,Z*)-)	Fatty acid	Anti-inflammatory, antiarthritic [11] and anticancer [22]
21	15.213	3.53	Octadecanoic acid Synonym: Stearic acid; Cetylacetic acid; *n*-Octadecanoic acid	Fatty acid	Hypocholesterolemic [5], antioxidant, antimicrobial [4], 5-alpha-reductase-inhibitor, cosmetic, flavour, lubricant, perfumery, propecic, suppository [12] and antifungal [16]
22	18.381	4.23	2-(2-Ethylhexoxycarbonyl) benzoic acid Synonym: Mono(2-ethylhexyl) phthalate; Phthalic acid, mono-(2-ethylhexyl) ester	Phthalic acid monoester	Antifungal, anti-retroviral, anti-tumour, anti-diabetic, anti-cancer, antioxidant, anti-scabies, anti-inflammatory, potent antimicrobial [15], plasticizer for PVC and other resins and used as dielectric fluid [23]
23	19.807	0.60	(6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene Synonym: Squalene; 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-*E*)-)	Triterpene	Anti-cancer, antioxidant, pesticidal, sunscreen [24-25], oxygen generator, power immune stimulator, antibiotic, anticoagulant, antihistamine and antiallergic [25]
24	20.659	0.32	Heptacosan-1-ol	Alcohol	Nematicidal, anticancer[26], antioxidant and antimicrobial [27]

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Table 2 (*continued*)

Peak No.	Retention Time (min)	Relative Peak Area (%)	IUPAC Name	Group	Biological Properties/ Reported Applications
25	21.276	0.51	(2R)-2,5,8-Trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol Synonym: β-Tocopherol; Cumotocopherol; p-Xylotocopherol	Vitamin E	Antioxidant [28]
26	22.375	2.25	2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydrochromen-6-ol Synonym: dl-α-Tocopherol; Vitamin E	Vitamin E	Anti-aging, analgesic, anti-diabetic, antioxidant, anti-inflammatory, antidermatitic, antileukemic, antitumor, anticancer, antibronchitic, vasodilator, anticoronary, antiulcerogenic, antispasmodic, antistroke, hypocholesterolemic and hepatoprotective [29]

[1] Kumar et al. (2010), [2] Yu et al. (2013), [3] Ban et al. (2007), [4] Saravanan et al. (2014), [5] Manorenjitha et al. (2013), [6] Vadivel and Gopalakrishnan (2011), [7] Rajeswari and Rani (2015), [8] Akpuaka et al. (2013), [9] Mujeeb et al. (2014), [10] Lalitharani et al. (2009), [11] Anbuselvi and Rebecca (2013), [12] Hema et al. (2011), [13] Kishore and Alluraiah (2013), [14] Arora et al. (2017), [15] Martins et al. (2010), [16] Phuong et al. (2018), [17] Violet Dhayabaran and Thangarathinam (2016), [18] Zayed et al. (2014), [19] Yu et al. (2005), [20] Ali et al. (2017), [21] Ravi Kumar et al. (2012), [22] Das (2006), [23] Waghmare and Kurhade (2014), [24] Ezhilan and Neelamegam (2012), [25] Ingole (2016), [26] Venkata Raman et al. (2012), [27] Renukadevi et al. (2011), [28] beta-Tocopherol (n.d.), [29] Promprom and Chatan (2017).
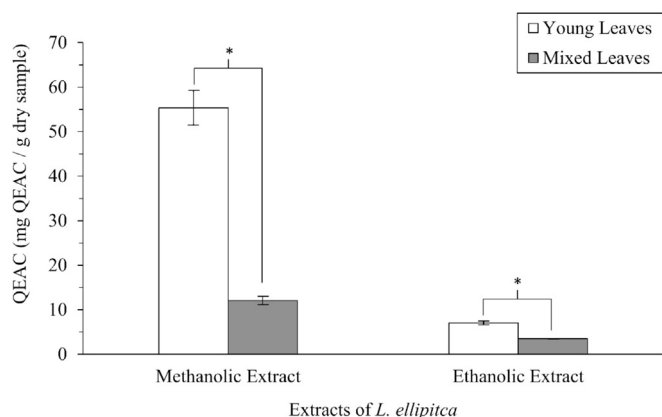

Fig. 1. Quercetin equivalent antioxidant capacities (QEAC) of the *L. elliptica* young-leaf extracts versus the mixed-leaves extracts determined from the DPPH radical scavenging activity assay. * indicates $p < 0.01$. The data presented are from the average of three independent tests ± SD.
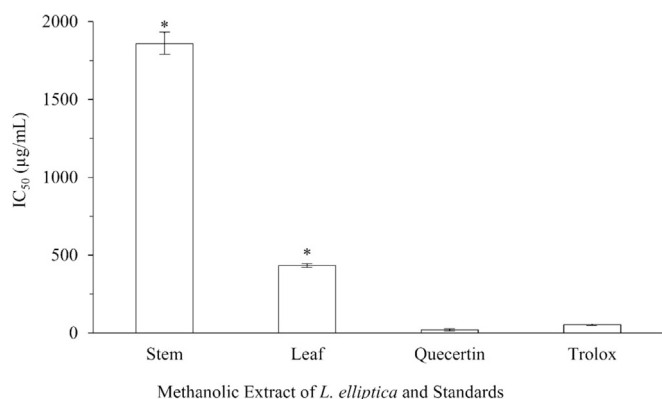

Fig. 3. Total phenolic content (TPC) and total flavonoid content (TFC) of 1 mg/mL and 20 mg/mL of the methanolic and ethanolic extracts of the young leaves of *L. elliptica* obtained by the soaking and shaking method respectively. The data presented are the average values of three independent experiments ± SD. * indicates $p < 0.01$.

Fig. 2. IC$_{50}$ value of the *L. elliptica* mixed-leaves extracts versus stem extracts determined from the DPPH radical scavenging activity assay. * indicates $p < 0.01$ between the extract and the standards quercetin and trolox. The data presented are from the average of three independent tests ± SD.

Fig. 4. Cytotoxicities of the *L. elliptica* young-leaf and mixed-leaves extracts against the A549 cell line following 24, 48 and 72 h of treatment. * indicates significant differences where $p < 0.05$.

fluorescence at a very high intensity on majority of the cells, such as that shown by the cell represented in Fig. 8-C2, demonstrating enhanced binding of the Annexin V dye. In comparison, no signs of apoptosis were detected in the untreated A549 cell population as a majority of the cells showed no signs of Annexin V or PI uptake.
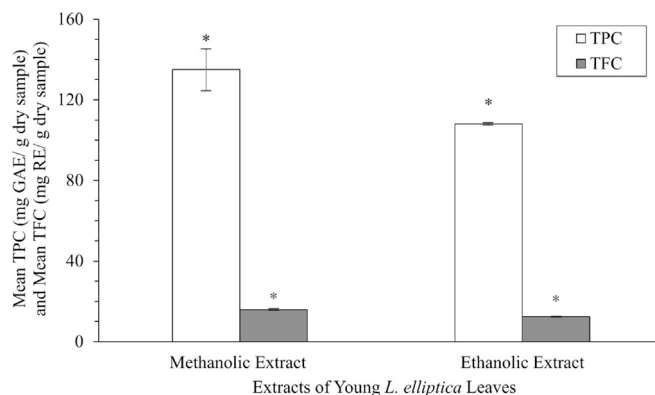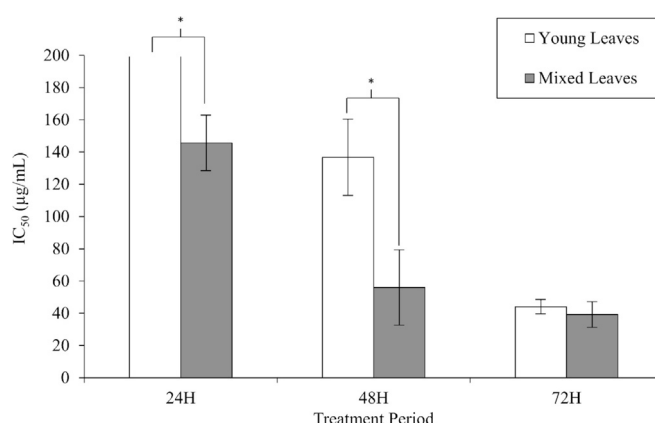
3.8. Mitochondrial involvement in the cytotoxicity of Litsea elliptica on A549

Changes in mitochondrial membrane potential of A549 cells as a result of treatment with *L. elliptica* mixed leaves extract was
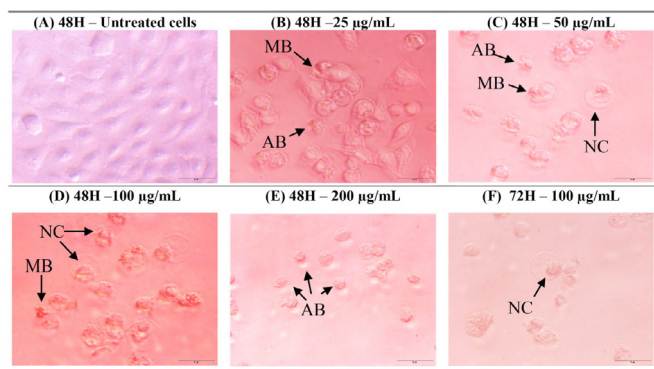
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Fig. 5. 40× magnification views of A549 cells treated wth *L. elliptica* (B to F) when compared with the untreated A549 cells (A) under an inverted light microscopy. Characteristics of apoptosis such as nuclear compaction (NC), apoptotic bodies (AB), and membrane blebbing (MB) were shown on the cells.
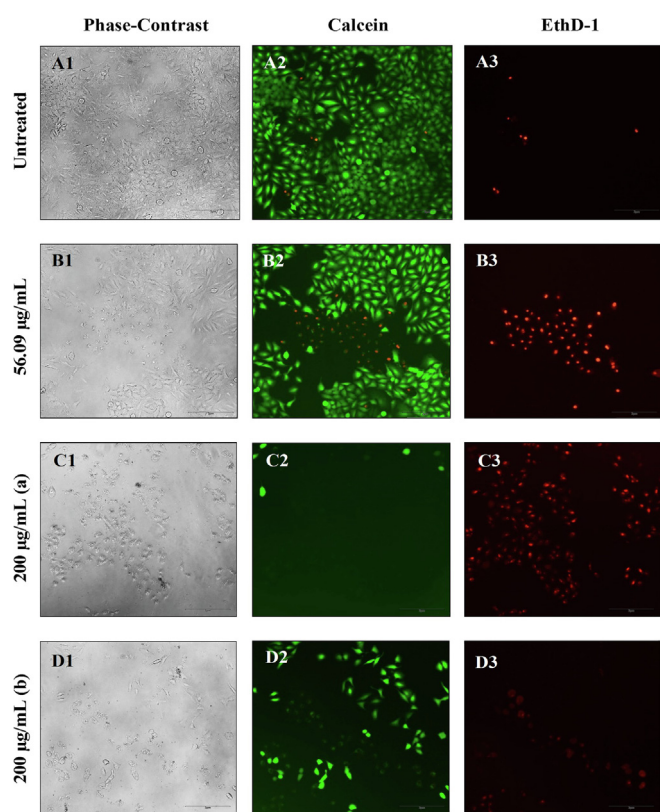

Fig. 6. Viability of A549 cells following 48 h treatment with the *L. elliptica* mixed-leaves (LEML) extract at doses of 56.09 μg/mL (IC$_{50}$ of LEML at 48 h) (row B) and 200 μg/mL (maximum treatment concentration) (row C and D) versus the untreated control cell population (row A). Lower number of viable cells (stained green with calcein) and a correspondingly higher number of dead cells (stained red with ethD-1) were detected at 200 μg/mL (row C). As shown in Row D, a dose of 200 μg/mL resulted in an overall lower number of cells compared to the untreated cells and cells treated with a dose of 56.09 μg/mL. The cells were simultaneously stained with calcein-AM and ethD-1 and images were taken from random fields.

assessed by MitoTracker Red CMXRos following incubation of the cells for 48 h with 200 μg/mL extract. As shown in Fig. 9, the population of untreated cells largely resembled healthy and proliferating cells with intact mitochondria as demonstrated by the bright red fluorescence. In comparison, the extract-treated cells showed relatively weaker fluorescence compared to the untreated cell population

4. Discussion

The phytochemical investigation of compounds in the methanolic extracts of the young and mixed *L. elliptica* leaves obtained *via* Soxhlet extraction was carried out using gas chromatography-mass spectrometry (GC-MS). Both extracts of the young and mixed leaves indicated the presence of the characterised compounds, catechol, palmitic acid, methyl ester, linoleic acid, methyl ester, phytol, mono(2-ethylhexyl) phthalate and *dl*-α-tocopherol. This shows that these six compounds were present throughout the early and late stages of development of the *L. elliptica* leaves. The young-leaf extract possessed the highest level of phytol (**6**), octacosanol (**9**), palmitic acid, methyl ester (**4**), mono(2-ethylhexyl) phthalate (**7**) and oleic acid isopropyl ester (**8**) whereas the mixed-leaves extract had the highest level of pyrogallol (**5, 6**), palmitic acid (**15**), palmitic acid, methyl ester (**14**) phytol (**10, 13**) and mono (2-ethylhexyl) phthalate (**22**). Interestingly, phytol, palmitic acid and mono(2-ethylhexyl) phthalate were commonly present at high levels in both young-leaf and mixed-leaves extracts of *L. elliptica*. This suggests that both young and mixed leaves of *L. elliptica* may have antimicrobial, anti-inflammatory, anticancer, diuretic, antioxidant, hypercholesterolemic, antifungal, antiandrogenic, haemolytic, anti-retroviral, anti-tumour, anti-diabetic, anti-scabies and cancer preventive potentials (refer to biological properties of phytol, palmitic acid, methyl ester and mono(2-ethylhexyl) phthalate in Tables 1 and 2). Additionally, we speculate that the mixed leaves of *L. elliptica* may also demonstrate antidermatic, antimutagenic, pesticidal and antimalarial activities due to their major compounds **5/6** and **15** (refer Table 2). As the mixed-leaves extract was characterised with a wide variety of active compounds, we hypothesise that the mixed leaves of *L. elliptica* may have a wide spectrum of potential biological properties. Such a vast array of pharmacological activities has also been reported for various plants of the Lauraceae family including *L. cubeba*, *L. glutinosa* and *Litsea coreana* (Wang et al., 2016; Jia et al., 2017).

The methanolic extracts of both the young and mixed *L. elliptica* leaves have demonstrated higher antioxidant capacities than their ethanolic counterparts. This suggests that methanol has higher efficiency at extracting compounds with higher proticities from the *L. elliptica* leaves compared to the ethanolic solvent, as protic compounds possess high antioxidant potentials to reduce DPPH radicals (Ahmed et al., 2015). In contrast, the low radical scavenging capacity of the ethanolic extracts also suggests the potential presence of phytochemicals with complex conformations which decreases their steric accessibility to reduce the DPPH nitrogen radical that is located within the centre of the DPPH molecule (Bazylak & Gryn, 2015; Ionita, 2005).

Our results further demonstrated that all of the extracts of the young *L. elliptica* leaves had significantly higher antioxidant capacities compared to the mixed-leaves extracts. This suggests the potential negative influence of maturity on the antioxidant properties of the *L. elliptica* leaves. The high antioxidant capacity of the young *L. elliptica* leaves may be due to their increased oxidative stress as a result of their higher exposure to light. As oxidative stress increases, flavonoids and antioxidant enzymes may accumulate to combat the detrimental effects of light-induced reactive oxygen species (ROS) (Agati and Tattini, 2010; Vidović et al., 2016). Additionally, high oxidative stress may also increase carotenoid and superoxide dismutase concentrations in the young leaves of *L. elliptica*, both of which possess high radical scavenging potentials and can catalyse the degradation of superoxide radicals respectively (Stahl and Sies, 2003; Merhan, 2017; Ariz et al., 2010; Younus, 2018). Therefore, we speculate that intensified activities and concentrations of the bioactive flavonoids, enzymes, carotenoids and superoxide dismutase following higher exposure
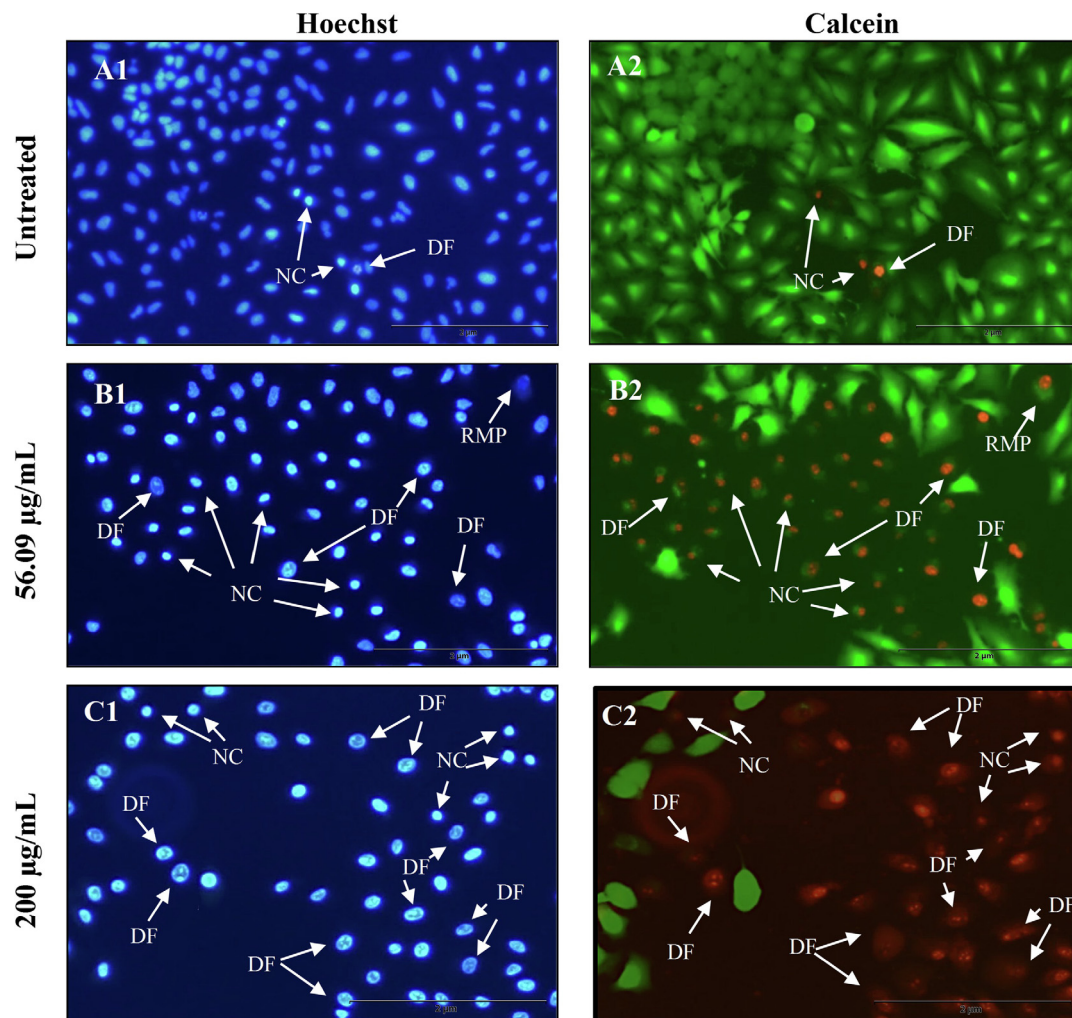
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Fig. 7. Fluorescent images of A549 cells stained with Hoechst 33342 (column 1) and Calcein/EthD-1 (column 2) following 48 h of treatment with the extract of *L. elliptica* mixed leaves at 56.09 µg/mL (row B) and 200 µg/mL (row C) versus the untreated cells (row A). Some hallmarks of apoptosis including nuclear compaction (NC), DNA fragmentation (DF) and reduced membrane potential (RMP) were observed in the treated cell population. The images were taken from random fields.

to direct sunlight have led to higher antioxidant capacities in the young *L. elliptica* leaves compared to the mixed leaves.

The methanolic young-leaf extract obtained *via* Soxhlet extraction demonstrated a DPPH radical scavenging activity of approximately five-folds higher compared to the methanolic extract of the young leaves obtained *via* soaking and shaking. This indicates that the methanolic extract of the young *L. elliptica* leaves acquired *via* Soxhlet extraction has higher antioxidant potential compared to the same extract which was obtained *via* soaking and shaking, possibly due to the higher level of active compounds extractable from the young *L. elliptica* leaves by the Soxhlet extraction method (Goh et al., 2021).

The antioxidant capacity of the Soxhlet extract of the young *L. elliptica* leaves was further demonstrated in the ABTS radical scavenging activity assay. In comparison to other potent standard antioxidant compounds such as ascorbic acid, butylated hydroxyanisole (BHA) and BHT which have demonstrated 86.3% to 100% inhibition of the ABTS radicals at a concentration of 45 µg/mL (Gülçin et al, 2012), the reactivity of the young-leaf extract against ABTS is considered moderate as it is able to inhibit the ABTS radicals by approximately 26.6% at the same concentration. The activity of the young-leaf extract against ABTS was about six-folds higher than the activity of trolox at the same concentration of 45 µg/mL as the same dose of trolox was

reported to inhibit ABTS by 4.4% in the study by Gülçin et al (2012).

The presence of electron donors in the extract would reduce the pale yellowish orange-coloured ferric ion (Fe^{3+}) in the ferric ion-TPTZ complex to the dark purple ferrous (Fe^{2+}) complex, and this colour change may be used as an indicator of potential antioxidant activity (Assefa et al., 2016). Although the young-leaf extract showed lower FRAP compared to the commercial antioxidants, sesamol, ellagic acid and BHA (385.13 ± 0.46, 585.19 ± 0.10 and 207.31 ± 2.02 g trolox/100 g dry weight, respectively), its ability to reduce the ferric-ion is more profound than that of the naturally occurring carotenoid, lutein (0.612 ± 0.01 g trolox/100 g dry weight) (Hayes et al., 2011). As opposed to the hydrogen atom transfer (HAT) mechanism involved in the DPPH radical scavenging assay, reactions of both the ABTS and FRAP assays are based on the single electron transfer (SET) mechanism between antioxidants and the ABTS radicals and ferric-TPTZ complex (Kaviarasan et al., 2007; Assefa et al., 2016). Thus, our ABTS and FRAP results suggest that the young-leaf extract has moderate radical scavenging activity *via* the SET mechanism. Overall, the methanolic extract of the young *L. elliptica* leaves obtained *via* Soxhlet extraction has demonstrated appreciable free radical reducing capacities.

The higher antioxidant activity of the *L. elliptica* leaves compared to the stem corroborates the preferential use of the *L. elliptica*
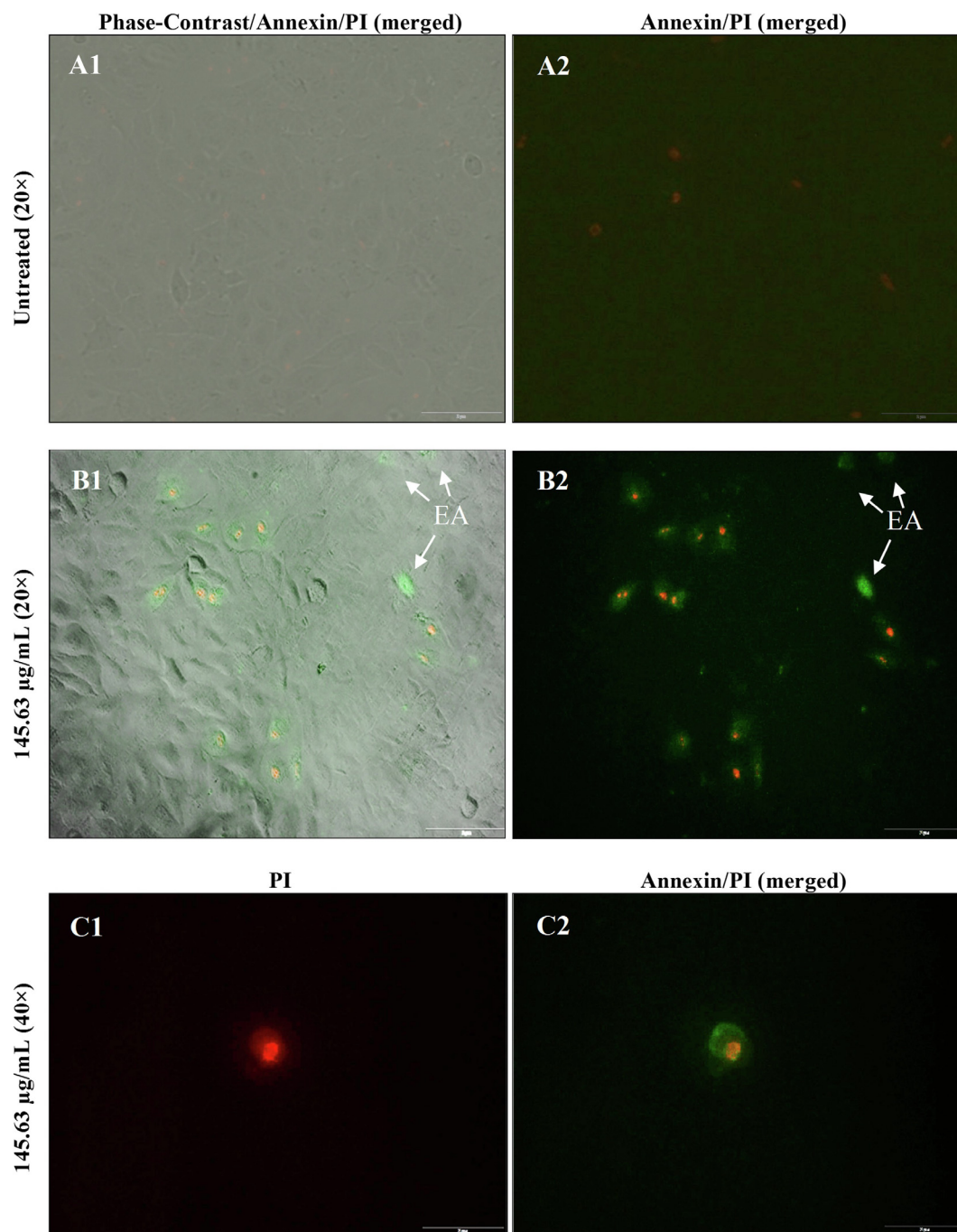
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Phase-Contrast/Annexin/PI (merged)

Annexin/PI (merged)

PI

Annexin/PI (merged)

Fig. 8. Phase-contrast and fluorescence images of Annexin/PI stained-A549 cells post 24 h treatment with 145.63 μg/mL of the *L. elliptica* mixed-leaves extract (row B and C) versus the untreated cells (row A). The cells in A1, A2, B1 and B2 were viewed at 20× magnifications whereas the cell in C1 and C2 was viewed at 40× magnifications. The white arrows in B1 and B2 highlights cells at early apoptosis (EA) (displaying only the green fluorescence of Annexin V) while the other fluorescent cells presented signs of late apoptosis (displayed both green Annexin V and red PI fluorescence). The binding of Annexin V to the externalised phosphatidylserine was clearly observed in a majority of the cells (shown here a representative cell with intense signal - C2), showing internalised PI attaching to the nucleic acids of the cell.

leaves by traditional medicine practitioners. The low antioxidant capacity of the stem may be ascribed to the absence of reactive phytochemicals such as flavonoids, carotenoids and superoxide dismutase to reduce DPPH radicals compared to the leaves (Agati and Tattini, 2010; Merhan, 2017; Younus, 2018). In addition to that, the low antioxidant capacity of the stem may also be due to the existence of antagonistic compounds in the stem of *L. elliptica* that would reduce the antioxidant activities of other bioactive compounds contained in the extract (Hidalgo et al., 2010; Pinelo et al., 2004).

The DPPH radical scavenging activity of the methanolic extract of the *L. elliptica* leaf was lower than that of quercetin and trolox. Nevertheless, despite demonstrating lower antioxidant capacity for the *L. elliptica* leaf extracts compared to previous studies, our study has shown that the highest antioxidant activity of *L. elliptica* lies in its young leaves whereas its stem posses modest antioxidant capacities.

The results from this study showed that more phenolic compounds were extracted from the young leaves of *L. elliptica* when using methanol as an extraction solvent compared to those
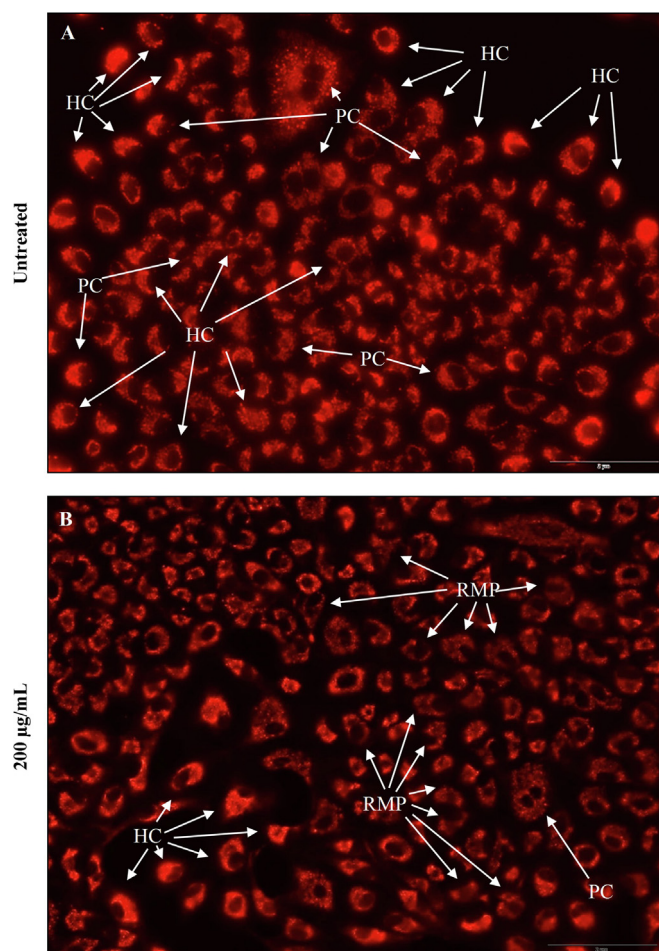
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Fig. 9. Fluorescent images of the untreated A549 cells (A) versus the cells after 48 h of treatment with 200 µg/mL of the *L. elliptica* mixed-leaves extract (B) stained with MitoTracker Red CMXRos. The mitochondria of cells going through apoptosis would experience reduced membrane potential (RMP) and thus produce weaker fluorescence compared to healthy cells (HC) when stained with MitoTracker Red CMXRos. The untreated cell population contained a higher proportion of healthy and proliferating cells (PC) whereas the population of treated cells possessed more cells with RMP. Cells were viewed at 10× magnification.

extracted using ethanol. As plants may comprise a wide array of phenolic compound with varying polarities (Dai and Mumper, 2010), different extraction solvents are expected to extract different phytochemicals based on the solubility of the compounds. Although the phenolic composition of the ethanolic extract of the young leaves was merely 20% lower compared to the methanolic extract, the antioxidant capacity of the ethanolic extract was almost eight-folds lower than that of the methanolic extract as shown in Fig. 1. The modest antioxidant activity of the ethanolic extract despite its high phenolic content shows that the ethanol-soluble phenols possess mild antioxidant capacity whereas those extracted in methanol may exert higher antioxidant activity. The ability of the ethanol-extracted phenols to scavenge radicals may be potentially hampered by their complex structures that would cause difficulties in reaching the DPPH radicals due to steric inaccessibility (Bazylak and Gryn, 2015). In comparison, as the methanolic extract demonstrated potent DPPH radical scavenging capacity, it suggests that the phenols and flavonoids present in the methanolic extracts may have substantial antioxidant potency which may have also contributed to the FRAP and ABTS radical scavenging activities of the methanolic young-leaf extract. Nevertheless, the antioxidant activity of the young *L. elliptica* leaves

may also be contributed by the other antioxidant compounds it constitutes in addition to phenols or flavonoid as various other antioxidative compounds including tocopherol, fatty acid esters, aromatic alcohol and phthalic acid monoester were also identified in the young leaves as listed in Table 1.

Our cytotoxicity study showed that the mixed-leaves extract had significantly higher cytotoxicities than the young-leaf extract against A549 especially at the 24 and 48 h treatment periods. The wide variety of phytochemical compounds in the mixed *L. elliptica* leaves may have an eminent contribution to the cytotoxicity of the mixed-leaves extract against A549 as several of the compounds identified in the extract including various flavonoids, terpenes, alcohols, fatty acids and their methyl esters and vitamin E have been reported to have anti-cancer or cancer preventive properties (Table 2).

The potent cytotoxicity of the mixed-leaves extract was despite its lower antioxidant activities as reported earlier. This was in agreement with the findings of Heo *et al.* who showed that plant extracts with high antioxidant activities did not correspondingly produce high cytotoxicities against tumour cells (Heo et al., 2007). Although the free radical scavenging properties of polyphenols have been claimed to contribute to the inhibition of carcinoma cells, various phytochemicals may also be synergistically involved in the therapeutic and protective actions against cancer cells (Sahpazidou et al., 2014; Maurya and Vinayak, 2015; Brglez Mojzer et al., 2016; Liu, 2003; Liu, 2014). Considering the lack of association between the antioxidant activity and cytotoxicity of the *L. elliptica* extracts against A549, we speculate that the cytotoxicity of the *L. elliptica* leaves may be due to the presence of other phytochemicals including the bulky phytochemicals that could not scavenge the DPPH radicals.

As compared to other known anticancer agents such as curcumin that has IC_{50} values of less than 4 µg/mL (Zhou et al., 2014), the *L. elliptica* leaf extracts have exhibited relatively lower cytotoxicities. Nonetheless, as cytotoxicities are generally classified as active with an $IC_{50} \leq 20$ µg/mL or inactive with $IC_{50} > 100$-µg/mL (Malek et al., 2011; Ahmed Hamdi et al., 2014), we conclude that the crude methanolic extracts of *L. elliptica* leaf were moderately cytotoxic against the A549 cancer cell line.

Morphological analysis and subsequent cytotoxicity evaluations *via* the fluorescence calcein/ethidium viability assay and Hoechst staining further showed that the cytotoxicity of the methanolic extract of the mixed *L. elliptica* leaves against the A549 cell line was concentration- and time-dependent. Observations of various apoptotic hallmarks including nuclear compaction, membrane blebbing and apoptotic bodies as shown in Fig. 5 as well as DNA fragmentation, which was highlighted *via* fluorescent staining by Hoechst as shown in Fig. 7, indicate that the methanolic *L. elliptica* leaf extract against the A549 cell line may be induced *via* the apoptotic programmed cell death.

As shown in Fig. 8, some cells that have been treated with 145.63 µg/mL of the *L. elliptica* extract for 24 h have commenced the early apoptotic event of phosphatidylserine externalisation as they emitted the green fluorescence of Annexin V while the majority of the cells have reached the late stages of apoptosis as they displayed both green and red fluorescence as a consequence of their compromised membranes. As shown by the cells that display intense green fluorescence, it implies that high levels of phosphatidylserine translocation to the outer surface of the cell, which allowed enhanced binding of the Annexin V dye. We found that both early and late apoptotic cells existed within the population of the *L. elliptica*-treated cells. Nevertheless, as majority of the treated cells have demonstrated moderate to strong fluorescence of both the Annexin V and PI dyes, it suggests that a modest dose of the mixed *L. elliptica* leaves extract was able to cause the A549 cells to enter the late stage of apoptosis within 24 h of treatment.

The findings from our study suggest that the cell death induced by the *L. elliptica* leaves was *via* the apoptotic pathway. The lack of inflammation that accompanies apoptosis indicates that the cytotoxicity of the *L. elliptica* leaves would offer minimal damage to surrounding cells as they subject the target cancer cells to their deaths. This infers an added advantage for the use of the *L. elliptica* leaves as a natural alternative to synthetic drugs against lung cancers. Further mechanistic investigations were required to validate its pharmacological effect and therefore, we have assessed the effects of the *L. elliptica* leaf extract on the mitochondrial membrane potential of A549 cells by incorporating MitoTracker Red CMXRos.

As shown in Fig. 9, the relatively weaker fluorescence demonstrated by the extract-treated cells compared to the untreated cell population following the staining with MitoTracker Red CMXRos is indicative of compromised mitochondrial membrane in the treated cells as a result of escalated mitochondrial outer membrane permeabilisation (MOMP) (Xiong et al., 2014; Frank et al., 2001). MOMP represents a standard event within the mitochondria-mediated caspase-activated apoptotic pathway regulated by the B-cell lymphoma 2 (Bcl-2) proteins (Xiong et al., 2014; Ly et al., 2003). When encountered with cellular stress, executioner proteins, BAK or BAX, are activated by BH3-only proteins (Bcl-2 family proteins) to oligomerise and permeabilise the outer membrane of mitochondria to cause MOMP (Shamas-Din et al., 2013). The mitochondria-mediated apoptotic pathway is irreversible as MOMP would release mitochondrial proteins such as cytochrome *c* into the cytoplasm to activate caspases that trigger non-inflammatory apoptotic cell deaths (Li and Dewson, 2015; Wang et al., 2014; Shamas-Din et al., 2013; Xiong et al., 2014; Waterhouse et al., 2001). As our study showed that the *L. elliptica* mixed-leaves extract could deteriorate mitochondrial membrane integrity in the A549 cells, it is thus speculated that the mixed *L. elliptica* leaves extract-induced apoptosis in the A549 cells was mediated by the Bcl-2 regulated mitochondrial pathway.

5. Conclusion

Antioxidant studies revealed that the methanolic extracts of both the *L. elliptica* young and mixed leaves had higher antioxidant capacities compared to their ethanolic extracts. The young leaves of *L. elliptica* showed an overall higher antioxidant capacity than the mixed leaves as demonstrated by both their methanolic and ethanolic extracts. As the young leaf extracts was also shown to contain high total phenolic content, it indicates that the phenolic compounds may contribute to the high antioxidant activity of the young *L. elliptica* leaves.

Despite the lower antioxidant potentials of the mixed-leaves extracts, the methanolic extract of the mixed leaves of *L. elliptica* demonstrated higher cytotoxicity against the A549 lung cancer cells than that of the young leaves. Based on morphological examinations, we speculate that the mixed-leaves extract may have induced apoptotic cell deaths in the A549 cells as various hallmarks of apoptosis including apoptotic bodies, nuclear compactions and membrane blebbings were observed in the group of treated cells. The *L. elliptica* mixed leaves-induced apoptosis on the A549 cells was further demonstrated by Annexin V/PI staining, which showed that cells at both early and late stages of apoptosis were present in the treated cell population where the latter was predominant. Additional downstream analysis on the mitochondria of the cells showed that the mode of cytotoxicity engaged by the leaves of *L. elliptica* against the carcinoma cells was likely *via* the mitochondria-mediated pathway. Overall, the results from this study have shown promising pharmaceutical benefits of both the young and mixed leaves of *L. elliptica* especially against the A549 lung cancer cell line.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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