HOSTED BY

ELSEVIER

CrossMark

Available online at www.sciencedirect.com

ScienceDirect

Food Science and Human Wellness 5 (2016) 65–75

Food Science and Human Wellness

www.elsevier.com/locate/fshw

Antioxidant, antimicrobial and cytotoxic potential of condensed tannins from *Leucaena leucocephala* hybrid-Rendang

Mazni Abu Zarin [a,*], Ho Yin Wan [a], Azizul Isha [b], Nurdin Armania [a,c]

[a] *Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[b] *Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[c] *Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

Received 4 December 2015; received in revised form 2 February 2016; accepted 10 February 2016
Available online 21 February 2016

Abstract

Condensed tannins (CTs) are one of the promising compounds due to their potentially health-promoting qualities. In this study, CTs were extracted from a *Leucaena leucocephala* hybrid-Rendang and subjected to various biological studies including antioxidant (using Ferric reducing antioxidant power (FRAP), DPPH and ABTS radical scavenging assay), anti-microbial (against different pathogens) and cytotoxic activities (toward human breast adenocarcinoma (MCF-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa) and human liver carcinoma (HepG2) cell lines) in cancer cells through *in vitro* experiments. The structural characteristics and purity of CTs extract were determined using ^{13}C NMR. The results showed that CTs exhibited higher *in vitro* antioxidant activities (2257.12 ± 80.55 mg TEAC/g extract, 605.3 ± 1.82 mg TEAC/g extract and 1014.03 ± 1.20 mg TEAC/g extract in FRAP, ABTS and DPPH assay, respectively) and demonstrated anti-microbial activities toward selected Gram's positive and Gram's negative bacteria tested with MIC and MBC value at 6.25–50 mg/mL. Furthermore, among other selected cancer cells, CTs also demonstrated cytotoxic activity toward human breast cancer cells (MCF-7) ($IC_{50} = 38.33 \pm 2.08$ μg/mL). Characteristic of apoptosis such as cell shrinkage, nuclear condensation and apoptotic bodies were shown in MCF-7. These preliminary investigations have provided scientific rationale to use CTs as an alternative therapy for various oxidative and inflammatory associated diseases.

Keywords: Antioxidant; Anti-microbial; Condensed tannins; Cytotoxic; *Leucaena leucocephala*

1. Introduction

Since natural product-derived dugs frequently seem to be less toxic and more effective, identification and investigation of antioxidant, antimicrobial and anti-cancer agents from natural substances have been one of the research interests in recent years. Antioxidants scavenge variety of free radicals and reactive oxygen species and it can be extremely important in inhibiting oxidative mechanisms that lead to degenerative diseases [1]. Free radicals have been implicated as playing a role in the etiology of cardiovascular disease, cancer, Alzheimer's disease, Parkinson's disease, etc. Although several modern drugs are used to treat this type of disorder, their prolonged use may cause severe adverse side effects on chronic administration [2]. Cancer is the largest single cause of death in both men and women. Cancer is a class of diseases characterized by out of control of cell growth. There are different types of cancer, and each is classified by the type of cell that is initially affected. Cancer harms the body when altered cells divide uncontrollably to form lumps or masses of tissue called tumors except for leukemia where cancer prohibits normal blood function by abnormal cell division in the blood stream.

Breast cancer is the most common cancer in women followed by cervical cancer; with about more than 55% of breast cancer related deaths occur in the developing world. The incidence of

* Corresponding author at: Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia. Tel.: +60 389472235.
E-mail address: mazniaz@upm.edu.my (M. Abu Zarin).
Peer review under responsibility of Beijing Academy of Food Sciences.

http://dx.doi.org/10.1016/j.fshw.2016.02.001

this disease is increasing in both industrialized and developing countries [3]. Considerable progress has been made in treating breast cancer through surgery, radiotherapy, chemotherapy, and hormone therapy [4]. However, those carcinomas that do not express the estrogen remain generally resistant to therapy. Almost all cases of cervical cancer were from persistent infection with one of about 15 genotypes of carcinogenic human papillomavirus (HPV) [5]. Cases are often detected at late stages due to non-existent or inadequate screening, and the standard treatment options are often absent or unaffordable. Colon cancer is cancer of the large intestine. Most cases of colon cancer begin as small, noncancerous (benign) clumps of cells called adenomatous polyps and over time, some of these polyps become colon cancers. Meanwhile liver cancer is one of the major causes of malignancy-related deaths worldwide, and its incidence is on the rise. Typical treatment approaches to liver and colon cancer include surgery, radiotherapy, chemotherapy and transplantation but cure rates are not satisfactory [6]. To date, many anticancer drugs have been developed and applied by clinical doctors but recently, resistance to anticancer drugs was discovered; therefore, there is a need to develop new anticancer agents with minimum side effects.

Leucaena leucocephala hybrid-Rendang, which is known as the 'miracle tree' available in large quantities and abundant resources as well underutilize in Malaysia. This tree is a thornless tree which may grow up to 18 m and has a wide variety of uses, especially as a protein supplement for animals [7]. In Malaysia, a *Leucaena* hybrid, *L. leucocephala* hybrid-Rendang, which was produced from crossing *L. leucocephala* and *Leucaena diversifolia* [8], was found to have high condensed tannin content and low digestibility [9]. Condensed tannins (CTs), also known as proanthocyanidins, are polymers of 2–50 (or more) flavonoid units that are joined by carbon-carbon bonds, which are not susceptible to being cleaved by hydrolysis and most CTs are water soluble [10]. Condensed tannins health benefits extend far beyond their antioxidant properties and also anti-inflammatory [11], anti-asthmatic [12], anticancer [13], anti-viral, anti-carcinogenic, anti-allergy, antimicrobial, antihypertension and cardiovascular system-protective [14]. Condensed tannins help wounds heal, reduce the pain from pancreatitis, reduce insulin resistance in diabetics, help protect from drug toxicity and also can help lower the levels of low-density lipoproteins, or the "bad" cholesterol. Antioxidants also decrease the oxidation of low density lipoproteins (LDL), which may lead to the buildup of plaque on the walls of arteries. Most of the previous study was done on reduction of methane and volatile fatty acid production in rumen digestion system using condensed tannins extract from *L. leucocephala* [15,16]. Thus, this study was conducted due to unexplored of condensed tannins from *L. leucocephala* on antioxidant, antimicrobial and cytotoxic activities.

The principal objective of the current exposition was therefore, to utilize and investigate the biological activities of CTs extract from *L. leucocephala* hybrid-Rendang including antioxidant using ferric reducing antioxidant power (FRAP), DPPH radical scavenging assay and ABTS radical scavenging assay, antimicrobial against *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Acinetobacter anitratus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Enterococcus faecium*, *Streptococcus faecalis*, *Candida albicans*, *Candida tropicalis*, and *Aspergillus niger* and cytotoxic activities toward human breast adenocarcinoma (Mcf-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa) and human liver carcinoma (HepG2) cell lines whereas purity of the compound were confirmed by ^{13}C NMR spectroscopy. These preliminary investigations will provide scientific rationale to use CTs from *L. leucocephala* as an alternative therapy for various inflammatory associated diseases.

2. Materials and methods

2.1. Chemicals

Acetone and sodium carbonate (Na_2CO_3) were obtained from R&M chemicals, diethyl ether, gallic acid and ethanol were purchased from Merck KGaA (Darmstadt, Germany), potassium ferricyanide, iron(III) chloride ($FeCl_3$), potassium persulphate were purchased from Pronadisa Chemie Brunschwig AG (Switzerland) trichloroacetic acid (TCA), Trolox (2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picryl hydrazyl (DPPH), nystatin, ampicillin, streptomycin, penicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), nutrient agar (NA), Potato Dextrose Agar (PDA), Sabouraud agar (SDA) were purchased from Difco (BD, USA), RPMI 1640 and fetal bovine serum were purchased from PAA Laboratories GmBH (Pasching, Austria). Methanol was purchased from Thermo Fisher Scientific Inc. (United State) and Folin–Ciocalteu reagent was purchased from Fluka Analytical, Sigma–Aldrich Co. (St. Louis, MO, USA). All other reagents were of analytical and HPLC grades.

2.2. Plant material

Young leaves (3–4 layers from shoot) of *L. leucocephala* hybrid-Rendang was collected from a farm at Universiti Putra Malaysia. These plant materials were identified by our botanist, Dr Shamsul Khamis, Institute of Bioscience, Universiti Putra Malaysia and same material was stored at Herbarium for further identification.

2.3. Extraction and purification of condensed tannin

The leaves were cut into small pieces; freeze dried and then ground using a 0.5 mm sieve. The condensed tannins from the ground leaves were extracted as previously described [17]. Briefly, 5 g of ground leaves was soaked in 200 mL aqueous acetone solution (70%, v/v) for 20 min. After centrifugation (3500 × *g* for 10 min), the supernatant was filtered under vacuum to remove any particulate plant residues and washed with diethyl

ether (ratio 1:1) to remove chlorophyll, pigments and low molecular weight phenolic acids. The bottom layer of the mixture (yellow) was collected and evaporated using a vacuum rotary evaporator at <40 °C. The crude extract was re-dissolved in 40% (v/v) of methanol (ratio 1:1) and stored at 4 °C. Purification of condensed tannins extract was carried out using a Sephadex LH-20 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 40% (v/v) methanol and 80% (v/v) acetone as the respective purification solvents. In the purification process, low molecular weight phenolics were eluted with 40% (v/v) methanol, and the condensed tannins were eluted with 80% (v/v) of acetone. Purified condensed tannins were evaporated using rotary evaporator prior to freeze dried and stored at −20 °C.

2.4. ^{13}C NMR analysis

25 mg of the purified CT were dissolved in 1 mL of D_2O/acetone-d_6 (1:1) mixture. Spectra were obtained using 400 MHz Jeol NMR spectrometer (ECX 400 II). Experiment was conducted at 45 °C and was carried out using 20,000 scans. The resulting spectra was manually phased and baseline correction. The chemical shifts were reported relative to tetramethylsilane (0 ppm), using acetone peak at 30.7 ppm as a secondary standard.

2.5. Total phenolic content

Total phenolic compounds were determined with Folin–Ciocalteu reagent using gallic acid as standard. One mL of crude extract or purified condensed tannins was diluted with 46 mL of distilled water. One mL of Folin–Ciocalteu reagent was added and the content in the flask were mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%, w/v) was added and the mixture were allowed to stand for 2 h with intermittent shaking. Blank was prepared by replacing 1 mL of condensed tannins extract with 1 mL of deionized water. The absorbance of the mixture was measured against blank at 765 nm by using UV-light spectrophotometer (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan). Gallic acid was used to calibrate the standard curve. Each crude extract was analyzed in triplicate and the results were expressed in milligrams of gallic acid equivalents per gram of extract sample (mg GAE/g extract).

2.6. Determination of antioxidant activity of condensed tannins

2.6.1. Ferric reducing antioxidant power (FRAP)

The reducing power of condensed tannins was determined as described by Amarowicz et al. [18]. The suspension of condensed tannins in 1 mL of distilled water was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. A total of 2.5 mL of trichloroacetic acid (TCA) was added and the mixture were then be centrifuged at 1750 × *g* for 10 min. About 2.5 mL aliquot of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$, and the absorbance was read at 700 nm. Trolox was used as a standard for construction of the calibration curve and the reducing power was reported as Trolox equivalent per gram of extract (mg TEAC/g extract).

2.6.2. DPPH radical scavenging assay

The free radical scavenging activity of the condensed tannin was evaluated by the DPPH according to the method previously proposed by Blois [19] with modifications. Briefly, 195 μL of 0.1 mmol/L DPPH solution were added into 50 μL of condensed tannin in a 96-well plate. The mixture was incubated in the dark at room temperature for 1 h. The absorbance was measured at 540 nm using Elisa reader (Bio Tek Instruments, Inc., Vermont, United States). The degree of scavenging activity was calculated by the following Eq. (1) and expressed as in milligram Trolox equivalent antioxidant capacity per gram of extract (mg TEAC/g extract).

$$\text{Scavenging activity}(\%) = \left(\frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}}\right) \times 100 \quad (1)$$

2.6.3. ABTS radical scavenging assay

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS+) was produced by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulphate in a ratio 1:1 (v/v) and allowing the mixture to stand in the dark at room temperature for 12–16 h before used. The absorbance of ABTS+ solution was equilibrated to an absorbance of 0.7 ± 0.02 at 734 nm using the spectrophotometer by diluting with ethanol before used. Two hundred microlitre of condensed tannins (6.25–100 μg) was mixed with 1.8 mL of diluted ABTS+ solution. The absorbance was taken at 734 nm after 7 min using spectrophotometer. Simultaneously, absorbance of negative control (1.8 mL of ABTS radical solution and 0.2 mL of ethanol) was also measured at 734 nm. The radical scavenging capacity of ABTS (%) was calculated as following Eq. (2) and expressed as mg Trolox equivalent per gram of extract (mg TEAC/g extract). Trolox solution was used to calibrate the standard curve.

$$\text{Scavenging activity}(\%) = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \times 100\% \quad (2)$$

where As = absorbance of sample at 734 nm and Ac = absorbance of negative control at 734 nm.

2.7. Determination of antimicrobial activity

2.7.1. Preparation of pathogenic microbes

Clinical isolates of the following organisms: *Methicillin Resistant Staphylococcus aureus* (MRSA), *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *A. anitratus*, *B. subtilis*, *E. coli*, *P. vulgaris*, *S. marcescens*, *E. faecium*, *S. faecalis*, *C. albicans*, *C. tropicalis*, and *A. niger* that were used in this study were obtained from Institute Medical of Research (IMR) and from the culture collection of the Institute of Bioscience, UPM. The following reference strains were also included in the study: *Pseudomonas aeruginosa* ATCC 60690, *Salmonella choleraesuis* ATCC S974, *Bacillus subtilis* ATCC B29 and *Saccharomyces*

cerevisiae ATCC 20341. The bacterial strains were grown and maintained on nutrient agar (NA) slant, while yeast and fungi on Potato Dextrose Agar (PDA) slant.

2.7.2. Disk diffusion assay

Preliminary screening was performed by the disk diffusion method. Antimicrobial activity is based on clear zone formed around the disk. Complete inhibition was indicated by a clear zone, while partial inhibition by a semi-clear zone. Nystatin (100 mg/mL) was used as the reference drug for antifungal and anti-yeast activity while streptomycin (100 mg/mL) for antibacterial activity. Sterile distilled water (dH_2O) was used as negative control. The test microorganisms (10^5 bacteria or yeast cells/mL or 10^5 spore/mL) were seeded onto respective medium by using steriled cotton swab. The filter paper discs impregnated with the extracts were placed on test microorganism-seeded plates. The plates were incubated at 37 °C for 24 h. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

2.7.3. Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

Only microbes which showed positive results in preliminary screening were selected for this study. The MBC and MIC value was determined by the liquid dilution method. Various concentrations of condensed tannins (ranging from 0.391 to 100 mg/mL) and 10 μL of standardized suspension bacteria were placed in each test tube. The tubes were incubated at 37 °C for 24 h. The lowest concentration which did not show any growth of the tested microorganism after macroscopic evaluation was determined as the MIC and the lowest concentration of condensed tannins that does not yield any growth is the MBC. The tests were performed in triplicate.

2.8. Determination of cytotoxic activity of condensed tannins

2.8.1. Cancer cells

Human breast adenocarcinoma (Mcf-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa) and human liver carcinoma (HepG2) cell lines purchased from the American Type Culture Collection (ATCC), USA were used for the cytotoxicity test. Non-tumorigenic Swiss mouse embryo fibroblast (3T3 F442A) was purchased from the Health Protection Agency (HPA) Culture Collection. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 μg/mL) and streptomycin (100 μg/mL). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.8.2. Cytotoxicity assay

The MTT assay was performed to evaluate the cytotoxicity of the condensed tannin. Briefly, 1×10^5 cell/mL were exposed to different concentration (15–150 μg/mL) of condensed tannin for 72 h. The untreated cells were also included. Subsequently, a total of 20 μL of MTT solution (5 mg/mL) were added to each well. After 4 h of incubation, the supernatant were discarded and 100 μL of DMSO were added to each well to dissolve the dark-blue formazan crystals. The absorbance was measured at 570 nm using Elisa reader (Bio Tek Instruments, Inc., Vermont, United States). Data were calculated as the percentage of inhibition and average of IC_{50} was reported.

2.8.3. Morphological studies

The cells were treated with different concentration of condensed tannin (25–100 μg/mL) for 24, 48 and 72 h. The morphological changes including characteristic of apoptosis or necrosis of the cells were observed under an inverted light microscope (Olympus, USA).

2.9. Statistical analysis

Analyses were performed in triplicates and the data were statistically evaluated using analysis of variance (ANOVA) with SPSS 15.0 version. Duncan's multiple range tests was carried out in order to test any significant differences between the results. Significance levels were defined using $p < 0.05$.

3. Results and discussions

3.1. ^{13}C NMR analysis

The purity of the tannin preparation can be determined by studying the ^{13}C NMR spectra. Various researchers have used ^{13}C NMR in their study of tannin [20–24]. Fig. 1 shows the ^{13}C NMR spectrum of condensed tannins from *L. leucocephala* leaves. The signal assignment was made based on the publication of Czochanska et al. [20]. Procyanidin (PC) and prodelphidin (PD) units were observed in the spectrum which is typical signals due to condensed tannins. The peaks at 116 ppm (C2′, C5′), 120 ppm (C6′), and 144 ppm (C3′, C4′) show the presence of PC units (catechin/epicatechin). PD units (gallocatechin/epigallocatechin) signals were detectable at 146 ppm. The peaks in region between 30 and 90 ppm were typical signals C2, C3, and C4 in flavan-3-ol units. The two signals at 76 and 81 ppm were recognizable as 2,3-*cis* and 2,3-*trans* isomers, respectively. This indicated that both the stereoisomers co-exist, while the signal at 78 ppm was detectable as *cis* form. The signals at 70 and 66 ppm were assignable to the C3 terminal and extension units, respectively. The ^{13}C NMR spectroscopy confirmed the purity of CT extract and the efficiency of the extraction.

3.2. Total phenolic contents

To determine the total phenolic content, calibration curves was obtained using known quantities of standard gallic acid. Total phenolic content of the crude extract compared to purified CTs were measured using the Folin–Ciocalteu method in terms of gallic acid equivalent. The total phenolic content of crude extract was 3.21 mg GAE/g extract while purified condensed tannins was obtained at 2.06 mg GAE/g extract. *Nepeta melissifolia*, *Phlomis lanata* and *Origanum vulgare* demonstrated the highest total phenol content with more than 15.0 mg

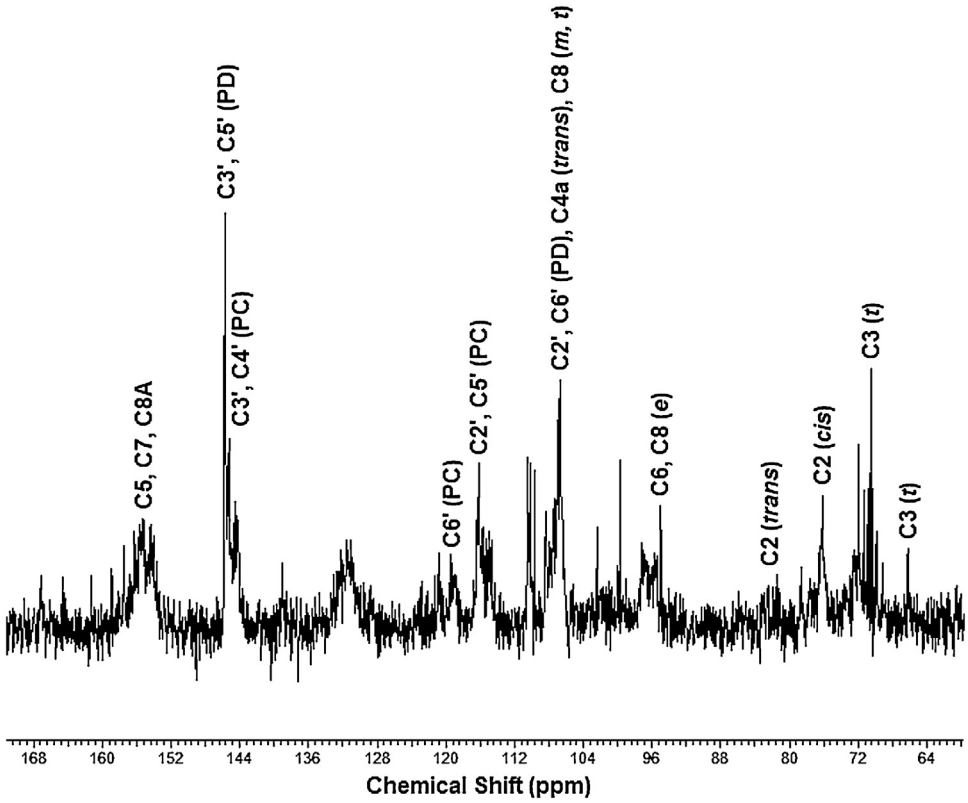

Fig. 1. ^{13}C NMR (500 MHz) spectrum of condensed tannins from *Leucaena leucocephala* leaves in acetone-d_6/D_2O (PC: procyanidin; PD: prodelphidin t: terminal unit; m: middle unit; e: extension unit).

GAE/g dried sample meanwhile *Geranium purpureum*, *Matricaria chamomilla* and *Lavandula vera* seemed to have less than 7 mg GAE/g dried sample [24]. These results have shown that higher phenolic content than the present result due to the methanol solvent used to removes sugar and low molecular weight of phenolic compound in the extraction and purification method of CTs [25].

Folin–Ciocalteu reagent determines total phenols, producing blue color by reducing yellow heteropolyphosphomolybate-tungstate anions [26]. Phenolic compounds are a class of antioxidant agents acting as free radical terminators and also plant metabolites characterized by the presence of several phenol [27]. Some of them are very reactive in neutralizing free radicals by donating a hydrogen atom or an electron, chelating metal ions in aqueous solutions. Previous studies were reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation [28]. Studies also have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced the risk of atherosclerosis and cardiovascular disease [29]. Besides, the phenolic compounds possess multiple biological properties including anti-tumor, and antibacterial properties, and these activities might be related to their antioxidant activity.

3.3. Antioxidant activities of condensed tannins

In this study, CTs exhibited highest antioxidant activity in FRAP (2257.12 ± 80.55 mg TEAC/g extract) compared to DPPH (1014.03 ± 1.20 mg TEAC/g extract) and ABTS (605.3 ± 1.82 mg TEAC/g extract) assay. Trolox was used as control for all antioxidant assays. Figs. 2–4 showed the trend of the activity for all assay including Trolox. According to the results, the antioxidant activities of CTs increased in concentration-dependent manner.

Condensed tannins showed significant different activities compared to Trolox in DPPH and ABTS assay with $p < 0.05$, excluded for 25 μg/mL of ABTS assay. Meanwhile, CTs was not significant different at concentration of 3.125–12.5 μg/mL in FRAP assay compared to Trolox. CTs also demonstrated higher or equivalent activities to Trolox which means that CTs could be one of the potential sources for antioxidant agent as well as Trolox.

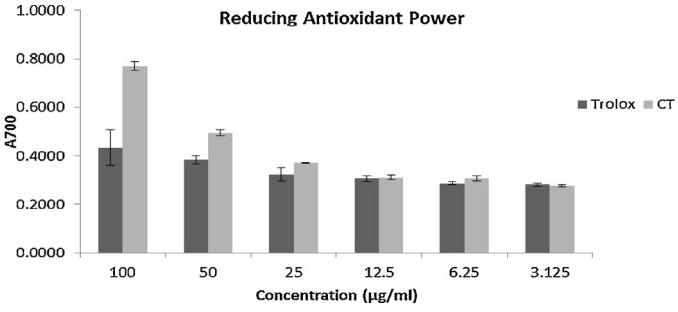

Fig. 2. Reducing antioxidant power activity of CTs and Trolox at different concentrations. No significant different was showed at concentration of 3.125–12.5 μg/mL.

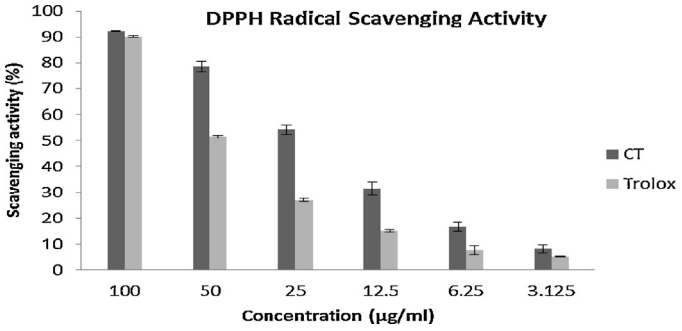

Fig. 3. DPPH radical scavenging activity of CTs and Trolox at different concentrations.

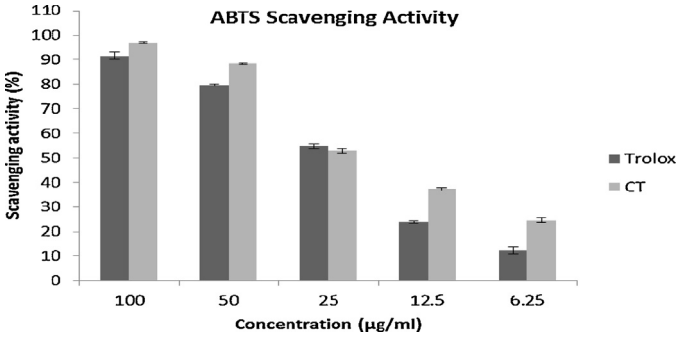

Fig. 4. ABTS scavenging activity of CTs and Trolox at different concentrations. There was no significant different at concentration of 25 μg/mL.

Ferric reducing antioxidant power (FRAP) is a measurement of Fe^{3+} reductive to Fe^{2+} by donating an electron, which is an important mechanism of phenolic antioxidant action. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm [30]. Increasing absorbance at 700 nm indicates an increase in the reductive ability. Extract from *Aloe vera*, *Bacopa monniera*, *Moringa oleifera* and *Zingiber officinale* showed reducing antioxidant power activity range 0.038 ± 0.015 to 0.119 ± 0.086 at 100 μg/mL compared to standard BHT and Vitamin C which were 0.702 ± 0.076 and 1.315 ± 0.030, respectively [31]. According to the result, CTs extract showed higher activity compared to previous study and can be one of the reductive potential and could serve as electron donor.

The electron donation ability of natural products can be measured by 2,20-diphenyl-1-picrylhydrazyl radical (DPPH) purple-colored solution bleaching where the method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants [32]. Meanwhile, ABTS radical scavenging assay involves a method that generates a blue/green ABTS+ chromophore via the reaction of ABTS and potassium persulfate. In the present study, the scavenging of the DPPH radical by the extracts was found to be much higher than that of ABTS+ radical which means that the CTs extract can be considered as radical scavenger. The present findings was in agreement with previous finding. Wei et al. [33] showed that the CTs extracted from *Machilus pauhoi* leaves inhibited the activity of DPPH radicals in a dose-dependent manner where at 100 μg/mL, the scavenging activity of *M. pauhoi* leaves (52.86%) was higher than that of ascorbic acid (50.65%) and BHA (45.63%).

Antioxidant is group of substances that are useful for fighting cancer and other processes that potentially lead to disease. Unlike cytotoxic agents that damage tumor cells, antioxidant act by preventing the onset of cancer during carcinogenesis and generally beneficial to cells [34]. Reactive oxidants (reactive oxygen and nitrogen species) can cause damage to macromolecules such as proteins, lipids, enzymes and DNA [35]. Therefore, living organisms produce enzymes such as catalase, superoxide dismutase and peroxides or rely on non-enzymatic molecules (e.g.: ascorbic acid, flavonoids and vitamin K) to combat this radicals. Various studies have emphasized specific classes such as flavonoids and tannins which were proven to possess antioxidant capabilities [36,37]. Therefore, since CTs from *L. leucocephala* hybrid-Rendang exhibited higher antioxidant activities, this extract can become one of the potential antioxidant agents.

3.4. Antimicrobial activity of condensed tannins

The CTs extract was tested on a Gram's positive and negative bacteria and yeasts as well. In the disk-diffusion test, CTs was exhibited antibacterial activity against certain Gram's positive and Gram's negative bacteria (Table 1). The highest zone of inhibition (12 mm) was observed at *S. epidermidis*, followed by *S. choleraesuis*, *P. vulgaris*, *E. faecium*, *B. subtilis*, *A. anitratus*, *S. aureus*, and the least was found with *S. faecalis* which produced 7.0 mm diameter inhibition zone. Additionally, the CTs demonstrated no inhibition toward yeast which suggested that bacteria are more sensitive to CTs than yeast. The resistance of fungal species against CTs could be due to their morphological

Table 1
Antimicrobial activities of condensed tannin extracted from *L. leucocephala* hybrid-Rendang.

Test microorganisms	CT	Streptomycin	Nystatin
Methicillin Resistant Staphylococcus aureus (MRSA)	–	21	ND
Staphylococcus aureus	8	22	ND
Staphylococcus epidermidis	12	20	ND
Pseudomonas aeruginosa	–	22	ND
Acinetobacter anitratus	8	22	ND
Bacillus subtilis	8	25	ND
Escherichia coli	–	21	ND
Proteus vulgaris	9	20	ND
Serratia marcescens	–	25	ND
Enterococcus faecium	9	25	ND
Streptococcus faecalis	7	22	ND
Salmonella choleraesuis S974	9	20	ND
Bacillus subtilis ATCC B29	9	22	ND
Pseudomonas aeruginosa ATCC 60690	–	22	ND
Candida albicans	–	ND	22
Candida tropicalis	–	ND	21
Saccharomyces cerevisiae ATCC 20341	–	ND	25
Aspergillus niger	–	ND	20

Antimicrobial activities were determined based on diameter of zone inhibition (mm); –, no inhibition, ND, not determined; a mean values from triplicate results.

Table 2
Minimum inhibition concentration and minimum bactericidal concentration of condensed tannin extract on selected bacteria.

Test microorganisms	Condensed tannins (mg/mL)	
	MIC	MBC
Staphylococcus aureus	12.5	25
Staphylococcus epidermidis	6.25	12.5
Salmonella choleraesuis ATCC S974	25	50
Acinetobacter anitratus	50	50
Bacillus subtilis	12.5	25
Bacillus subtilis ATCC B29	12.5	25
Proteus vulgaris	12.5	25
Enterococcus faecium	50	50
Streptococcus faecalis	25	25

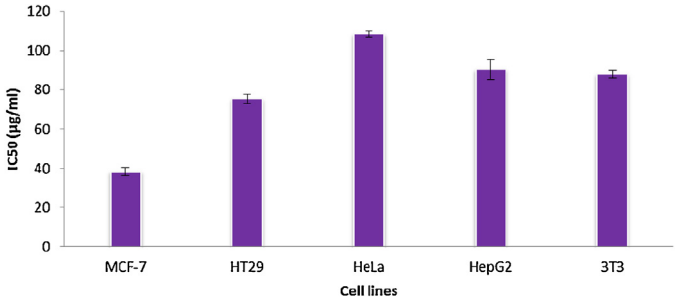

Fig. 5. The IC_{50} of selected cancer cell lines treated with CTs of *L. leucocephala* hybrid-Rendang as compared to non-tumorigenic (3T3) after 72 h of incubation as determined by MTT assay. Values are presented as mean ± standard deviation (S.D.). $p < 0.05$ as compared to the 3T3 cell lines.

structure where fungi have thicker walls and contain high percentage of chitin [38].

The MIC values of the selected bacteria were ranged from 6.25 to 50.0 mg/mL while MBS was between 12.5 and 50 mg/mL (Table 2). Since each bacterium showed different MIC/MBC value, it was suggests that different bacteria possess different degrees of sensitivity to antimicrobial compounds such as CTs. Previous study showed CTs had antimicrobial activity toward *C. albicans*, *S. cerevisiae*, *S. marcescens* and *P. aeruginosa* with MIC/MBC/MFC value range 3.15–25.0 mg/mL but in this study the results showed otherwise [39–41]. This was probably due to the different sources of plants which make the CTs had different degrees of biological activity where it depends on their chemical structure and concentration [33].

Almost all selected bacteria were categorized as Gram's positive group and related to skin disease. *S. epidermidis* strains are

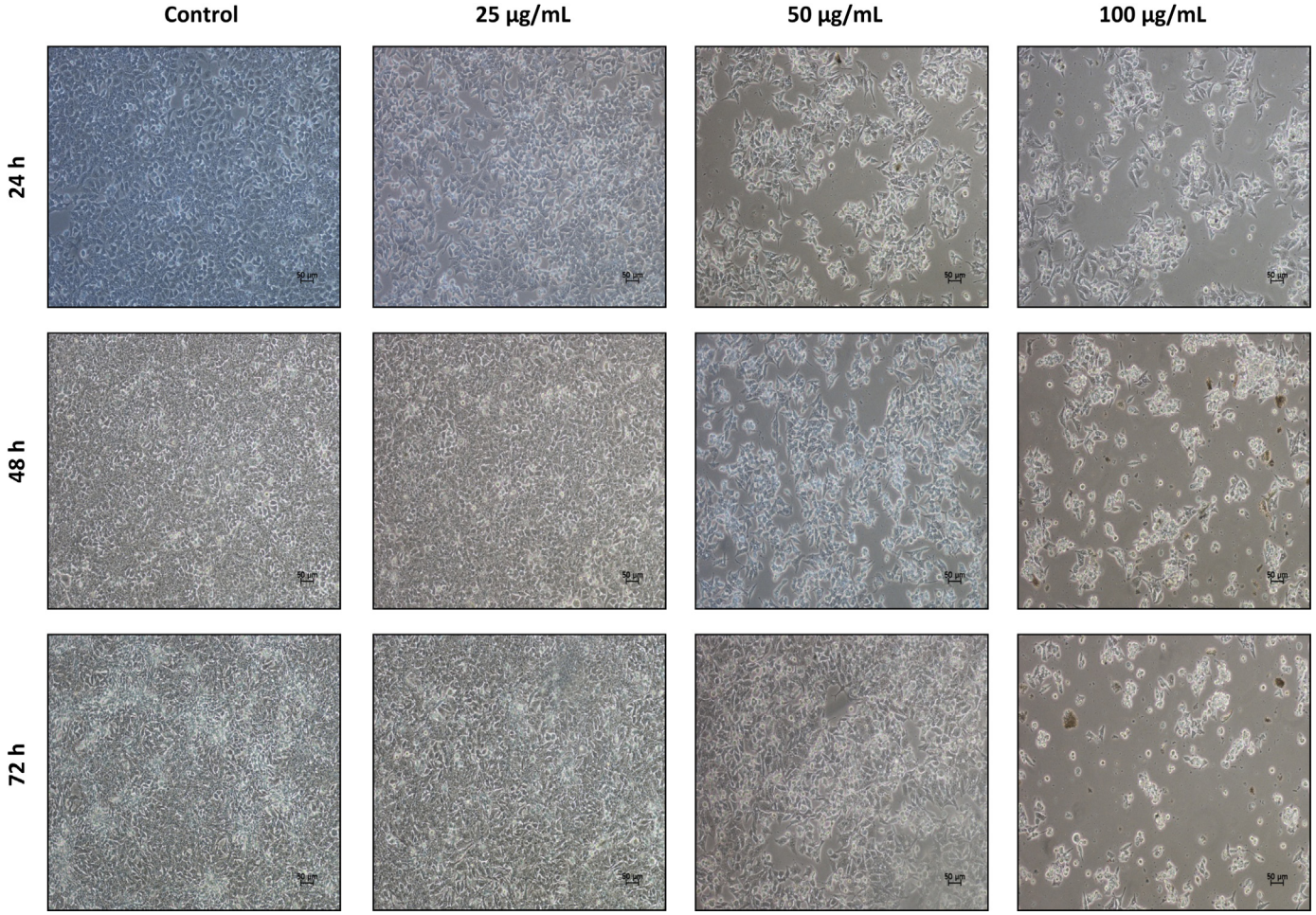

Fig. 6. Morphological changes of HepG2 cells treated with different concentration CTs of *L. leucocephala* hybrid-Rendang for 24, 48 and 72 h. The cells population was reduced in concentration-dependent manner as compared to untreated cells (100× magnification).

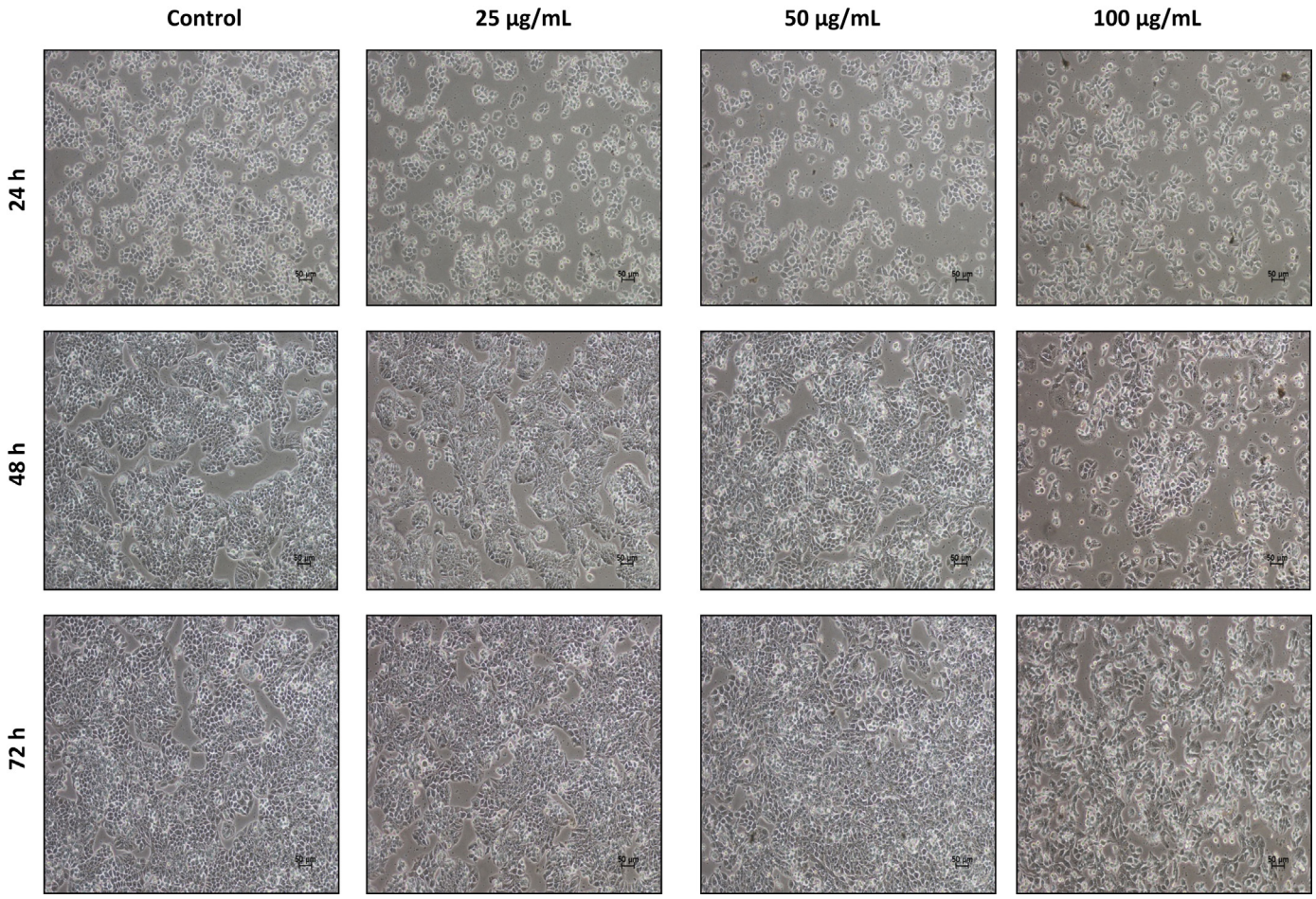

Fig. 7. Morphological changes of HT29 cells treated with different concentration of CTs of *L. leucocephala* hybrid-Rendang for 24, 48 and 72 h. The cells population was reduced in concentration-dependent manner as compared to untreated cells (100× magnification).

often resistant to antibiotics, including penicillin, amoxicillin and methicillin. The ability to form biofilms on plastic devices is a major virulence factor for *S. epidermidis*. In the present study, CTs showed inhibition against *S. epidermidis* which means that CTs can be one of the new potential compound to produce antibiotics for skin disease infection. Antibiotics are one of the most important weapons in fighting bacterial infections. However, over the past few decades, many commonly used antibiotics have become decreasingly effective against certain illnesses due to emergence of drug-resistant bacteria and most of the bacteria produced toxic reactions as well [42].

The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. The antibacterial and antifungal activities of extracts from leaves of *Cassia fistula* Linn showed *S. pyogenes* and *S. aureus* were more sensitive as compared with *E. coli* and *P. aeruginosa*, and for fungal activity, *C. albicans* shows good result as compare with *A. niger* and *A. clavatus* [42]. The growth inhibition zone measured ranged from 11 to 20 mm for all the sensitive bacteria, and ranged from 14 to 20 mm for fungal strains. This previous study demonstrated higher zone of inhibition compared to CTs, probably due to different content of compound in the extracts and had different degrees of biological activity.

3.5. Cytotoxicity of condensed tannin of L. leucocephala hybrid-Rendang

The cytotoxicity of CTs on selected cancer cell lines was determined using MTT assay and depicted in Fig. 5. Human breast adenocarcinoma (Mcf-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa) and human liver carcinoma (HepG2) cell lines were chosen in this study due to the cancer-related death in Malaysia and worldwide as well. Currently, breast cancer and cervical cancer are the leading cause of cancer-related death in women. Therefore, there is an urgent need to develop alternative therapeutic measures against this deadly disease.

As shown, CTs was found to be most cytotoxic toward MCF-7 with IC_{50} 38.33 ± 2.08 µg/mL as compared to other cell lines. Besides that, CTs was also less toxic to normal cell (3T3 F442A) with IC_{50} of 88.00 ± 2.00 µg/mL whereby 2 fold higher than the IC_{50} of CT toward MCF-7 cell lines.

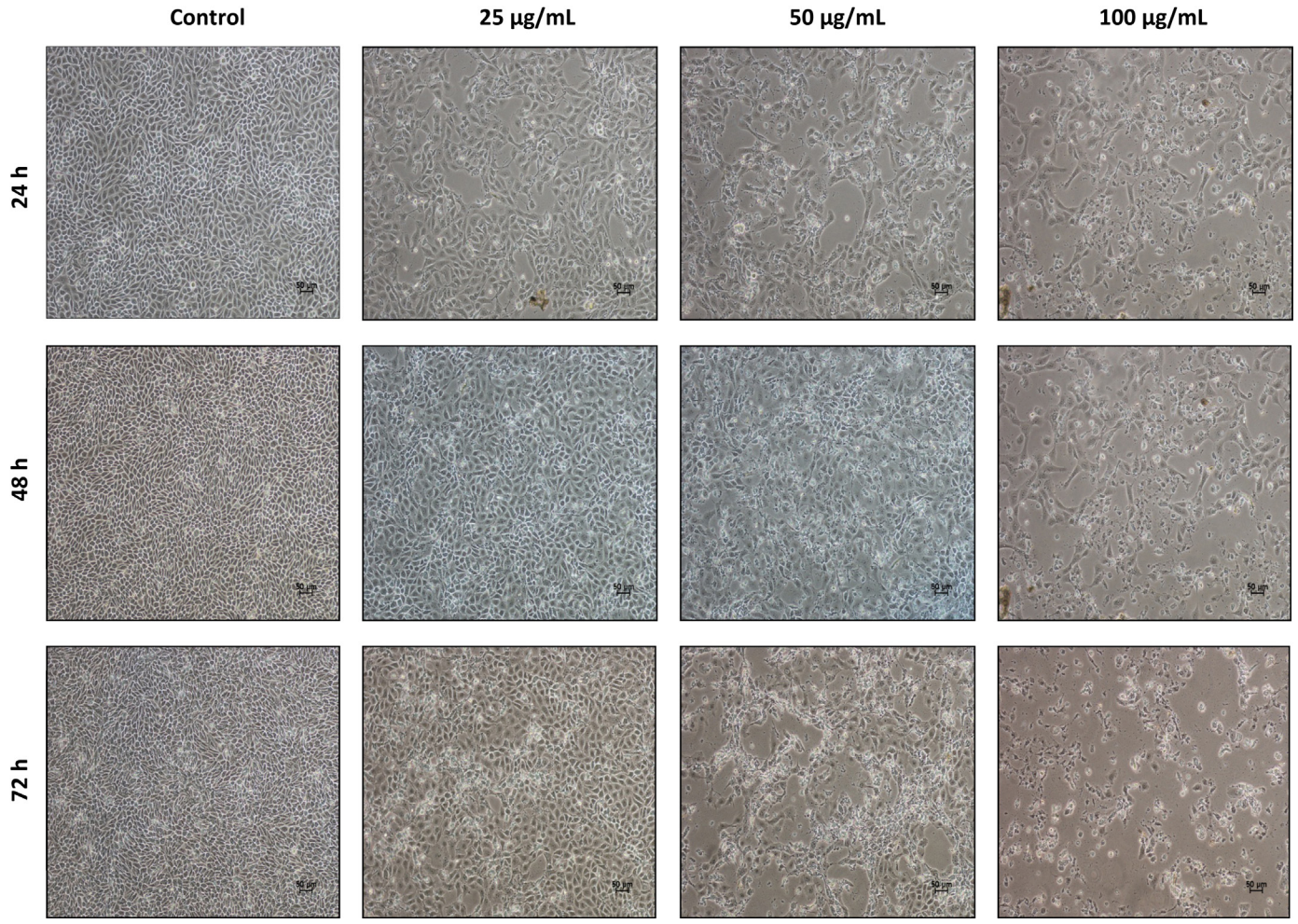

Fig. 8. Morphological changes of MCF-7 cells treated with different concentration of CTs of *L. leucocephala* hybrid-Rendang for 24, 48 and 72 h. The cells population was reduced in concentration-dependent manner as compared to untreated cells (100× magnification).

The present findings was in agreement with previous findings in which the plant extract extracted from various sources have shown to possess antitumor-promoting effects especially on human breast cancer cells. The condensed tannins from catechu (a traditional astringent) potently suppress the growth of MCF-7 breast cancer cells, and the effect was related to their activity of fatty acid synthase (FAS) inhibition [43]. The inhibition of both FAS activity and MCF-7 growth was exhibited by low concentrations of condensed tannins without FAS being precipitated. The results was similar with the present findings (cytotoxicity towards MCF-7) and suggests that CTs could be used as valuable resources for bioactive substances related to its cytotoxic properties.

3.5.1. Morphology of cancer cells treated with CTs of L. leucocephala hybrid-Rendang

As illustrated, the untreated cells (HepG2, MCF-7 and HT29) were distributed evenly with increase in the proliferation of the cells in 24, 48 and 72 h of incubation period (Figs. 6–8). In contrast, the most prominent changes including detachment of the cells from substratum, irregular in shape and size and cell shrinkage were observed in the cells treated with CTs. Besides that, some of the cells showed membrane blebbing and as well as formation of apoptotic bodies which regarded as one of the characteristic of apoptosis (Fig. 9). These cellular changes are the characteristics of the apoptotic induction of cell death which suggested that programmed cell death (apoptosis) could be the major factor contributing to the inhibition of cancer cell growth [44].

Apoptosis is a programmed cell death that removes or eliminates targeted unwanted or dead cells. Other than shrunken cells, characteristics of apoptotic cells include condensation of the cytoplasm and nucleus, aggregation of chromatin, and formation of membrane-bound vesicles known as apoptotic bodies [45]. Most of the cancer cells including MCF-7 which treated with plant extract showed apoptosis formation [46–48]. Unlike antioxidant agents act by preventing the onset of cancer during carcinogenesis and generally beneficial to cells, cytotoxic agents will damage the tumor cells which produced apoptotic bodies. Further studies on the pathway of cell death would help to find a novel active natural compound for cancer.

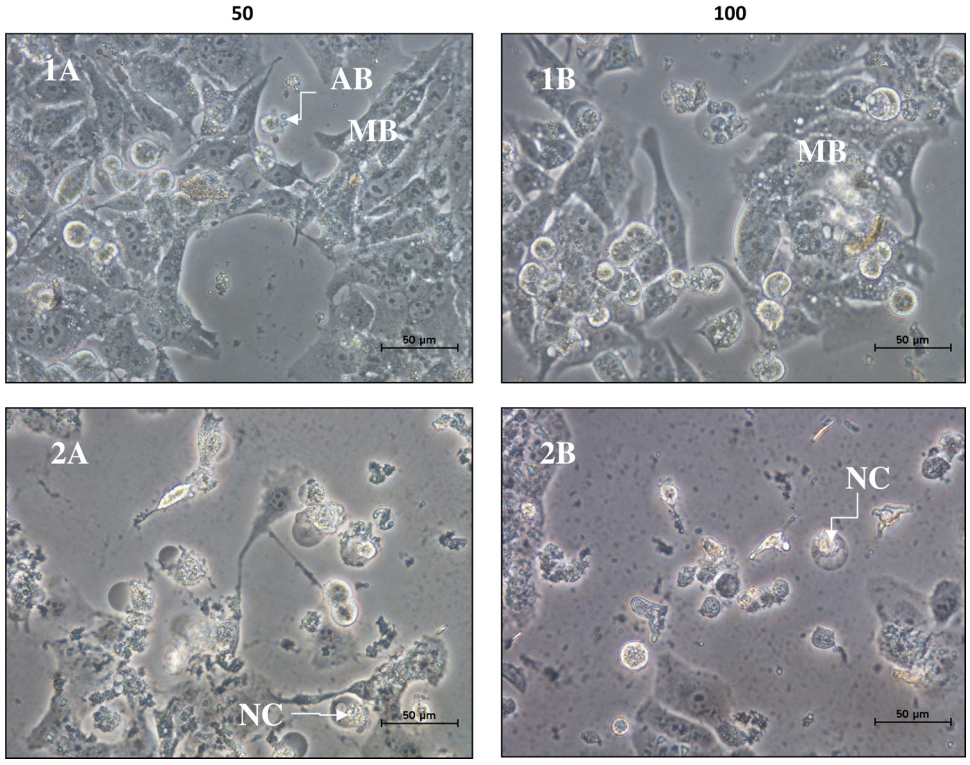

Fig. 9. Close-up views of HepG2 (1A and 1B) and MCF (2A and 2B) cells treated different concentration of condensed tannins of *L. leucocephala* hybrid-Rendang for 72 h viewed under an inverted light microscope. The cells showed characteristics of apoptosis such as nuclear compaction (NC) and membrane blebbing and as well as formation of apoptosis bodies (AB) (400× magnification).

4. Conclusion

The capability of the CTs extracts to scavenge free radicals in different systems, indicating that they may be useful therapeutic agents for treating various chronic diseases which are more related to oxidative stress associated diseases. The CTs extract showed significant antimicrobial activity, against different pathogens and inhibited cell proliferation and induced apoptosis in MCF-7, Hep G2 and HT29 cancer cells. These preliminary investigations have suggested that the CTs extracted from *L. leucocephala* hybrid-Rendang can be used to discover the bioactive natural product that may serve as a leads in the development of new pharmaceuticals in food and as potent antimicrobial and cytotoxic agent. Further studies will be designed to investigate its biomedical applications with a detailed mechanism through appropriate experimental model.

Conflict of interests

The authors declared that they had no conflict of interests.

Acknowledgements

This study was financially supported by the Universiti Putra Malaysia (RUGS:9199879). Special thanks to one of the author; the late Dr Sieo Chin Chin.

References

[1] L.A. Pham-Huy, H. He, C. Pham-Huy, Free radicals, antioxidants in disease and health, Int. J. Biomed. Sci. 4 (2) (2008) 89–96.
[2] J.R. Vane, R.M. Botting, A better understanding of anti-inflammatory drugs based on isoforms of cyclooxygenase, Adv. Prostaglandin Thromboxane Leukot. Res. 23 (2005) 41–48.
[3] R. Jakesz, Breast cancer in developing countries: challenges for multidisciplinary care, Breast Care 3 (2008) 4–5.
[4] S. Manoharan, R.B. Singh, S. Balakrishnam, Chemopreventative mechanisms of natural products in oral, mammary and skin carcinogenesis, Open Nutraceut. 2 (2009) 52–63.
[5] M. Schiffman, P.E. Castle, J. Jeronimo, A.C. Rodriguez, S. Wacholder, Human papillomavirus and cervical cancer, Lancet 370 (2007) 890–907.
[6] C.C. Lu, J.S. Yang, A.C. Huang, T.C. Hsia, S.T. Chou, C.L. Kuo, H.F. Lu, T.H. Lee, W.G. Wood, J.G. Chung, Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells, Mol. Nutr. Food Res. 54 (2010) 967–976.
[7] A.B. Archana, M.J. Varsha, V.J. Kadam, Potential of tannins: a review, Asian J. Plant Sci. 9 (4) (2010) 209–214.
[8] M.A.C. Torras, C.A. Faura, F. Schonlau, P. Rohdewald, Antimicrobial activity of Pycnogenol, Phytother. Res. 19 (7) (2005) 647–648.
[9] B.H.S. Lau, S.K. Riesen, K.P. Truong, E.W. Lau, P. Rohdewald, R.A. Barreta, Pycnogenol as an adjunct in the management of childhood asthma, J. Asthma 41 (8) (2004) 825–832.
[10] S.D. Ray, H. Parikh, D. Bagchi, Proanthocyanidin exposure to B6C3F1 mice significantly attenuates dimethylnitrosamine-induced liver tumor induction and mortality by differentially modulating programmed and unprogrammed cell deaths, Mutat. Res. 579 (1–2) (2005) 81–106.
[11] T. Kohama, N. Suzuki, S. Ohno, M. Inoue, Analgesic efficacy of French maritime pine bark extract in dysmenorrhea – an open clinical trial, J. Reprod. Med. 49 (10) (2004) 828–832.

[12] H.M. Shelton, J.L. Brewbaker (Eds.), *Leucaena lecocephala* – The Most Widely Used Forage Tree Legume, CAB International, Wallingford, U.K., 1994.
[13] C.C. Wong, C.P. Chen, E.M. Hutton (Eds.), Development of Acid/Psyllid Tolerant *Leucaena* Hybrids for Ruminant Production, ACIAR Proceedings No. 86, *Leucaena* – Adaptation, Quality and Farming System, 1998.
[14] (Degree of Doctor of Philosophy Thesis) B. Khamseekhiew (Ed.), Characteristics and protein binding affinity of condensed tannins in *Leucaena* species, Universiti Putra Malaysia, Malaysia, 2006.
[15] M. Saminathan, N.P. Nurul, H.Y. Tan, C.C. Sieo, N. Abdullah, C.M.L.V. Wong, J.B. Liang, Y.W. Ho, Effects of *Leucaena* condensed tannins of differing molecules weights on in vitro methane and volatile fatty acid production, in: 33rd Annual Conference of Malaysia Society of Animal Production, 2012, pp. 82–83.
[16] H.Y. Tan, C.C. Sieo, A. Norhani, J.B. Liang, X.D. Huang, Y.W. Ho, Effect of condensed tannins on bovine rumen protist diversity based on 18S rRNA gene sequences, J. Eukaryot. Microbiol. 60 (1) (2012) 98–100.
[17] T.H. Terrill, A.M. Rowan, G.B. Douglas, T.N. Barry, Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains, J. Sci. Food Agric. 58 (3) (1992) 321–329.
[18] R. Amarowicz, M. Naczk, F. Shahidi, Antioxidant activity of crude tannins of canola and rapeseed hulls, J. Am. Oil Chem. Soc. 77 (9) (2000) 957–961.
[19] M.S. Blois, Antioxidant determination by the use of a stable free radical, Nature 181 (4617) (1958) 1199–1200.
[20] Z. Czochanska, L.Y. Foo, R.H. Newman, L.J. Porter, Polymeric proanthocyanidins: stereochemistry, structural units and molecular weight, J. Chem. Soc. Perkin Trans. 1 19 (1980) 2278–2286.
[21] L.L. Zhang, Y.M. Lin, HPLC, NMR and MALDI-TOF MS analysis of condensed tannins from *Lithocarpus glaber* leaves with potent free radical scavenging activity, Molecules 13 (2008) 2986–2997.
[22] L. Zhang, G. Gellerstedt (Eds.), 2D Heteronuclear (^{1}H–^{13}C) Single Quantum Correlation (HSQC) NMR Analysis of Norway Spruce Bark Components, Blackwell, Oxford, 2008.
[23] P.N. Diouf, C.M. Tibirna, M.E. García-Pérez, M. Royer, P. Dubé, T. Stevanovic, Structural elucidation of condensed tannin from *Picea mariana* Bark, J. Biomater. Nanobiotechnol. 4 (2013) 1–8.
[24] P. Charalampos, L. Konstantina, K.M. Olga, Z. Panagiotis, J.S. Vassileia, Antioxidant capacity of selected plant extracts and their essential oils, Antioxidants 2 (1) (2013) 11–22.
[25] Z. Wissam, B. Ghada, A. Wassim, K. Warid, Simple and fast method for the extraction of polyphenol and the separation of proanthocyanidins from carob pods, Scholars Acad. J. Pharm. 2 (5) (2013) 375–380.
[26] Y. Athukorala, K.N. Kim, Y.J. Jeon, Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*, Food Chem. Toxicol. 44 (7) (2006) 1065–1074.
[27] F. Shahidi, P.K.J.P.D. Wanasundara, Phenolic antioxidants, Crit. Rev. Food Sci. Nutr. 32 (1) (1992) 67–103.
[28] I. Gülçin, D. Berashvili, A. Gepdiremen, Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinensis decne*, J. Ethnopharm. 101 (1–3) (2005) 287–293.
[29] C. Manach, A. Mazur, A. Scalbert, Polyphenols and prevention of cardiovascular diseases, Curr. Opin. Lipidol. 16 (1) (2005) 77–84.
[30] M.A. Ebrahimzadeh, S.F. Nabavi, S.M. Nabavi, B. Eslami, Antihemolytic and antioxidant activities of *Allium paradoxum*, Cent. Eur. J. Biol. 5 (3) (2010) 338–345.
[31] P. Padmanabhan, S.N. Jangle, Evaluation of DPPH radical scavenging activity and reducing power of four selected medicinal plants and their combinations, Int. J. Pharm. Sci. Drug Res. 4 (2) (2012) 143–146.
[32] N. Saeed, R.K. Muhammad, S. Maria, Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L., BMC Complement. Altern. Med. 12 (2012) 1–12.
[33] S.D. Wei, R.Y. Chen, M.M. Liao, N.W. Tu, H.C. Zhou, Y.M. Lin, Antioxidant condensed tannins from *Machilus pauhoi* leaves, J. Med. Plants Res. 5 (5) (2011) 796–804.
[34] M. Valco, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (1) (2007) 44–84.
[35] I.S. Young, J.V. Woodside, Antioxidants in health and disease, J. Clin. Pathol. 54 (3) (2001) 176–186.
[36] C.A. Rice-Evans, N.J. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends Plant Sci. 2 (4) (1997) 152–159.
[37] V. Koleckar, K. Kubikova, Z. Rehakova, K. Kuca, D. Jun, L. Jahodar, L. Opletal, Condensed and hydrolysable tannins as antioxidants influencing the health, Mini Rev. Med. Chem. 8 (5) (2008) 436–447.
[38] M.T. Madigan, J.M. Martinko (Eds.), Brock Biology of Microorganisms, Pearson Prentice Hall, Upper Saddle River, United States, 2006.
[39] S. Suraya, I. Darah, K. Jain, S.H. Lim, Antimicrobial and antioxidant activities of condensed tannin from *Rhizophora apiculata* barks, J. Chem. Pharm. Res. 3 (4) (2011) 436–444.
[40] S.H. Lim, I. Darah, K. Jain, Antimicrobial activities of tannins extracted from *Rhizophora apiculata* barks, J. Trop. For. Sci. 18 (1) (2006) 59–65.
[41] C.C.S. Andréia, C.L. Gisely, V.N. Celso, P.D.F. Benedito, C.P.M. João, Antioxidant and antifungal activities of extracts and condensed tannins from *Stryphnodendron obovatum* Benth, Braz. J. Pharm. Sci. 41 (1) (2005) 101–107.
[42] R.B. Nayan, V.J. Shukla, Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* l.: an ethnomedicinal plant, J. Adv. Pharm. Technol. Res. 2 (2) (2011) 104–109.
[43] S.Y. Zhang, C.G. Zheng, X.Y. Yan, W.X. Tian, Low concentration of condensed tannins from catechu significantly inhibits fatty acid synthase and growth of MCF-7 cells, Biochem. Biophys. Res. Commun. 371 (4) (2008) 654–658.
[44] P. Sirinet, U.T. Wanlaya, T.W. Benjamas, Anticancer and apoptosis-inducing activities of microbial metabolites, J. Biotechnol. 13 (2010) 1–12.
[45] M.Z. Majid, M.Z. Zuraiza, A.R. Fathilah, Apoptosis-inducing effect of three medicinal plants on oral cancer cells KB and ORL-48, Sci. World J. 2014 (2014) 1–8.
[46] N.N. Farshori, E.S. Al-sheddi, M.M. Al-Oqail, J. Musarrat, A.A. Al-Khedhairy, M.A. Siddiqui, Anticancer activity of *Petroselinum sativum* seed extracts on MCF-7 human breast cancer cells, Asian Pac. J. Cancer Prev. 14 (10) (2003) 5719–5723.
[47] H. Hongbu, A. Nam-Shik, Y. Xinlin, L. Yong-Soon, K. Kyung-Sun, *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell, Int. J. Cancer 102 (3) (2002) 250–253.
[48] S. Sumathi, B. Dharani, J. Sivaprabha, R.K. Sonia, P.R. Padma, Cell death induced by methanolic extract of *Prosopis cineraria* leaves in MCF-7 breast cancer cell line, Int. J. Pharm. Sci. Invent. 2 (1) (2013) 21–26.