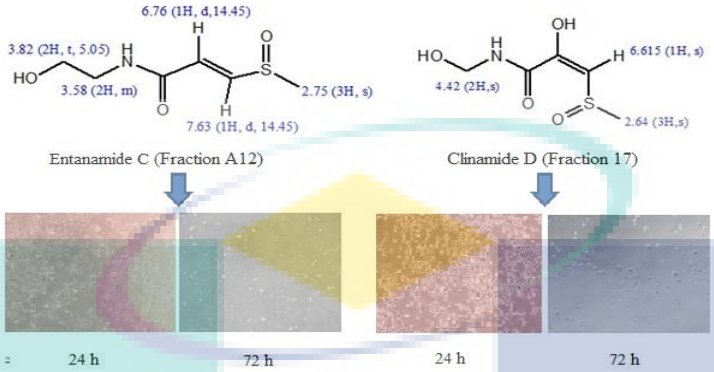

Development of chemical markers and chromatographic fingerprint of Clinachantus nutans for evaluation of therapeutic products

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ABSTRACT (120 words)

Clinacanthus nutans has attracted Malaysian public interest due to its high medicinal values in the prevention of cancer. The extraction was done by MeOH at room temperature while chromatography was done on a silica gel RP-18. Six fractions collected from column chromatography were evaluated by the MTT assay against two breast cancer cell lines: MDA-MB-231 and MCF-7. Amongst the fractions, A12 and A17 were shown to exhibit the most activity, and the compounds were mostly isolated from these fractions. Two sulphur-containing compounds, entadamide C and clinamide D were isolated. Molecular docking simulation studies revealed that entadamide C and

clinamide D could bind favourably to the caspase-3 binding site with the binding energy of -4.28 kcal/mol and -4.84 kcal/mol, respectively.

1. INTRODUCTION

Clinacanthus nutans (Belalai gajah) has been extensively used as primary sources of complementary and alternative healthcare for cancer patients in Malaysia but the scientific proof of its anti-cancer property remains insufficient. To date, there is little to no research investigating the effects on pure bioactive compounds from *C. nutans* that contribute to the specific therapeutic effect. *C. nutans* products available in the marketplace but most of the products were unlabelled active ingredients. Currently there are no chemical markers for identifying active ingredients used in *C. nutans* as herbal products. As a result, the marketplace is prone to contamination and possible product substitution, which dilute the effectiveness of useful remedies, lowering the perceived value of the products because of a lack of consumer confidence in them. Therefore, the scientific study should be designed to provide individual compounds as chemical markers and fingerprint chromatogram of *C. nutans* extract is develop for quality control of herbal medicines. Then, the effectiveness of bioactive compounds isolated from *C. nutans* as a therapeutic medicine need to be studied. Chemical marker compounds will be purified by chromatographic procedure and the structure will be elucidated by spectroscopic techniques. Chromatographic fingerprint of *C. nutans* will be developed by using UPLC-QTOF/MS. Quantitative analysis of bioactive contents and in-vivo biological activities will be determined. The chemical fingerprint analysis will provides comprehensive quality evaluation and control for *C. nutans* products. This finding will be useful in

setting the baseline for chemical marker development for *C. nutans* commercial products and also contributes to safer drug administration. This approach would support the need to address considerable health risks to consumers who expect to have access to high quality herbal products that promote good health.

2. RESEARCH METHODOLOGY

2.1 Plant Material

The leaves of *C. nutans* (4.0 kg) were collected from the TKC Herbal Nursery, Pusat Pertanian Pantai, Seremban, Malaysia and identified by Dr. Shamsul Khamis, a botanist at the Institute of Bioscience, University Putra Malaysia. A voucher specimen number (SK 2874/15) of the plant was deposited at the Herbarium Unit of University Putra Malaysia, Malaysia.

2.2 Extraction and Chromatography Fractionation

The dried powdered leaves of *C. nutans* (4.0 kg) were left to percolate at room temperature in hexane (10 L) and methanol (20 L) consecutively. The extract of *C. nutans* was sonicated for 30 minutes at a temperature of 60 °C. The process was replicated three times. The extracts of hexane (Hx) and methanol (MeOH) were filtrated and allowed to evaporate in the vacuum rotary evaporator at 60 °C, consecutively. The reduced extract of MeOH was chromatographed on a silica gel RP-18 column. The column was eluted successively with water, water-methanol (1:1), methanol, and ethyl acetate. The total phenolic content of all collected fractions were determined using Folin-

Ciocalteu method and the total flavonoid content was measured using aluminium calorimetric method. Fraction A12 (28.5 mg), were re-chromatographed with $CHCl_3$-MeOH (80:20) to yield compound 1 (1.71 mg), while fraction A17 (25.3 mg) was chromatographed with $CHCl_3$-MeOH (85:15) to produce compound 2 (1.75 mg). The structures of the isolated compounds were identified by spectroscopic means (MS, 1D, and 2D NMR).

2.3 Optimization of UPLC-QToF/MS System

UPLC-QTOF/MS method will be optimized to ensure the constituents are well separate and sufficient information can be obtained from chemical peaks, each chromatogram exhibit excellent characteristics with good repeatability, precision and stability. 1 g of each extracts were dissolved in 1 mL methanol and subjected to ultra-high performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QToF/MS) for the determination of active composition present in the extracts. Different extracts were subjected to UPLC-QToF/MS using an HSS BEH C18 column (2.1 × 100 mm). HPLC data are obtain via Waters Xevo G2-S QToF. The polarity is change by adjusting the ratio of MeOH. The QToF/MS was operated in the range 100 – 1500 Da, in both the positive and negative modes. All operations, acquisitions, and data analyses are monitor by the Natural Products Application Solution with UNIFI featuring the Traditional Medicine Library. Isolated compounds will be used as reference as chemical markers for quantitative analysis of commercial products of *C.nutans*.

2.4 Cell Culture for Cytotoxicity

A cryovial consisting of cancer cells was thawed in a water bath at 37 °C with light swirling. The cells then were transferred into a 15 mL centrifuge tube and centrifuged at 3000 rpm for 5 minutes to remove the dimethylsulfoxide (DMSO), a cryopreservative agent. The cells were introduced into a T-25 falcon flask and were cultivated in DMEM medium supplemented with 10% (v/v) foetal serum and 1% (v/v) penicillin-streptomycin to increase and stimulate cells survival and proliferation. Penicillin-streptomycin was added to preferentially kill any present bacteria (*Streptococcus lactic* and *Bacillus cereus*) that might contaminate the cells. The growth medium was supplemented with phenol red, a pH indicator. The maintenance of pH is crucial and a requisite for the sustenance of exponential growth of cells. Cells were incubated in the CO_2 incubator at 37 °C and supplemented with 5% CO_2. The medium was replaced every alternate day until the cells were confluent and ready to be sub-cultivated.

The media in the flask was discarded when the cells have reached its confluence. The cells were then washed several times with 2 mL of phosphate-buffered saline (PBS). This is to ensure the removal of any residue of spent culture media and dead cells. Cells were detached by adding 500 µL of trypsin and incubated for 5 minutes. The process of trypsinisation was enhanced by gentle tapping of the flask a few times. Once the cells appear rounded and singly under the microscope, 4 mL of 10% FCS-DMEM was added to the cells to inactivate the trypsin. The cell suspension was transferred to a 15 mL falcon tube and centrifuged at 3000 rpm for 5 minutes at 4 °C. Resuspension of the cells was done in the culture media.

2.5 Cell Viability Assay

The effect of *C. nutans* extract and fractions on the viability of various cancer cell lines was determined by an MTT-based assay. Cells growing in the exponential phase were collected and transferred to a 96-well microtiter plate (5×10^4 cells per well). The cells were incubated in the presence of different concentrations of the extract and fractions for three days. The positive control experiment involved tamoxifen, while the negative control was untreated media. At the end of the incubation period, 10 µL MTT solution was added into each well, and the plate was further incubated at 37 °C for 4 h. The procedure was conducted either in the dark or in minimal light due to the photosensitivity of the MTT solution. The medium with the MTT solution was carefully aspirated from each well. Sequentially, 110 µL of DMSO was added to each well to solubilise the formazan product. The absorbance was recorded at 490 nm using a microplate reader. The IC_{50} value of *C. nutans*, which is the concentration of the extract and fractions that inhibited cell growth by 50%, was established in triplicates for each well.

A stock solution (10 mg/mL) was prepared for all *C. nutans* extract, fractions, and tamoxifen (positive control). The concentrations of the test solutions were prepared from 10 µg /mL to 100 µg /mL (10 µg/mL, 50 µg/mL, and 100 µg/mL). The determination of cell concentration was conducted using trypan blue before the plating process began. Cells at 80%–90% confluence were detached from the flask with 0.05% (v/v) trypsin and 0.02% (v/v) EDTA. The cells were then plated onto 96-well plates at the initial density of approximately 5×10^4 cells per well and were left overnight to attach. On the following day, the medium was discarded from each well, and different concentrations of *C. nutans*

fractions were added into the wells (100 µL/well of fresh media with fractions). Tamoxifen dissolved in DMSO functioned as the control drug. The same concentration of solvent was used for the negative control of culture cells. The final concentration of DMSO was 0.1% (v/v) or less. The assay for each concentration of the extract was done in triplicates. Incubation of the cells was done in the CO_2 incubator at 37 °C and 5% CO_2. The assay plates were incubated for 72 h. The MTT staining assay was used to determine the number of surviving cells. A volume of 10 µL MTT solution was added to each well after incubation. The plates were then incubated for another 4 h. Then, the MTT assay was discarded, and 110 µL of DMSO was used to dissolve the purple formazan crystal that formed at the bottom of the wells. After 10 minutes, the plates were read at 490 nm on a microplate reader. The IC_{50} values were obtained through the construction of the dose-response curves. The same methods had been repeated for the viability effects of isolated compounds.

2.6 Morphological Study

A 12-well plate was used to plate the cells with a density of 5×10^5 cells per well and incubated for 24 h. The exposure duration of the cells is 24, 48, and 72 h with an optimum concentration of extract and fractions (100 µg/mL). The positive control used was tamoxifen. The inverted light microscope (Olympus, USA) was used to observe the morphological changes such as the presence of apoptosis or necrosis in the cells.

2.7 Molecular Docking Study

Autodock4 (version 4.1.2) was employed for the docking simulation, while its calculation was carried out by AutoDock tools 1.5.6 and MGL tools 1.5.6 packages. In docking calculations, the receptor-ligand poses obtained are ranked using an energy-based scoring function. In this research, the protein crystal structure of caspase-3 (PDB Code: 1RE1) – a well-known cancer-related structure was retrieved from the Protein Data Base (PDB, www.rcsb.org) and the Lamarckian-Genetic Algorithm (LGA) was employed in this docking study. For the docking simulation, the integration of the grid box was 40(x) × 54(y) × 48(z) and centred at 41.3(x) × 92.9(y) × 21.9(z) for the active site of caspase-3 (Pandurangan, Enkhtaivan, & Kim, 2016). The highest binding activities were computed, and the Protein-ligand interactions were analysed using the Discovery Studio Visualizer.

3. LITERATURE REVIEW

3.1 Pharmacological Activities of *C. nutans*

C. nutans has been reported previously to have numerous phenolics, terpenoids, benzenoids, cerebrosides, glycoglycerolipids, glycosylglycerides, fatty acids, chlorophyll derivatives, phytosterols, and sulfur-containing glucosides that contribute to this plant possesses a diverse range of pharmacological activities.

Phytopharmaceuticals are increasingly popular as alternative medicines, but poorly regulated in many countries. The manufacturers of these products should be subject to strict controls regarding each product's quality and constituents. Routine testing

and identification of raw materials should be performed to ensure that the raw materials used in pharmaceutical products are suitable for their intended use (Osathanunkul, Suwannapoom, Osathanunkul, Madesis, & de Boer, 2016).

Recently, *C. nutans* has received much attention due to its therapeutic applications related to its biological activities (Mustapa, Martin, Sanz-Moral, Rueda, & Cocero, 2016). Chloroform extract from *C. nutans* leaves revealed a bioactive compound which exhibits anti-inflammatory, in-vitro wound healing, and anti-biofilm activities (Roeslan, Ayudhya, Yingyongnarongkul, & Koontongkaew, 2019). *C. nutans* also had been found as for its potential as natural nutraceuticals for cancer prevention and treatment (Yong et al., 2013).

C. nutans displayed an important role in the treatment of skin diseases. A cream and tincture form of *C. nutans* extract are indicated to be used for relieving herpes simplex and herpes zoster; its lotion form for healing dermatitis and urticaria; and its ointment form for relieving swelling from insect bites (Neamsuvan & Bunmee, 2016).

Extracts from the leaves were reported to possess analgesic and anti-inflammatory activities, antiviral activities against varicella-zoster virus and herpes simplex virus type-2 (Kunsorn et al., 2013). Clinical trials have been carried out to investigate the efficacy of *C. nutans* extract in treating herpes (HSV and VZV) patients. Findings from these studies revealed faster recovery (crusting time within 3 d and healing time within 7 d) in patients treated with the extract compared to the placebo group (crusting time 4-7 d and healing time 7-14 d). Additionally, the extract exhibited similar efficacy as the antiviral drug, Acyclovir, which is chiefly prescribed to treat herpes infections. Besides, patients receiving the extract as treatment described minimal pain and no side effects.

C. nutans is commonly used in traditional Malaysian medicine for its nourishing and antioxidant properties. Recently, the extracts from leaves of *C. nutans* have been used extensively as primary sources of complementary and alternative healthcare or as economical in-housing regimens for cancer patients (P'ng, Akowuah, & Chin, 2013).Patients have claimed that they have recovered from cancer illness after consuming *C. nutans* leaves over certain period. However, the inclination among researchers to conduct research on *C. nutans* in Malaysia remains lacking. To date most of the published results are from the research in Thailand. Most of the publications reported the bioactivity of the extract from *C. nutans*. Recently, there are many *C. nutans* products available in market in the form of herbal tea, capsules, tablets and concentrated plant extracts. However, formulation of *C. nutans* as commercially packaged fresh drinks remains rare. Moreover, the popularity of these products remains low due to lack information of these products and promotion initiative.

3.2 Phytochemical Constituents of *C. nutans*

Medicinal plants contain bioactive phytochemicals, defined as secondary metabolites that are produced to protect the plants. Since plants are stationary autotrophs, they need to develop survival strategies against number of challenges, including engineering their own pollination and seed dispersal, nutrient deprivation, solar radiation and the coexistence of herbivores and pathogens in their immediate environment. Therefore, plants have evolved biochemical pathways production of secondary metabolites in vegetative (e.g. leaf, stem and root) and reproductive (e.g. flower, fruit and seed) regions in response to specific environmental stresses. They also serve as an

attractant (e.g. colour, pheromones) for pollinating insects or fruit-dispersing animals. These secondary metabolites are unique to specific plant species or genera and are not involved in the plants' primary metabolic requirements.

Plant secondary metabolites are classified into three main groups based on their biosynthetic origin: i) terpenes, ii) nitrogen-containing alkaloids and iii) phenolic compounds. These secondary metabolites are synthesised from important building blocks (shikimic acid, acetyl coenzyme A, mevalonic acid and 1-deoxyxylulose-5-phosphate) via different pathways, such as shikimate, acetate, mevalonate and deoxyxylulose.

Phytochemical compounds are beneficial elements (phenolic, vitamin, amino acids, minerals) in the various plant constituents, which show a potential therapeutic in biological activities to be used as additives in food, cosmetics, medicine, and others fields (Muhamad et al., 2017; Volf & Popa, 2018). Representatives of Acanthaceae, particularly *C. nutans* and *A. paniculata*, possessed a wide range of phytochemicals including terpenoids, alkaloids, flavonoids, glycosides, steroids, saponins, phenolic acids and tannins.

C. nutans contains terpenoids, phenolics and other potentially bioactive compounds, including sulphur-containing glucosides, lipids, chlorophyll derivatives and benzonoids. The vital and representative phytochemical found from *C. nutans* extract are shown below.

C. nutans has been phytochemically investigated previously for its phytosterol: stigmasterol **(1)** and β-sitosterol **(2)**, and triterpenoid: lupeol **(3)** and betulin (**4)** (Le et al., 2017). Six known C-glycosyl flavones: vitexin **(5)**, isovitexin **(6)**, Apigenin-6-C-β-D-

glucopyranosyl-8-C-α-L-arabinopyranoside (shaftoside) **(7)**, isomollupentin-7-O-β-glucopyranoside **(8)**, orientin **(9)**, and isoorientin **(10)** had been isolated from *C. nutans* extract.

Early phytochemical studies of *C. nutans* revealed the presence of five sulfur-containing glycosides: clinacoside A **(11)**, clinacoside B **(12)**, clinacoside C **(13)**, cycloclinacoside A1 **(14)**, and cycloclinacoside A2 **(15)** (Teshima et al., 1998).

Recently, four new sulfur-containing compounds: clinamides A **(16)**, clinamides B **(17)**, clinamides C **(18)** and 2-cis-entadamide A **(19)**, were isolated and identified along with three known sulfur-containing compounds named entadamide A **(20)**, entadamide C **(21)**, and trans-3-methylsulfinyl-2-propenol **(22)** (Tu et al., 2014). A mixture of cerebrosides **(23)**, monoacylmonogalactosylglycerol (2S)-1-*O*-linolenoyl-3-*O*-b-digalactopyranosylglycerol **(24)** (Tuntiwachwuttikul, Pootaeng-on, Phansa, & Taylor, 2004) and two glycoglycerolipid were isolated from the leaves extract of *C. nutans*, namely monogalactosyl diglycerol **(25)** and digalactosyl diglyceride **(26)** (Janwitayanuchit et al., 2003; Satakhun, Suwanborirux, Lipipun, Suttisri, & Pummangura, 2001)

The chlorophyll derivatives (phaeophytins) of *C. nutans* chloroform extracts contained 13^2-hydroxy-(13^2-R)-phaeophytin b **(27)**, 13^2-hydroxy-(13^2-S)-phaeophytin a **(28)**, 13^2-hydroxy-(13^2-R)- phaeophytin a **(29)**, and purpurin-18 phytyl ester **(30)**, were isolated from *C. nutans* chloroform extract (Roeslan et al., 2019; S Sakdarat, Shuyprom, Ayudhya, Waterman, & Karagianis, 2006).

The presence of various phenolic and fatty acid compounds in leaf extracts of *C. nutans* were reported (Mustapa, Martin, Gallego, Mato, & Cocero, 2015; Sulaiman et al.,

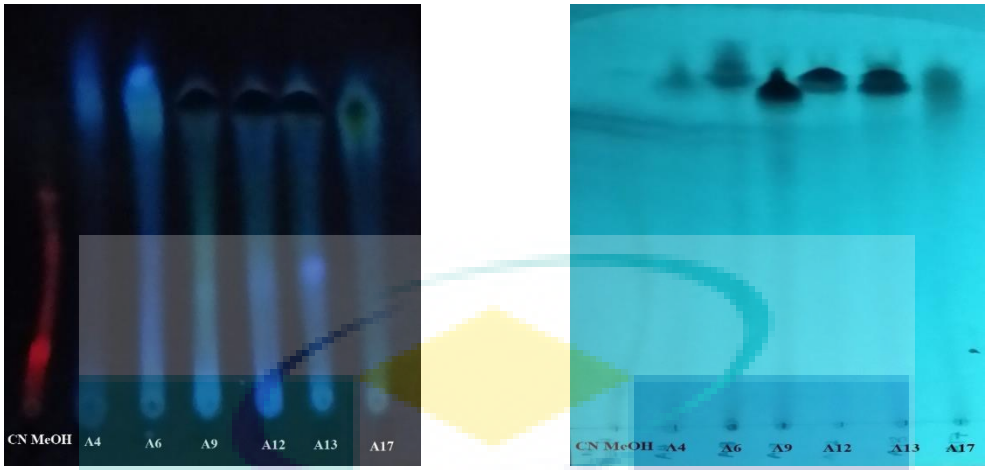
2015). Representative of major phenolic and fatty acid compounds identified in extraxt of *C. nutans*, are alpha-tocopherol **(31)**, sinapic acid **(32)**, 4-hydrophenylacetic acid **(33)**, 4-hydroxybenzoic acid **(34)**, coumalic acid **(35)**, quercetin hydrate **(36)**, ρ-coumaric acid **(37)**, vanilic acid **(38)**, stearic acid **(39)**, linoleic acid or omega-3 fatty acid **(40)**, palmitic acid **(41)**, oleic acid **(42)** and myristic acid **(43)**.

4. FINDINGS

The leaves of *C. nutans* were extracted yielded MeOH extract. Fractionation of MeOH extract from *C. nutans* on RP-18 silica gel eluted successively with water, water-methanol (1:1), methanol, and ethyl acetate bring forth to 25 different fractions.

4.1 Extraction and Fractionation of *C. nutans*

The leaves of *C. nutans* was productively extracted via maceration extraction method with hexane and MeOH to give dark yellow gums of Hx extract and dark green gums of MeOH extract. Methanol extract of *C. nutans* was chromatographed on RP-18 silica gel column eluted successively with water, water-methanol (1:1), methanol, and ethyl acetate yielded 25 fractions. Figure 1 shows thin layer chromatography of *C. nutans* MeOH extract and selected fraction dejected under UV light 365 nm and 254 nm.



a **b**

Figure.1 Thin-layer Chromatography of *C. nutans* MeOH extract and selected fractions, dejected under UV light (**a**:365 nm and **b**:254 nm)

4.2 Structure Identification

The leaves of *C. nutans* extracted via maceration with methanol (MeOH) yielded dark green gums extract. The methanol extract of *C. nutans* chromatographed on an RP-18 silica gel column and eluted successively with water, water-methanol (1:1), methanol, and ethyl acetate yielded 25 fractions. Fractions with the same profiles on the thin layer chromatography (TLC) plate were grouped, whereby six different fractions with different polarity, good separation and promising active compounds were obtained. From these six fractions, three fractions labelled A4, A6, and A9 were classified as the polar fraction, while the other three fractions labelled A12, A13, and A17 were classified as moderately polar. The results obtained from the analysis of TPC and TFC in all fractions were shown in Table 1. The analysis demonstrates methanol extract of *C. nutans* contained significantly higher TPC and TFC than other fractions. Flavonoids are a subclass of

phenolic compounds; therefore, the results show that TFC values is lower than TPC values in all fractions. Cytotoxic activity tests were carried on breast cell lines for all the fractions. The active fractions (A17 and A12) were subjected to further purification techniques. The structures of the isolated compounds were clarified by spectroscopic analysis, mainly, 1H NMR, 13C NMR, 13C DEPTQ, 1H–13C HSQC, 1H–13C HMBC, and mass spectrometry (MS). Comparison of all spectral data was made with the published data (Hamid, Yahya, Yusoff, & Zareen, 2016; Tu et al., 2014) and the compounds were identified as entadamide C (1) and clinamide D (2). The purity of the isolated compounds was confirmed by UPLC/QTOF MS chromatograms, as shown in Figure 2

Entadamide C (1), IUPAC, (E)-N-(2-hydroxyethyl)-3-(methylsulfinyl)acrylamide: pale yellow oil UV (MeOH) λmax (log ε) 254, 365 nm; IR (KBr) νmax 3328 cm-1 (OH), 2944.09 cm-1 (C-H), 1657.08 cm-1 (C=O) and 1051.11 cm-1 (S=O); positive ESIMS m/z 178.0532 [M + H]+ (calcd for, $C_6H_{11}NO_3S$, 177.05) 1H NMR (500 MHz, in CDCl3) δ 6.76 (1H, d, 14.45, H-2), 7.63 (1H, d, 14.45, H-3), 2.75 (3H, s, H-4), 3.58 (2H, m, H-5), 3.82 (2H, t, 5.05, H-6); 13C NMR (125 MHz, in CDCl3) δ 166.4 (C-1), 128.5 (C-2), 147.7 (C-3), 40.0 (C-4), 43.1 (C-5), 61.6 (C-6)

Clinamide D (2), IUPAC, (E)-2-hydroxy-N-(hydroxymethyl)-3-(methylsulfinyl)acrylamide: pale yellow oil; UV (MeOH) λmax (log ε) 240 nm; IR (KBr) νmax 3332.90 cm-1 (OH), 2945.39 cm-1 (C-H), 1666.08 cm-1 (C=O) and 1031.11 cm-1 (S=O); positive ESIMS m/z 179.9899 [M + H]+ (calcd for, $C_5H_9NO_4S$,179.03); 1H NMR (500 MHz, in CDCl3) δ 6.615 (1H, s, H-3), 2.64 (3H, s, H-4), 4.42 (2H, s, H-5); 13C NMR (125 MHz, in CDCl3) δ 162 (C-1),133.5 (C-3), 40.40 (C-4), 61.80 (C-5

4.3 UPLC-QToF/MS Analysis

The results of good match plot of MeOH *C. nutans* extract by UPLC-QTOF/MS was shown in Figure 4.2. The compounds were identified by Traditional Chinese Medicine (TCM) library search for flavonoids and phenolics groups in UNIFI software. Based on previous studies, these two classes of compounds were abundant in *C. nutans* extracts. The presence of phenolic compounds was determined based on their mass fragmentation pattern, low mass error within the acceptance range of ± 5 mDa and ion response

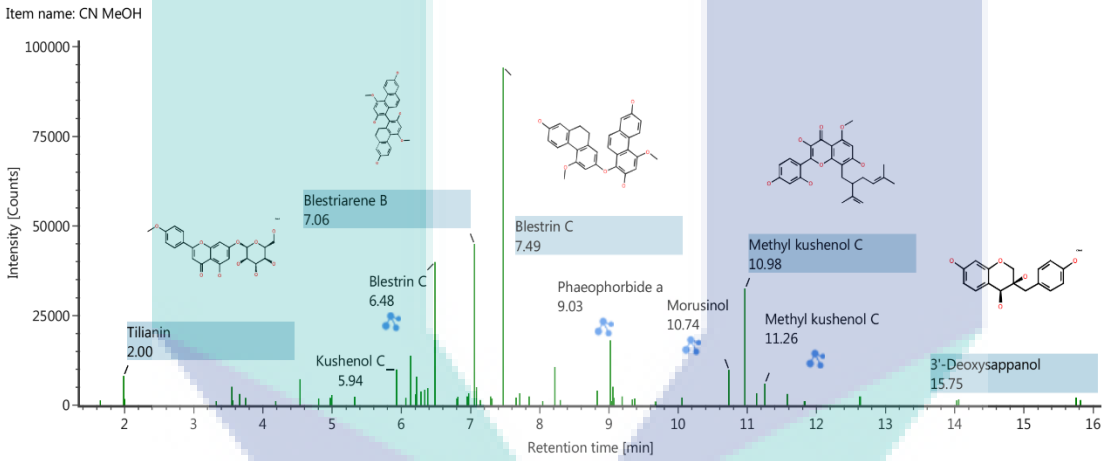
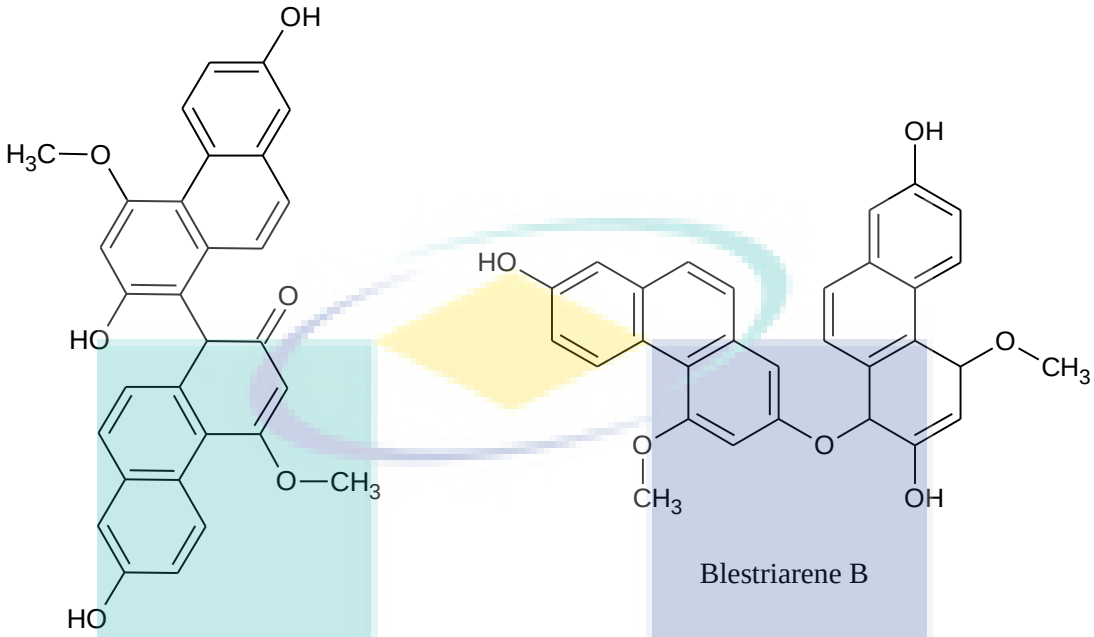


Figure 2 Good match plot of methanol *C. nutans* extract identified by UPLC-QTOF/MS

The ion response of blestrin C and blestriarene B, structures shown in Figure 3 are higher compared to other good match compounds. The identified compounds are not similar with compounds reported by previous study, Quah et al. (2017) due to different extraction techniques. In this study we used sonication technique for 30 min at a temperature of 60 °C while their study employed the maceration technique in methanol at room temperature for 48 h.



Blestrin C

Blestriarene B

Figure 3 Structure of blestrin C **(68)** and blestriarene B **(69)**

Research conducted by Deng et al. (2017) had shown that sonication extraction technique was able to extract more phenolic compounds compared to maceration technique. In addition, the difference of identified compounds also depends on the sensitivity of the advanced spectrometric and spectroscopic analytical tools used (L. W. Khoo et al., 2018).

4.4 Cytotoxicity of *C. nutans*

The cytotoxicity of *C. nutans* on MDA-MB-231 and MCF-7 breast cancer cell line were evaluated by MTT assay. Treatment of MDA-MB-231 and MCF-7 cancer cell line with *C. nutans* MeOH extract and selected fractions at various concentrations inhibited cell viability in a dose dependent manner. The extracts concentration (6.25 – 100 µg/mL) was based on suggestions by (Houghton et al., 2007) and previous cytotoxicity studies of medicinal plants on MTT assay with some minor modifications (Dahham et al., 2018; Haron et al., 2019; Vajrabhaya & Korsuwannawong, 2016).The preliminary process of cytotoxic screening showed that *C. nutans* MeOH extract and selected fractions (A4, A6, A9, A12, A13, and A17) for 24 h, 48 h, and 72 h possessed anticancer promoting activity against MDA-MB-231 and MCF-7 breast cancer cell line (Figure 4).

Figure 5a shows viability of MDA-MB-231 and 5b MCF-7 cancer cell lines on *C. nutans* MeOH extract and selected fractions (A4, A6, A9, A12, A13, and A17) after exposure for 3 days, at a concentration of 100 µg/ mL. At day 1, the percentage of cell death did not differ significantly between all treated samples and non-treated controls. Nonetheless, by day 2 and day 3, the percentage of MDA-MB-231 and MCF-7 cells had decreased more than 50 % in the treated cells compared to that seen in non-treated controls. The A9 fraction of *C. nutans* caused maximum inhibition of MDA-MB-231 cells with values of 87.16 % ± 0.077, while tamoxifen (positive control) showed 65.66 % ± 0.071 inhibition. Meanwhile, the *C. nutans* MeOH extract showed maximum inhibition of the MCF-7 cell line with 82.38 % ± 0.034, while tamoxifen showed maximum inhibition at 66.12 % ± 0.079.

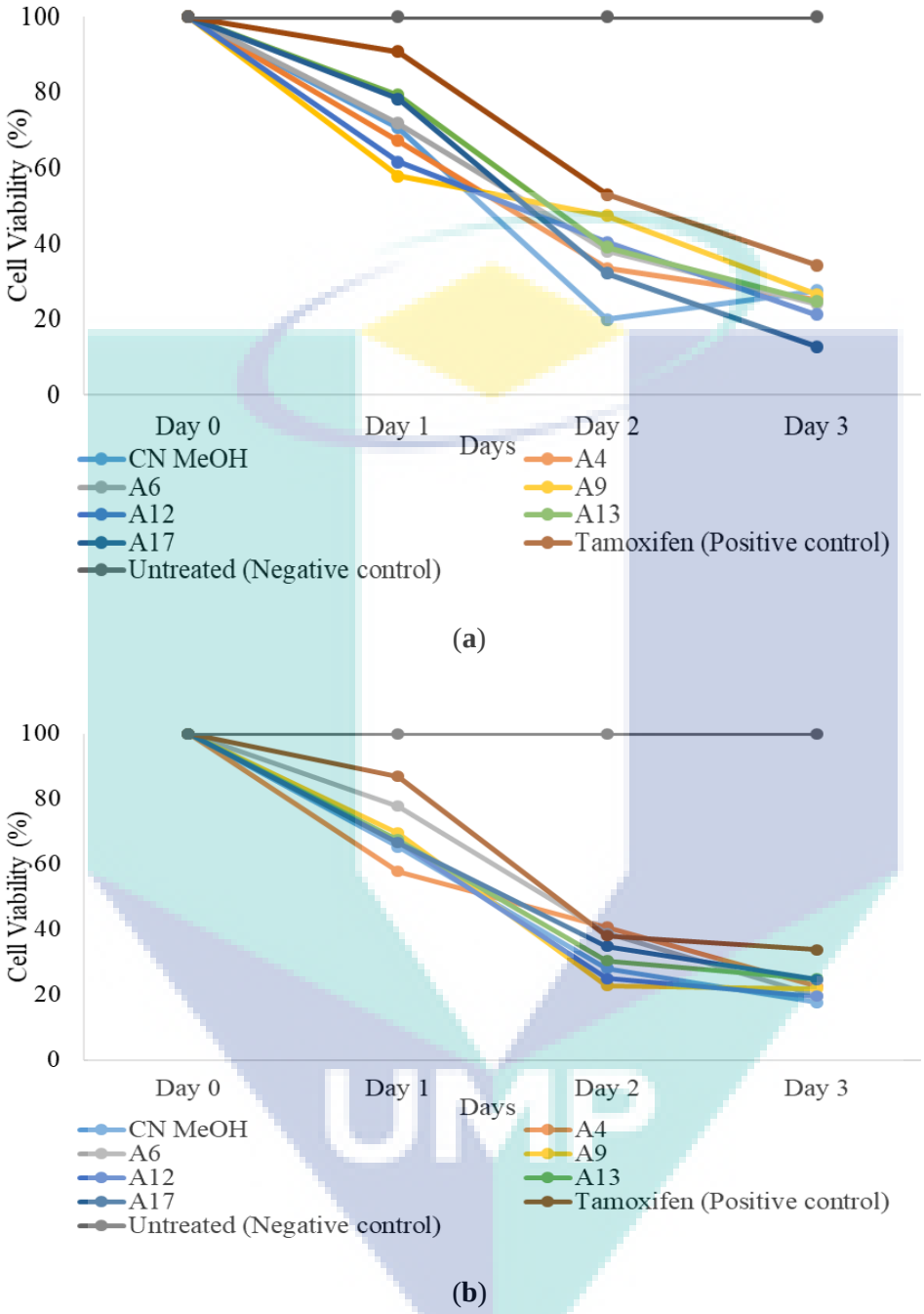


Figure 4 Viability of (**a**) MDA-MB-231 and (**b**) MCF-7 cancer cell lines on *C. nutans* MeOH extract and selected fractions after exposure for 3 days, at a concentration of 100 µg/ mL

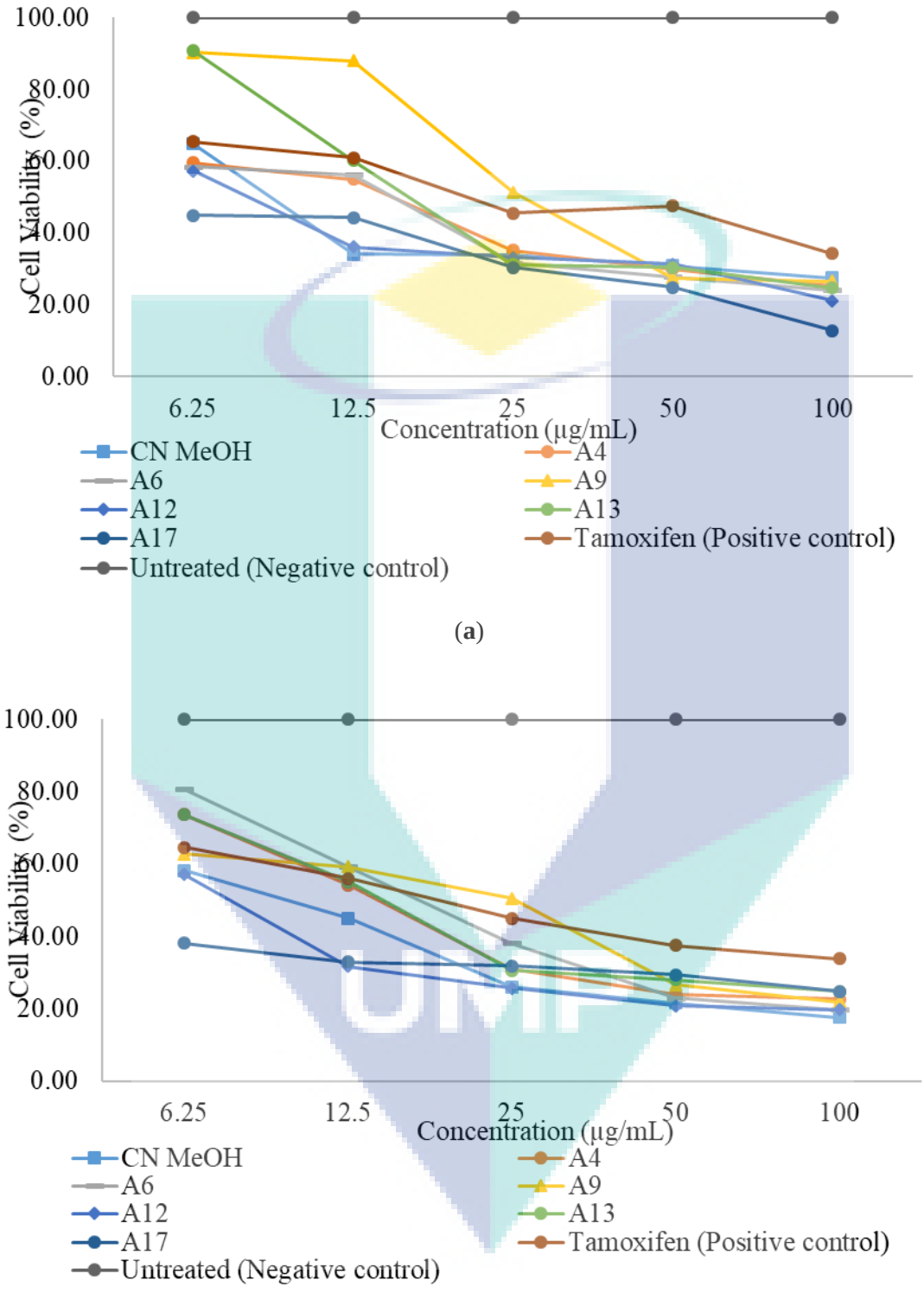


Figure.5 Dose-dependent cytotoxicity of *C. nutans* extract and fractions on (**a**) MDA-MB-231 and (**b**) MCF-7 cancer cell line under 72 hours

The cell viability of MDA-MB-231 and MCF-7 decreased with the increase of extract and fractions concentration. The concentration of 12.5 µg/mL was observed to be the most active extract concentration in inhibiting the proliferation of MDA-MB-231 and MCF-7 cancer cell lines as follows; *C. nutans* MeOH extract (MDA-MB-231: 65.92 ± 0.001%; MCF-7: 55.06 ± 0.014%), fraction A12 (MDA-MB-231: 63.85 ± 0.018%; MCF-7: 68.30 ± 0.021%), and fraction A17 (MDA-MB-231: 55.67 ± 0.016%; MCF-7: 67.17 ± 0.036%).

The cytotoxicity results of MeOH extract and selected fractions from leaves of *C. nutans* on MDA-MB-231 and MCF-7 cell lines at different concentrations show significant ($p < 0.05$) decrease in the IC_{50} values in a concentration-dependent manner after a 24, 48 and 72 h incubation period (Table 1). Tamoxifen was used as positive control which was able to inhibit cancer cell proliferation. The criterion of cytotoxicity for crude extract established by U.S National Cancer Institute, is $IC_{50} < 30$ µg/mL in the preliminary screening assay (Roslen, Alewi, Ahamada, & Rasad, 2014) while Houghton et al. (2007) suggested $IC_{50} < 40$ µg/mL as a suitable concentration for anticancer effect of either extract or compound.

Table 1 IC_{50} µg/mL values after 48 and 72 hours of incubation for *C. nutans* MeOH extract and selected fractions against MDA-MB-231 and MCF-7, breast cancer cell lines

Sample	MDA-MB-231 (IC_{50} µg/mL)		MCF-7 (IC_{50} µg/mL)	
	48	72	48	72
CN MeOH	35.335 ± 0.063	9.261 ± 0.023	21.184 ± 0.093	10.121 ± 0.043
A4	15.143 ± 0.086	15.602 ± 0.064	25.044 ± 0.050	14.749 ± 0.093
A6	21.881 ± 0.046	15.671 ± 0.028	21.482 ± 0.089	17.988 ± 0.058
A9	49.942 ± 0.075	26.516 ± 0.065	43.801 ± 0.091	25.485 ± 0.093
A12	24.720 ± 0.076	8.394 ± 0.086	22.036 ± 0.041	8.007 ± 0.043
A13	29.860 ± 0.054	16.906 ± 0.023	29.365 ± 0.073	15.105 ± 0.036
A17	6.045 ± 0.065	5.683 ± 0.064	6.232 ± 0.021	5.048 ± 0.083
Tamoxifen	-	21.408 ± 0.071	38.660 ± 0.081	19.288 ± 0.091

According to the results of IC_{50} in MeOH extract and each selected fraction from *C. nutans* (Table 1) exhibit significant activities in inhibiting MDA-MB-231 and MCF-7, cancer cell lines under 72 h incubation with IC_{50} value < 30 µg/mL. The comparisons of IC_{50} values of *C. nutans* MeOH extract and selected fractions on cancer cell lines after incubation under 72 h is summarized in Figure 6.

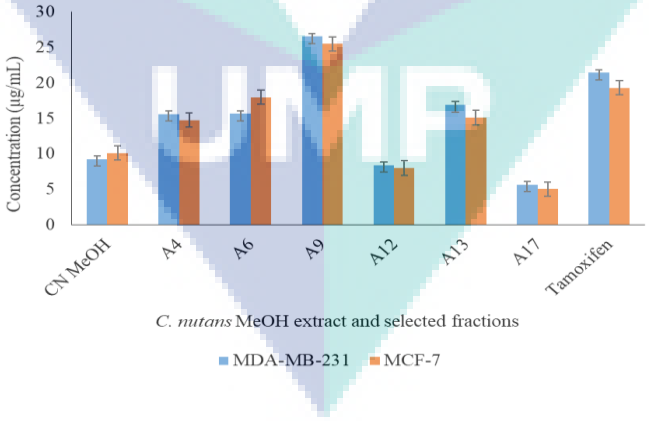


Figure **Error! No text of specified style in document.**6 IC_{50} values of *C. nutans* MeOH extract and selected fractions on MDA-MB-231 and MCF-7 cancer cell lines under 72 h incubation

Tamoxifen act as positive control, with IC_{50} values of 21.408 µg/mL ± 0.148 (MDA-MB-231) and 19.288 µg/mL ± 0.091 (MCF-7). MeOH extract of *C. nutans* and all selected fractions except A9 (MDA-MB-231: 26.516 µg/mL ± 0.065; MCF-7: 25.485 µg/mL ± 0.093), show IC_{50} values lower than Tamoxifen. Thought fraction A9 still exhibit significant anticancer activities due to its IC_{50} for both cell lines are less than 30 µg/mL. Fraction A17 exhibits the lowest IC_{50} value among MeOH extract and fractions from *C. nutans* with IC_{50} values of 5.683 µg/mL ± 0.064 for MDA-MB-231 cell line and 5.048 µg/mL ± 0.083 for MCF-7 cell line. Hence, fraction A17 from MeOH extract of *C. nutans* is the most active in inhibiting MDA-MB-231 and MCF-7 cancer cell lines.

Previous studies on PET extract from the leaves of *C. nutans* suggested that the plant has significant anticancer signs against HeLa cancer cell line with IC_{50} of 18 µg/mL (Arullappan et al., 2014). Both tested extracts of *C. nutans* demonstrated cytotoxic on the breast MCF-7 cancer cell line with IC_{50} value of 24.04 µg/ml for EtOAc extracts and 28.90 µg/mL for EtOH extract (Sulaiman et al., 2015).

The difference between the study conducted by Arullapan et al. (2014) and Sulaiman et al. (2015) with recent research indicates that MeOH extract of *C. nutans* possess lower cytotoxicity values with IC_{50} values of 9.261 ± 0.023 µg/mL and 10.121 ± 0.043 µg/mL against MDA-MB-231 and MCF-7 cancer cell line, respectively. The present study also contributes to the evidence that MeOH extract of *C. nutans* and fractions does indeed possess anticancer importance. This finding suggests that *C. nutans* could be used as valuable resources for bioactive substances related to its cytotoxic properties. Collectively, the finding from both recent and previous studies, suggests that the anticancer activities of extracts from different parts of *C. nutans* are largely

influenced by the types of extraction and solvents applied. The presence of bioactive compounds differs with chloroform, ethyl acetate, methanol and petroleum ether extraction.

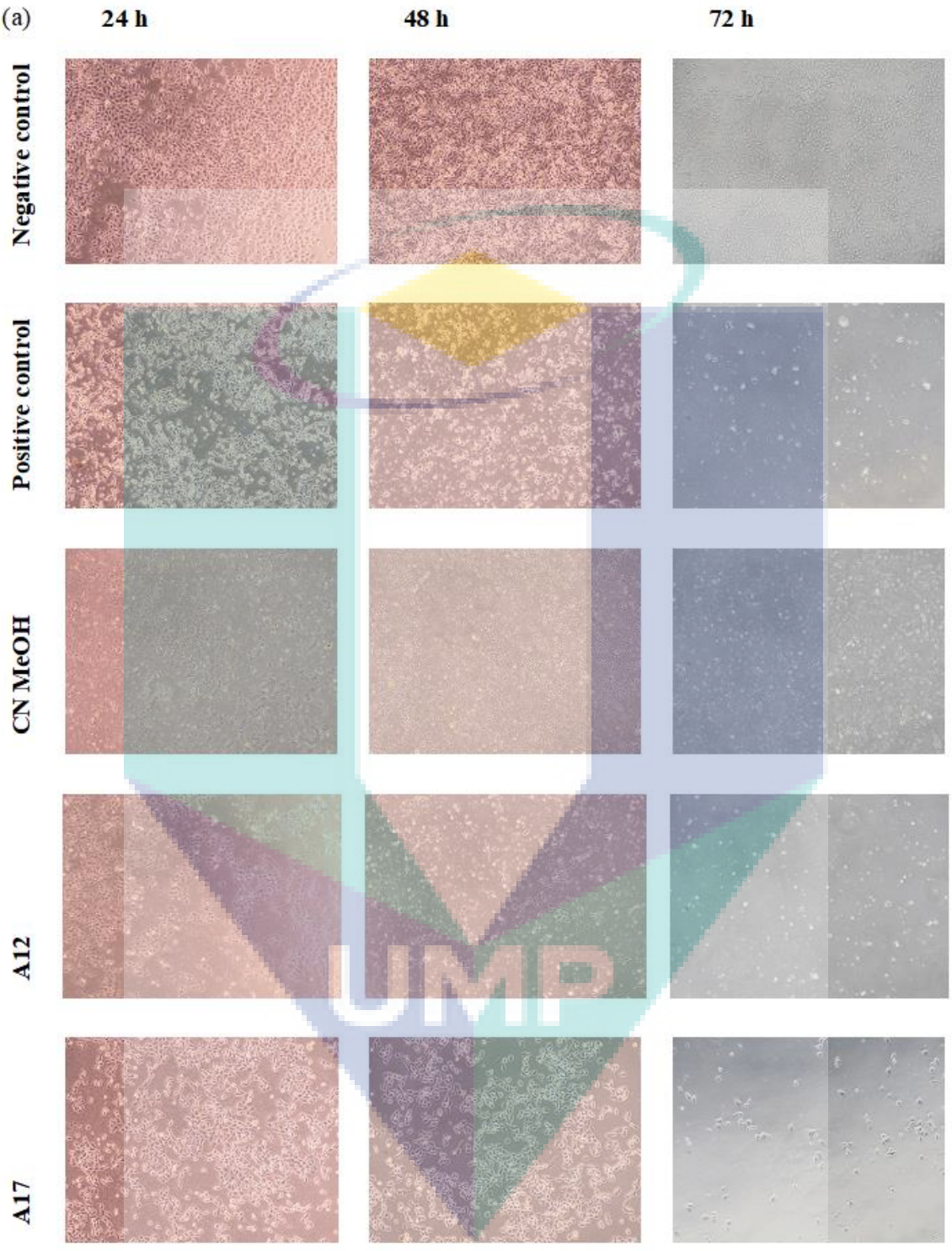
Quantitative evaluation in present study revealed that certain targeted phenolics and fatty acids (alpha tocopherol, quercetin, 4-hydrophenylacetic acid, coumalic acid, linoleic acid, stearic acid, and palmitic acid) found to be abundant in *C. nutans* extract. Squalene compound among other compounds that have been reported in this plant that are claimed to have anti-cancer, anti-tumor and chemo-preventive properties.

The anticancer activity of the compounds is dependent on the synergistic effect. Due to the immense amounts of bioactive compounds present in the *C. nutans* extract and its selected fractions, the synergistic effect could have taken place leading to the cytotoxic activity and therefore reduced cell viability of MDA-MB-231 and MCF-7. Another mechanism that may responsible for the cytotoxic effect could be related to the balance between its lipophilicity and hydrophilicity solubility. Several studies on different potential antitumor agents have shown that a maximum cytotoxic activity is often achieved for intermediate lipophilicity and water solubility values. The synergistic effect of *C. nutans* extract and its selected fractions can be correlated to lipophilicity of compound and their ability to penetrate the cell membrane.

4.5 Morphology of cancer cells treated with *C. nutans*

As illustrated in Figure 7a and 7b, the untreated MDA-MB-231 and MCF-7 cells were dispersed evenly with an increment in cell proliferation within the period of 24, 48,

and 72 h of the incubation period. Treatment of MDA-MB-231 cells and MCF-7 with *C. nutans* MeOH extract and selected fractions caused marked alterations on the cell morphology, such as cell size, shape, and plasma membrane changes, when compared with both positive and negative control. The most prominent changes between the untreated cell (negative control) and treated cells (*C. nutans* MeOH extract, fractions, and positive control) were observed in the cells treated with *C. nutans* MeOH extract and selected fractions after 72, where the cells were detached from the substratum, the occurrence of irregular size and shape, and decrease volume of cells. Fractions A12 and A17 of *C. nutans* induced morphological effects prominently on MDA-MB-231and MCF-7 cells at 72 hr of treatment.


(a)
24 h
48 h
72 h
Negative control
Positive control
CN MeOH
A12
A17

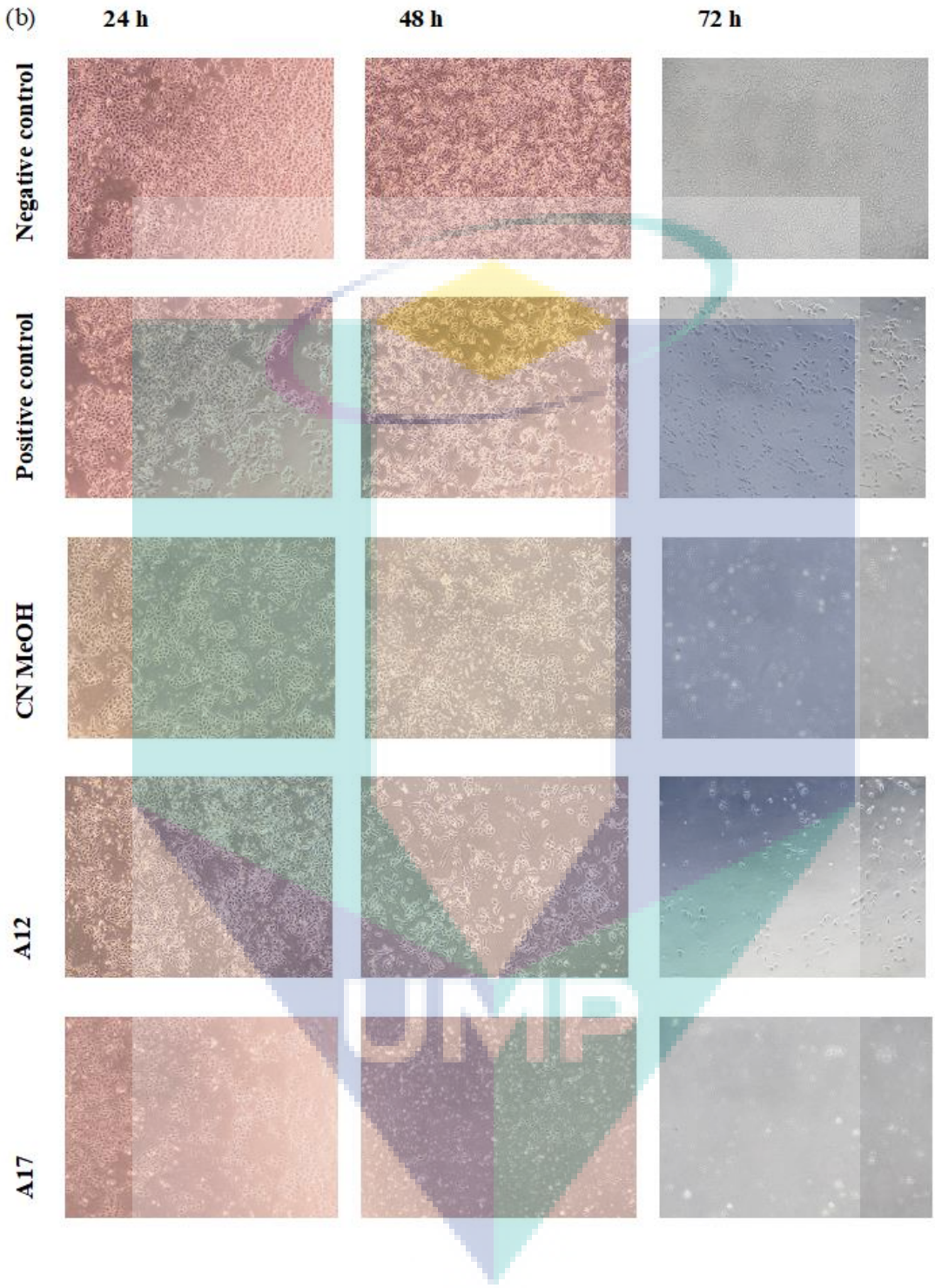


Figure 7 (a) Morphological changes of MDA-MB-231 and (b) MCF-7 cells treated with MeOH extract of C. nutans and selected fractions for 24, 48 and 72 h (10 × magnification).

4.7 Molecular Docking Study

In silico simulations studies have been widely employed in the current design and discovery of drugs to understand the gene pathway and drug-receptor interaction (Enkhtaivan, Kim, & Pandurangan, 2017). The cytotoxicity effect of some drugs is thought to be mediated by their ability to induce apoptosis. Activation of caspases seems to be a common intracellular pathway in many apoptotic pathways. Caspase-3 is an important marker of the cell's entry point into the apoptotic signalling pathway. Caspase 3 has been shown to cleave and activate numerous effectors including SREBPs, Caspase 6, Caspase 7, and Caspase 9 (Biosciences, 2012). Caspase-3 is activated by the upstream caspase-8 and caspase-9, and since it serves as a convergence point for different signalling pathways, it is well suited as a read-out in an apoptosis assay.Caspase-3 is essential for cell death processes and the formation of apoptotic bodies (Porter, Jänicke, & differentiation, 1999).

Investigation of possible binding modes of entadamide C (1) and clinamide D (2) against caspase-3 active sites was performed via *in silico* binding analysis using the AutoDock program, which is a public and academic standard program in molecular docking studies (Adeniyi & Ajibade, 2013). Molecular docking simulation study revealed that that entadamide C (1) and clinamide D (2) could bind favourably to a caspase-3 binding site with the binding energy of -4.28 kcal/mol and -4.84 kcal/mol, respectively; indicating the high binding affinity of these compounds to caspase-3. Detailed binding interaction study showed that entadamide C (1) participates in several important binding interactions with the caspase-3 active sites. The major interactions from the entadamide C (1) and clinamide D (2) are polar interaction. The hydroxyl and amide moiety from

entadamide C were found to form hydrogen bonds with Arg179 (2.09 Å), Ser236 (3.00 Å), His237 (2.52 Å), and Ser339 (2.32 Å) as illustrated in Figure 8a. Meanwhile, similar binding interaction was shown by clinamide D (2) in which the hydroxyl and amide moiety interacted with His237 (2.85 Å), Ser236 (2.60 Å), Gln283 (2.04 Å), Ser339 (2.00 Å), and Ser339 (2.19 Å) by hydrogen bond (Figure 8b). Such interactions are almost essential for the activation of caspase-3 based on binding interaction displayed by ligand-co-crystal of NA3 in the caspase active site (Becker et al., 2004). The sulfohydryl group of Cys285 and the imidazole ring of His237 are responsible as the catalytic site of caspase-3. His237 is vital to stabilise the carbonyl group of the key aspartate residue, while Cys285 play a role in cleaving the peptide bond (Lavrik, Golks, & Krammer, 2005).The critical element in the inhibition of entadamide C (1) and clinamide D (2) are through hydrogen bonding formation with one of the catalytic residues, His237, which might prevent the accessibility of this catalytic dyad to the solvent, that can effectively ablate protease activity.

In this study, the molecular docking was concrete enough to discover the binding interaction between entanamide C and clinamde D which are the ligands with breast cancer cell line protein caspase 3. Higher binding affinity interaction between ligand and receptor leads to activation of receptors. Activation of caspase-3 leads to induction of apoptosis, making it a potential target for anticancer drug design. The recognition of binding site between the ligand and the receptor is the beginning point for future drug designing and development of novel compounds. However, it is necessary to validate the cell death pathway of these compounds in wet lab studies for establishing them as potential novel candidates.

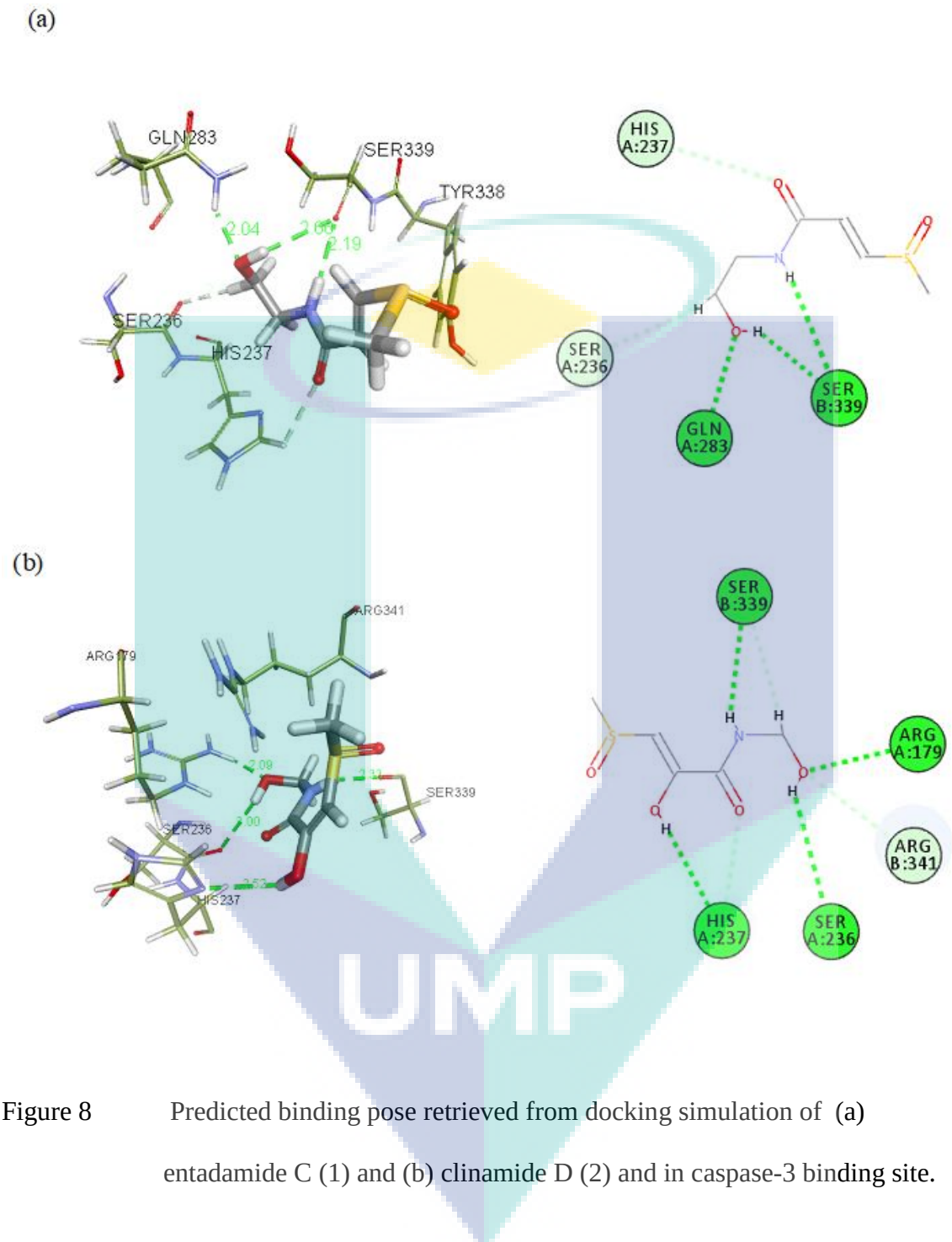


Figure 8 Predicted binding pose retrieved from docking simulation of (a) entadamide C (1) and (b) clinamide D (2) and in caspase-3 binding site.

5. CONCLUSION

In conclusion, under the present experimental conditions, the cytotoxic activity of *C. nutans* Fraction A17 exhibits the lowest IC_{50} value the other samples, with an IC_{50} values of 5.683 µg/mL ± 0.064 and 5.048 µg/mL ± 0.083 followed by fraction A12 with an IC_{50} values of 8.394 ± 0.086 and 8.007 ± 0.043 against MDA-MB-231 and MCF-7 cancer cell line, respectively. Moreover, the bio-assay guided isolation of cytotoxic active fractions from *C. nutans* extract resulted in the identification of sulphur-containing compounds, namely the entadamide C (1) and clinamide D (2). *In silico* result showed that entadamide C (1) and clinamide D (2) could bind to the caspase-3 active sites. These preliminary investigations have suggested that *C. nutans* possess the potential as a potent cytotoxic agent in the advancement of new pharmaceuticals from edible plants.

ACHIEVEMENT

i) Name of articles/ manuscripts/ books published

This project produced four journal papers
(WoS index Journal. RDU 160156(university reference number) represents the FRGS grants no. (FRGS/1/2016/WAB13/UMP/03/1)

Mutazah, R., Hamid, H. A., Ramli, A. N. M., Aluwi, M. F. F. M., Yusoff, M. M. J. F., & Toxicology, C. (2019). In vitro cytotoxicity of *Clinacanthus nutans* fractions on breast cancer cells and molecular docking study of sulphur containing compounds against caspase-3. 110869.

Zamri, N., & Hamid, H. A. J. P. F. f. H. N. (2019). Comparative Study of Onion (Allium cepa) and Leek (Allium ampeloprasum): Identification of Organosulphur Compounds by UPLC-QTOF/MS and Anticancer Effect on MCF-7 Cells. 74(4), 525-530.

Hamid, H. A., Ramli, A. N. M., Zamri, N., & Yusoff, M. M. (2018). UPLC-QTOF/MS-based phenolic profiling of Melastomaceae, their antioxidant activity and cytotoxic effects against human breast cancer cell MDA-MB-231. *Food chemistry*, *265*, 253-259.

Ukaegbu, C. I., Shah, S. R., Hamid, H. A., Normaiza, Z., & Alara, O. R. (2019). Extracts of Hypsizygus tessellatus (white var.) caps inhibited MCF-7 and MDA-MB-231 cell lines proliferation. *Journal of Food Measurement and Characterization*, *13*(1), 368-382.

ii) Title of Paper presentations (international/ local)

Antioxidant and antiproliferative properties of methanol extract of *Clinacanthus nutans* and identification of chemicals profile by UPLC-QTOF-MS. 2nd International Symposium on Phytochemicals in Medicine and Food (2-ISPMF) to be held in Fuzhou, China from April 7-10, 2017.

iii) Human Capital Development

The following student works for this project
Roziasyahira Binti Mutazah (PKD17003)
Development of Chemical Markers and Chromatographic Fingerprint of Clinacanthus Nutans For Evaluation Of Therapeutic Products
Ongoing

Chia Shi Yi (SA13043)
Synthesis of Silver Nanoparticles By Using Clinacanthus Nutans Extract, Characterization And Their Antimicrobial Activity
Graduated on year 2017

Lim Xiao Shan
SA14022
Chemical Constituents and Antioxidant Activity of *Clinacanthus nutans* and its Commercial Products
Graduated on year 2018

iv) Awards/ Others

Awards Title	*Date/Year*	*Organizer*	*Level*
Gold medal dalam Creation, Innovation Technology & Research Exposition (CITREX) 2017 Myrtle Mats	15-16 Mac 2017	UMP	Universiti
Silver medal dalam International Invention, Innovation & Technology Exhibition (ITEX) 2017 bagi inovasi Biodegradable Myrtle Mats	11-13 May 2017	Kementerian Sains, Teknologi dan Inovasi, Malaysia	Antarabangsa

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APPENDIXES

Food Chemistry 265 (2018) 253–259

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UPLC-QTOF/MS-based phenolic profiling of Melastomaceae, their antioxidant activity and cytotoxic effects against human breast cancer cell MDA-MB-231

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ARTICLE INFO

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Cytotoxicity
Melastomacea malabathricum
Melastoma decemfidum

ABSTRACT

Eleven compounds were identified during profiling of polyphenols by UPLC-QTOF/MS. In abundance was quercetin-3-O-α-L-arabinofuranoside in *M. malabathricum* ethanolic leaves extract while 6-hydroxykaempferol-3-O-glucoside was present in the leaves extract of *M. decenfidum* (its rare variety). TPC and TFC were significantly higher in *M. decemfidum* extract than *M. malabathricum* extract. During DPPH, FRAF and β-carotene bleaching assays, *M. decemfidum* extract exhibited greater antioxidant activity compared to *M. malabathricum* extract. Effect of *M. malabathricum* and *M. decemfidum* extracts on viability of MDA-MB-231 cell at concentrations 6.25–100 μg/mL were evaluated for 24, 48 and 72 h. After 48 and 72 h treatment, *M. malabathricum* and *M. decemfidum* leaves extracts exhibited significant activity in inhibiting MDA-MB-231 cancer cell line with *M. malabathricum* extract being more cytotoxic. *M. malabathricum* and *M. imbricatum* serves as potential daily dietary source of natural phenolics and to improve chemotherapeutic effectiveness.

1. Introduction

Oxidative stress has been implicated as one of the lead factors contributing to the onset of chronic and degenerative pathophysiologies which includes atherosclerosis, diabetes mellitus, cancer, cardiovascular and neurodegenerative diseases and ageing (Young & Woodside, 2001). When subjected to stress, the human body tends to produce excessive amounts of reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide and superoxide anion radicals. Living cells have antioxidant protective systems, namely, enzymatic and non-enzymatic antioxidants which prevent excessive formation of ROS or enable its inactivation (Finkel & Holbrook, 2000). Oxidative stress leads to an imbalance between the formation of ROS and the body's natural antioxidants. This imbalance then results in cell damage and severe health problems (Steer et al., 2002).

Antioxidants play a vital role in combating this issue by preventing cellular damage and are commonly used to treat cancer and age-related pathology. Plant-based foods tend to be high in naturally occurring polyphenols. Polyphenols are antioxidants and have been associated with their ability to combat cell damage (Bouayed & Bohn, 2010). Polyphenols may be classified into different groups include phenolic acids, flavonoids, stilbenes and lignans (Pandey & Rizvi, 2009). The flavonoids are the most important dietary polyphenols and the frequently reported of their antioxidant potential and inhibition of digestive enzyme. Different type of natural flavonoids form demonstrated different biological benefits and pharmacokinetic behaviours (Khan et al., 2018; Xiao, Capanoglu, Jassbi, & Miron, 2016). In most cases, *C*-glycoside showed higher antioxidant and anti-diabetes potential than *O*-glycoside and aglycones (Xiao, 2017). Metabolic features and mechanisms of antioxidant capacity of naturally occurred flavonoids can be altered by modification of flavonoids ring including hydroxylation, o-methylation, and glycosylation (Chen et al., 2018). Our antioxidant protective systems are incomplete without non-enzymatic antioxidants such as plant polyphenols in foods. Epidemiological data over certain cancer sites has shown that the presence of increased levels of exogenous antioxidants prevented the types of damage that have been associated with cancer development in laboratory animals (Bouayed & Bohn, 2010). Many observational studies, including case -control studies and cohort studies, have been conducted to investigate whether the use of certain dietary antioxidant supplements is associated with reduced risks of cancer in humans (Chandel & Tuveson, 2014; Conklin, 2000).

Melastomataceae is classified as one of the largest flowering plant family with over 4000 species and 166 genera in the world. In the Southeast Asian region alone, the genus Melastoma comprises 22 species, 2 subspecies, and 3 varieties (Rajenderan, 2010). *Melastoma*

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ORIGINAL PAPER

Extracts of *Hypsizygus tessellatus* (white var.) caps inhibited MCF-7 and MDA-MB-231 cell lines proliferation

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Abstract
Cancer management is associated with serious side-effects due to the harmful nature of radiation and chemotherapy on the body cells. These side-effects have necessitated the need for diversifying the alternative or complementary sources of cancer therapy. Natural products have been on the front line as alternative sources of anticancer agents and have attracted much attention in recent times. In this study, the anticancer activity of *Hypsizygus tessellatus* (white var.) caps (also known as Bunapi shimeji) extracted with acetone and ethyl acetate was evaluated in vitro against MDA-MB-231 and MCF-7 (breast cancer cell lines) and MCF-10a (Vero or normal breast cells). Likewise, the free radical scavenging and metal reducing activities of the extract were evaluated through in vitro chemical-based methods. Furthermore, the phytochemical compositions of the extracts were determined through LC–MS-QTOF-assisted mass spectroscopy. The results of this study indicated that acetone fraction had better radical scavenging activity against DPPH (IC_{50} = 0.76 mg/mL) and H_2O_2 (IC_{50} = 0.84 mg/mL) than ethyl acetate fraction against DPPH (IC_{50} = 1.10 mg/mL) and H_2O_2 (IC_{50} = 1.26 mg/mL) ($p < 0.05$). Additionally, the acetone fraction was observed to have more antiproliferative effects against MCF-7 (IC_{50} = 0.051–0.055 mg/mL) and MDA-MB-231 (IC_{50} = 0.122–0.131 mg/mL) compared to the ethyl acetate fraction against MCF-7 (IC_{50} = 0.075–0.096 mg/mL) and MDA-MB-231 (IC_{50} = 0.161–0.164 mg/mL) ($p < 0.05$). Both extracts generally had less effect on MCF-10a cells. Thus, these results suggested that Bunapi shimeji caps is a potential good natural source of anticancer agents.

Keywords MCF-7 · MDA-MB-231 · Antioxidant · Antiproliferation · Bunapi shimeji · *H. tessellatus* · Mushroom phytochemicals

Introduction

Cancer, diabetes, immune-system decline, cardiovascular diseases, and brain dysfunction are some of the degenerative diseases associated with reactive oxygen species (ROS) and aging [1]. Their linkage to free radicals is associated with the postulation that ROS-mediated damages to macromolecules and deoxyribonucleic acid (DNA) tend to accumulate with time and has been considered as a major form of endogenous damage that results to aging [2]. Mutagens such as hydroxyl radicals or hydrogen peroxide are also side-products of normal human body metabolism [3]. Humans have always embarked on the consumption of plant foods (fruits, red wines, fruits, and juices) and some mushrooms (to an extent) with the belief that they can confer them with some levels of protection against oxidative damages and diseases such as cancer [4]. This presumed protection was due to the ability of the antioxidants contained in these natural foods to scavenge the generated free radicals and keep their body healthy [5].

Before now, chemically-sourced antioxidants such as beta hydroxy acid (BHA) and butylated hydroxytoluene (BHT) have been commonly used in the stabilization of foods. However, these synthetic antioxidants were reported to be carcinogenic in experimental animals [6]. Consequently, efforts are geared towards finding alternative sources of natural antioxidants, especially from natural sources. In this respect,

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ORIGINAL PAPER

Comparative Study of Onion (*Allium cepa*) and Leek (*Allium ampeloprasum*): Identification of Organosulphur Compounds by UPLC-QTOF/MS and Anticancer Effect on MCF-7 Cells

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Abstract

Onion (*Allium cepa)* and leek (*Allium ampeloprasum* var. *porrum*) are common herbs and vegetables found in our daily life. It belongs to the genus *Allium*, which is usually known for their high antioxidant and anticancer properties. Medical researchers highly recommend the exploitation of herbs and plants as alternative ways in the treatment of cancer. This research was designed to study the anticancer effects of onion and leek extracts on MCF-7 human breast cancer cell. Crude extracts of ethanol, methanol, and water of onion and leek were obtained by maceration. MCF-7 cells were cultured in complete media at 37 °C and subjected to different treatments that involved varying concentrations (10, 50, and 100 μg/mL) of onion and leek extracts for 24, 48, and 72 h of incubation. The percentage of cell viability and the concentration of extracts on MCF-7 cells were determined using MTT assay. The water leek extract proved to be the most effective extract at 50 μg/mL, whereby it showed a significant inhibition ability due to the presence of entadamide A-β-D-glucopyranoside as identified by ultra-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC-QTOF/MS). Further studies about the mechanism of both extracts in causing cell death and the determination of the presence of other bioactive compounds in the extracts are needed.

Keywords Onion (*Allium cepa*) · Leek (*Allium ampeloprasum*) · Organosulphur compounds · UPLC-QTOF/MS, anticancer · MCF-7 cells

Introduction

Chemoprevention is a cancer preventive strategy to inhibit, delay, or reverse carcinogenesis. Altering dietary habits by reducing consumption of processed foods while increasing the intake of vegetables may decrease cancer risks. There is an increasing public health demand to identify those dietary patterns, bioactive foods, and components that may decrease cancer risks. Approximately 30–40% of cancers are preventable by appropriate food and nutrition intake, physical activity, and maintenance of healthy body weight [1]. One particular group of foods that has raised considerable interest in their putative cancer-preventive properties is the genus Allium.

Allium is a large genus of onion or garlic-scented bulbous herbs of the Amaryllidaceae family. Allium vegetables, such as garlic (*Allium sativum*), onion (*Allium cepa*), and leek (*Allium ampeloprasum* var. *porrum*) are widely consumed for their characteristic flavours (as spices) and their health-promoting effects [2]. Garlic is one of the most extensively studied functional species among Alliums, and it has been considered a medicinal food for centuries, being used as a traditional remedy for common disorders. Garlic consumption is associated with decreased risk of some types of cancer, cardiovascular diseases, and neurodegenerative disease [3]. Besides, various other biological activities have been reported for garlic, including antimicrobial, antioxidant, and anti-inflammatory properties [4]. Onion has also been utilised as a medicinal agent according to sources dating from ancient times. This broad spectrum of health-benefits is mainly attributed to the presence of organosulphur compounds, a distinct characteristic of garlic and other Allium species. It must be

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In vitro cytotoxicity of *Clinacanthus nutans* fractions on breast cancer cells and molecular docking study of sulphur containing compounds against caspase-3

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ABSTRACT

Clinacanthus nutans has attracted Malaysian public interest due to its high medicinal value in the prevention of cancer. Currently, the specific compound or compounds giving rise to the anticancer potential of *C. nutans* has not been investigated thoroughly. The extraction was carried out by MeOH at room temperature using the powdered bark of *C. nutans*, while chromatography was carried out on a silica gel RP-18 column using the crude methanolic extract. Six fractions collected from column chromatography were evaluated by MTT assay against two breast cancer cell lines: MDA-MB-231 and MCF-7. Amongst the fractions, A12 and A17 were shown to exhibit the highest activity. Two sulphur-containing compounds, viz., entadamide C (1) and clinamide D (2), were isolated from these fractions. Molecular docking simulation studies revealed that entadamide C and clinamide D could bind favourably to the caspase-3 binding site with the binding energy of −4.28 kcal/mol and −4.84 kcal/mol, respectively. This study provides empirical evidence for the presence of sulphur-containing compounds in the leaves of *C. nutans* that displayed anticancer effects which explains its ethnomedicinal application against breast cancer. The docking simulation study showed that both compounds could serve as important templates for future drug design and development.

1. Introduction

Cancer is presently considered a substantial public health issue in the world. One of the primary cancers inflicting women worldwide is breast cancer. The treatment of cancer is a significant challenge. Cancer is classified and treated solely according to the organs of origin or simplistic histomorphologic features (Zugazagoitia et al., 2016). The clinical modalities presently employed in the treatment of cancer such as surgical removal, radiotherapy, and specialised chemotherapy or hormone therapy are systemic anti proliferative proxies that distort cell division (Huang et al., 2017). These drugs do not limit themselves to cancerous cells, and their therapeutic efficiency is restricted as they can cause damage to healthy cells and tissues as well. Consequently, there is an essential need for new natural anticancer compounds in chemotherapeutics. In the past few years, many biological properties of several potential plants and herbs have been studied by various researchers.

However, to date, the potential chemotherapeutic properties of many compounds present in vegetables, fruits and traditional herbs that are ingested by humans are not well understood. The incidence of chemoprevention, a condition when apoptosis is induced in pre-cancerous and cancerous cells caused by these agents, instigate the interest of researchers to study their properties. The advancement in the development of novel chemopreventive agents might be possible with better and concise knowledge of the molecular mechanisms that cause these effects. Sulphur-containing compounds (OSC) such as garlic constituents (GCs) and isothiocyanates (ITCs) are some of the naturally occurring dietary chemopreventive agents that have shown anticancer effects (Wu et al., 2005). *Clinacanthus nutans* possesses sulphur-containing compounds that exhibit anticancer effects.

The potential use of *C. nutans* as an anticancer agent has been reported by Yong et al. (2013), whereby the chloroform extract of the plant was capable of inhibiting cell proliferation of seven tested cancer cell lines, namely, the HepG2, IMR32, NCL-H23, SNU-1, HeLa, LS-174 T, K562, and Raji cells. Although several compounds such as C-glycosyl flavones, sulphur-containing glycosides, cerebrosides, monoacyl-monogalactosylglycerol, and chlorophyll derivatives (phaeophytins) have been discovered in the leaves of *C. nutans* (Alam et al., 2016),

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OL12: Antioxidant and antiproliferative properties of methanol extract of *Clinacanthus nutans* and identification of chemicals profile by UPLC-QTOF/MS

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The leaves extract of *C. nutans* have been used extensively as primary sources of complementary and alternative healthcare or as economical in-housing regimens for cancer patients [1]. Patients have claimed that they have recovered from cancer illness after consuming C. nutans leaves over a period of time. It has been proved that many antioxidant substances have anticancer or anticarcinogenic properties [2]. A study had pointed to the necessity of chemicals profiling and evaluation of antioxidant and antiproliferative properties of *C. nutans* extract.

The whole plant of *C. nutans* were dried and extracted with methanol. The content of the active components in the extracts was determined by ultrahigh performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QTOF/MS). The compounds were tested for antiproliferative property on HT29, HepG2 and MCF-7 cell lines while antioxidant activity was monitored by radical scavenging assay (DPPH) and ferric reducing power (FRAP).

The content of the active components in the extracts were determined by UPLC-QTOF/MS.Six compounds were labelled as confirmed component from methanol extract of *C. nutans*. Peaks with retention times (RT; min) of 4.36, 4.94, 6.63, 7.60, 8.69 and 11.27 were identified as the following: 5,7-dihydroxychromone-7-β-D-glucoside, smiglanin, glabrol, corymboside, viscumneoside II and kushenol U (Figure 1). Based on the component confirmed plot, the major compound found was corymboside. Methanol extract show significant antioxidant activities in DPPH (IC_{50} = 127.09± 0.042 µg/mL) and total reduction capability (IC_{50} 469.13 ± 0.05 µg/mL) (Table 1) According to the IC_{50} obtained, the methanol extracts showed significant antiproliferative activity on HT29 (IC_{50} 36.19 ± 1.06 mg/mL), HepG2 (IC_{50} 48.37 ± 0.026 mg/mL) and MCF-7(IC_{50} 54.16 ± 0.99 mg/mL) after 72 h of treatment (Table 2).

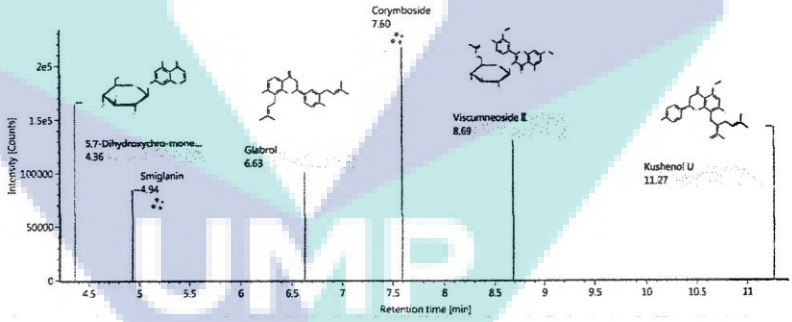

Figure 1 Confirmed component plot of *C. nutans* extract

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