

Full Length Research Paper

***In vitro* antioxidant, cytotoxic and phytochemical studies of *Clinacanthus nutans* Lindau leaf extracts**

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An increasing demand for natural additives has drawn more interest from consumers due to their relatively safe and wide acceptance. The present work examines the potential of different solvent system of *Clinacanthus nutans* Lindau leaves as a source of natural antioxidant. We also screened for its phytochemical constituents and cytotoxicity toward breast cancer cell. *In vitro* antioxidant activity of the *n*-hexane, dichloromethane, ethyl acetate, and ethanol extracts were assessed via 1,1-diphenylpicrylhydrazyl (DPPH) radical scavenging activity, oxygen radical absorbance capacity and β -carotene bleaching activity assays, whereas the cytotoxic effect was tested on tumorigenic breast cancer estrogen positive cell (MCF-7) and normal fibroblast cell (3T3) using the tetrazolium assay. Liquid chromatography mass spectrometry equipped with an electrospray ionization source liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI/MS) was used to analyze the amount of targeted phenolic and fatty acids in ethanol and ethyl acetate extracts. Several phenolic compounds and fatty acids were identified and quantified from ethanol and ethyl acetate extracts of this plant. Ethanol and ethyl acetate extracts demonstrated stronger antioxidant activity than *n*-hexane and dichloromethane extracts ($p < 0.05$). Thus, ethanol and ethyl acetate extracts of *C. nutans* may be explored as new sources of antioxidants in herbal medicines research.

Key words: Cytotoxicity, polyphenols, polyunsaturated fatty acid (PUFA), Sabah snake grass, anticancer.

INTRODUCTION

Antioxidant activity is associated to many biological functions in human body. One of the most important

properties of antioxidants is to defend body against free radical damage. Free radicals, especially reactive oxygen

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species (ROS), are harmful radicals produced by physiological and biochemical processes (Cai et al., 2004). ROS, when reacted with other substances in the body, may result into cell or tissue injury. Natural antioxidant compounds purified from plant products are gaining more interest than synthetic antioxidants due to their relatively safe and wide acceptance by consumers. Therefore, attention has been focused toward exploring natural antioxidants from plant sources. Phenolics are common compounds which contribute to antioxidant properties and also exhibit pro-oxidant properties. Pro-oxidant action of rich phenolic plant may be an important character associated with their anticancer properties (Cai et al., 2004; Azam et al., 2004; Rodríguez et al., 2007). Thus, phenolic compounds when absorbed into bloodstream will undergo chemical modification to result in antioxidant or pro-oxidants. Association of rich phenolic plant toward cytotoxicity of tumor cell is due to their pro-oxidative activity which accelerated a good effect of oxidative damage (Gomes et al., 2003; Rodríguez et al., 2007).

Native to tropical Asian countries, *Clinacanthus nutans* Lindau (*C. nutans*) (Acanthaceae) is a small shrub, locally known in Malaysia as Sabah Snake Grass or Belalai Gajah. Traditionally, paste from fresh leaves of *C. nutans* are being consumed as a remedy for bites and stings, especially by snake, mosquitos, millipedes, catfish, centipedes, hornets, jellyfish, ants, bees, scorpions and wasps in Thai folk medicine (Sakdarat et al., 2009; P'ng et al., 2012). An ethnobotanical survey in Singaporean communities revealed that *C. nutans* fresh herbs are consumed as a traditional medicine for its general detoxification purposes (Siew et al., 2014; Uawonggul et al., 2006). Moreover, *C. nutans* is reported to exhibit better protection to plasmid DNA against riboflavin photoreaction in comparison to green tea (Yuann et al., 2012). Chloroform extracts of *C. nutans* are reported to have the anti-proliferative effect on cancer cell lines of human erythroleukemia (K-56), and human Burkitt's lymphoma (Raji) at 100 µg/ml (Yong et al., 2013). In Malaysia, local traditions claim that *C. nutans* has a unique properties to cure cancer (Yong et al., 2013; Yuann et al., 2012), hence drawn public interest and its leaves been commercialized as herbal tea.

However *C. nutans* is well documented for its anti-inflammatory and anti-herpes simplex virus (Wanikiat et al., 2008; Kunsorn et al., 2013; Farooqui et al., 2015). Thus, cytotoxicity of *C. nutans* extracts toward cancer cells and phytochemical assessment are still limited. The main objectives of this study were to determine the antioxidant properties of four different solvent extracts from the leaves of *C. nutans*, screen their phytochemical constituents and cytotoxicity toward breast cancer cell estrogen positive (MCF-7). The total phenolic content (TPC), total tannin content (TTC) and total flavonoid

content (TFC) of the four different extracts were also evaluated.

MATERIAL AND METHODS

Chemicals

All solvents and reagents were of analytical or high performance liquid chromatography (HPLC) grade. *n*-Hexane, dichloromethane, ethyl acetate, ethanol, folin-ciocalteu reagent, gallic acid, 1,1-diphenyl-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), linoleic acid, rutin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 2,2-Azobis (2-methyl propionamide) dihydrochloride (AAPH), fluorescein sodium salt, β-carotene and alpha tocopherol were obtained from Sigma-Aldrich (Germany). Sodium carbonate (Na₂CO₃), polyvinylpyrrolidone (PVPP), aluminum chloride (AlCl₃), acetonitrile, iron chloride (III) (FeCl₃), hydrochloric acid (HCl) and Tween 20 were purchased from Merck (Darmstadt, Germany).

Plant

Fresh leaves of whole *C. nutans* were collected from botanical farm in Jelebu, Negeri Sembilan, on January, 2014. The plant was authenticated at Department of Biology, Faculty of Science, Universiti Putra Malaysia, Malaysia by biologist Associate Prof. Dr. Rusea Go. The voucher specimen number (RG5125) is deposited at the Herbarium Unit of Universiti Putra Malaysia.

Preparation of extracts

The leaves of *C. nutans* were air-dried under sun shade and grounded to fine powder using lab blender (Waring MX7011S). The fine powder was sequentially soaked (3 days, repeated twice) in *n*-hexane, dichloromethane, ethyl acetate and ethanol. The extracts in all four solvents were collected separately, filtered through Whatman filter paper No. 1, concentrated by using rotary evaporator (Rotavapor R-210, Buchi, Switzerland) at approximately 40°C and stored at -20°C prior to further analysis.

Total phenolic content (TPC)

Poor solubility of *n*-hexane and dichloromethane extracts in aqueous reagents limited the measurement of TPC. Therefore, *n*-hexane and dichloromethane extracts were purified (Ramadan et al., 2003) prior to TPC determination. TPC of *C. nutans* extracts was determined by a method developed by Negi (2012). An aliquot of sample was prepared in methanol and pipetted out into a test tube and mixed with 2.5 ml of diluted Folin-Ciocalteu's reagent (10-fold). Then, 2 ml of 7.5% of Na₂CO₃ was added. The test tube was allowed to stand for 30 min at room temperature before absorbance was measured at 760 nm using UV-Visible spectrometer Shimadzu UV-1601. Methanol was used as a blank and gallic acid was used as standard. TPC values were determined from a calibration curve prepared from graph absorbance against a series of gallic acid concentrations (0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml). All measurements were performed in triplicate and the results were expressed as milligram gallic acid equivalent per hundred grams of extract (mg GAE/100 g extract).

Total tannin content (TTC)

TTC of CN extract was estimated by using insoluble polyvinylpyrrolidone (PVPP), a method determined by Makkar (2003). Aliquot parts (1.0 ml) containing a 10 mg/ml sample was mixed with 100 mg of PVPP in test tube. The mixture was vortexed and left for 15 min at 4°C, and centrifuged for 10 min at 3000 rpm. Non-tannin phenolic content appeared as clear supernatant on top of the test tube, removed by pipetted and determined with the same method as TPC (Negi, 2012). The difference between TPC and non-tannin phenolic content is an estimation of the amount of TTC in the extract. Results are expressed as milligram gallic acid equivalent per hundred grams of extract (mg GAE/100 g extract).

Total flavonoid content (TFC)

Total flavonoid content (TFC) was assessed by aluminium colorimetric method (Iqbal et al., 2005). An aliquot of 0.5 mL of sample and 2% AlCl₃ were mixed in test tube. The mixture was vortexed and allowed to stand for 10 min at room temperature, and the absorbance of the reaction mixtures was measured at 435 nm by using a UV-Visible spectrophotometer Shimadzu UV-1601. Rutin

was used for the standard and methanol was used as a blank. Results were expressed as milligram rutin equivalent per hundred grams of extract (mg RE/100 g extract).

Antioxidant assays

1,1-Diphenyl-picrylhydrazyl scavenging activity

Radical scavenging activity was determined according to Ramadan et al. (2003) by reduction of 1,1-diphenyl-picrylhydrazyl (DPPH) radical in methanol. A methanolic solution of DPPH radical was freshly prepared at concentration 0.2 mM. Initially, an aliquot of sample was mixed with 2.34 ml of DPPH solution. After being vortexed for 20 s, the resulting mixture was allowed to stand for 30 min in the dark. Finally the absorbances of reaction mixture were recorded at 515 nm using UV-Visible spectrophotometer Shimadzu UV-1601. Trolox was used as a standard and the DPPH scavenging activity of *C. nutans* extracts was expressed as milligrams trolox equivalent per hundred grams of extract (mg TE/100g extract). The inhibition percent was calculated according to the following equation (Lee et al., 2002).

$$\% \text{Inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100 \quad (1)$$

Oxygen radical absorbance capacity

The antioxidant properties of the extract of *C. nutans* were measured *in vitro* using oxygen radical absorbance capacity (ORAC) method. ORAC was measured using 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxy radicals source and fluorescein sodium salt as a molecular probe (Huang and Ou, 2002). ORAC values were calculated using the regression equation between trolox concentration and the net area under the curve (AUC). This dynamic curve combines both inhibition time and inhibition percentages of the free radical damage by the antioxidant into a single value. The assay was analyzed with FLUOstar OMEGA (BMG LABTECH, Germany) microplate reader utilizing fluorescence filters for an excitation wavelength 485 nm and emission wavelength 520 nm. Results are expressed as micromole trolox equivalent per hundred grams of extract ($\mu\text{mol TE}/100 \text{ g extract}$).

β -carotene bleaching activity

β -Carotene bleaching activity (BCB) activity was determined according to the protocol described by Wettasinghe and Shahidi (1999). β -Carotene-linoleic acid emulsion was prepared by adding 40 mg of linoleic acid and 400 mg of Tween 20 to a test tube containing 3 ml of β -carotene solution (5 mg β -carotene/50 ml chloroform). The mixture was vortexed and dried under a stream of nitrogen. After that, 100 ml of distilled water was added to the mixture to form a β -carotene-linoleic acid emulsion. In a different test tube, 1.5 ml of prepared emulsion was pipetted to 20 μl of sample (5 mg/ml). Methanol was used as a negative control and distilled water as a blank. Absorbances at 470 nm UV-Visible spectrometer Shimadzu UV-1601 of the mixture were recorded prior

to incubation for 1 h at 50°C. Results were expressed as milligrams α -tocopherol equivalent per hundred grams of extract (mg Teq/100 g extract). Percentages of antioxidant activity of *C. nutans* extracts were calculated by using the formula (El-Ghorab et al., 2007):

$$AA\% = \frac{100 (DR_C - DR_S)}{DR_C} \quad (2)$$

Where, AA= antioxidant activity; DR_C= degradation rate of control= [(a/b)/60]; DR_S=degradation rate of sample = [(a/b)/60]; a= initial absorbance; b= absorbance after incubation.

Liquid chromatography-mass spectrometry (LC-MS) analysis of phenolic and fatty acid compounds

LC-MS targeted analysis was performed to quantify phenolic and fatty acid compounds in two selected extract of *C. nutans* with good antioxidant activity. Liquid chromatography (LC) analysis was carried out using UPLC Waters ACQUITY UPLC, followed by single quadrupole mass spectrometry equipped with an electrospray ionization source (LC-ESI/MS). For fatty acid analysis, LC analysis was performed with an ACQUITY UPLC BEH C18 column (100 mm \times 2.1 mm \times 1.7 μm), and the mobile phase temperature was set to 30°C. The solvent gradient was 75% A and 25% B in 15 min in the negative ionization mode. The mobile phase was composed of solvent A: acetonitrile (LC-MS grade) and solvent B: 2-propanol (LC-MS grade). A flow rate of 0.15 ml/min was used and 1 μl of sample were injected. For phenolic analysis, liquid chromatography mobile phase temperature was set to 35°C. The solvent gradient

Table 1. Extraction yields of *C. nutans* in four different systems.

Sample extracts	Weight of extracts (g)	Percentage of extract (%)
<i>n</i> -Hexane	7.85±0.07	1.78
Dichloromethane	9.18±0.30	2.09
Ethyl acetate	4.36±0.10	0.99
Ethanol	9.30±0.24	2.11

Values are expressed as mean ± standard deviation (n=3).

was initiated with 75% A and 25% B for 8.5 min, and then increased to 100% B, held for 11.5 min and reinitialized to 75% A and 25% B in 5 min at both ionization modes. The mobile phase was composed of solution A: Water (LC-MS grade) + 0.1% formic acid and solution B: Methanol (LC-MS grade) + 0.1 % formic acid. A flow rate of 0.15 ml/min was used and 1 µl of sample were injected. Samples were filtered through a 0.22 µm Millipore filter, type GV (Millipore, Bedford, MA) prior to UPLC injection. The mass raw data was analyzed by Masslynx MS Software version 4.1. Automated quantification with MassLynx targeted quantitative analysis was performed by QuanLynx (Waters Technologies). The identification of phenolic standard and fatty acid compounds in ethanol and ethyl acetate extracts of *C. nutans* were obtained by comparing the retention times to reference standards. The results are expressed as mg/100 g of extract.

Cytotoxicity assay

The two previously selected extracts of *C. nutans* were tested further for its cytotoxicity activity. Extracts were test on MCF-7 human breast cancer estrogen positive and 3T3 normal fibroblast cells. All cells were maintained in RPMI medium supplemented with 10% fetus calf serum (FCS), 100 unit/ml penicillin and 0.1 mg/ml streptomycin. Cytotoxicity of the extracts were determined with MTT assay according to protocol by Mosmann (1983) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. Initially, cell culture with the concentration of 1×10^5 cells/ml was prepared and plated (100 µl/well) onto 96-wellplates. The cell was incubated for 24 h at 37°, 5% CO₂. After incubation, the diluted ranges of samples (1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) were added to each well and incubated for another 24 h. Then MTT solution at 2 mg/ml was added to each well and left to stand for 1 h before measured at 570 nm. The cytotoxicity result was expressed as IC₅₀, defined as the concentration of sample that caused inhibition of 50% cell growth.

Statistical analysis

Values are expressed as mean ± SD of three replicates. Statistical analyses were performed by one-way ANOVA with $P < 0.05$.

RESULTS AND DISCUSSION

Extraction yield

Table 1 presents the extraction yields of *C. nutans* leaves

by using four different solvent systems. The yields obtained were extracted from total dry weight of 440 g *C. nutans*. Among the extracts, the highest and the lowest yields were obtained by ethanol and ethyl acetate extracts, respectively. Total percentage of all extracts obtained is 6.97% from the dry weight *C. nutans*. The high yield in ethanol was probably due to the high solubility of major components of *C. nutans* such as phenolic group in ethanol (Ismail et al., 2010).

Total phenolic content (TPC), total tannin content (TTC) and total flavonoid content (TFC) analysis

Phenolic, flavonoid and tannin compounds derived from plants are small molecules which play important roles as antioxidants. Phenolic compounds are widely distributed in plants and possess ultraviolet protection, pigmentation, and disease resistance (Prior et al., 2005; Maestri et al., 2006). Harmful oxidation caused by free radicals in the human body can be blocked by phenoxide ions that are delocalized in these antioxidants, which scavenge the free radicals and detoxify the organism (Chang et al., 2001; Maestri et al., 2006). The results obtained from TPC in the different extracts were summarized in Figure 1. The highest TPC in *C. nutans* was quantified in ethanol extracts (1850 mg GAE/100 g extract) whereas the lowest content was measured in *n*-hexane extracts (60 mg GAE/100 g extract) ($p < 0.05$). TTC (Figure 2) of *C. nutans* extracts varied from 55.4 to 1737 mg GAE/100 g extract. The highest tannin content was detected in ethanol (1737 mg GAE/100 g extract) whereas the lowest content was determined in *n*-hexane (55.4 mg GAE/100 g extract) ($p < 0.05$).

TPC of *C. nutans* extracts exhibited good correlation to its TTC. Ethanol had the highest in TPC and TTC followed by ethyl acetate, dichloromethane and *n*-hexane extracts, respectively. These findings are in agreement with the observations of Robards et al. (1999) which observed that polar solvents such as ethanol are a good choice for extracting phenolic compounds. Moreover, a few plant species belong to same family of Acanthaceae (*Lepidagathis anobrya*, *Hygrophila auriculata* and

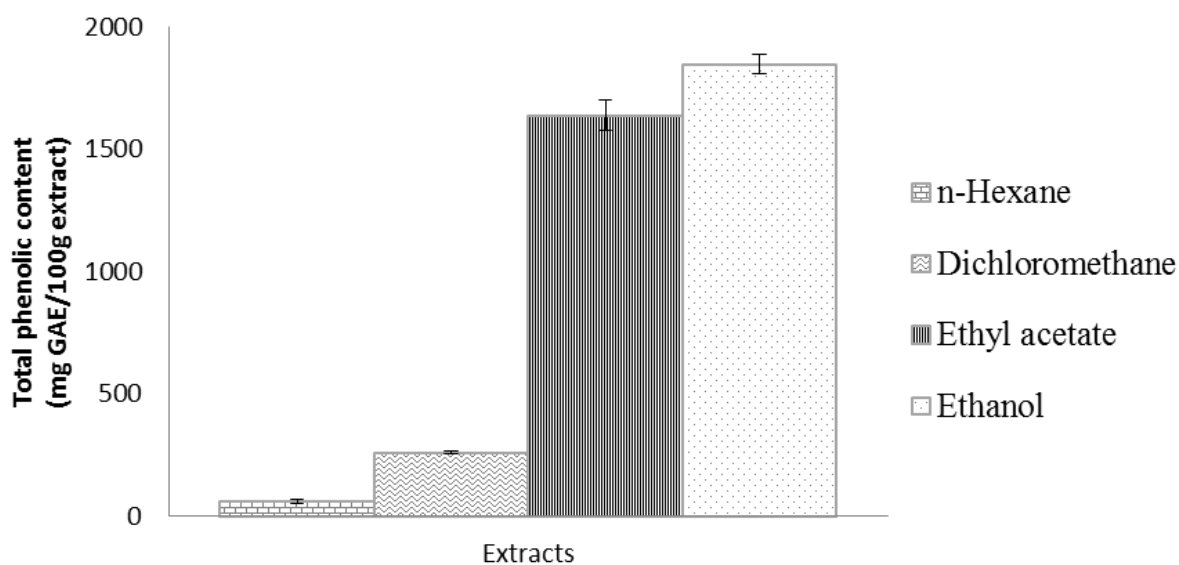


Figure 1. Total phenolic content (TPC) of *C. nutans* leaves from four different solvent systems. Error bars show the variations of three determinations in terms of standard deviation ($p < 0.05$).

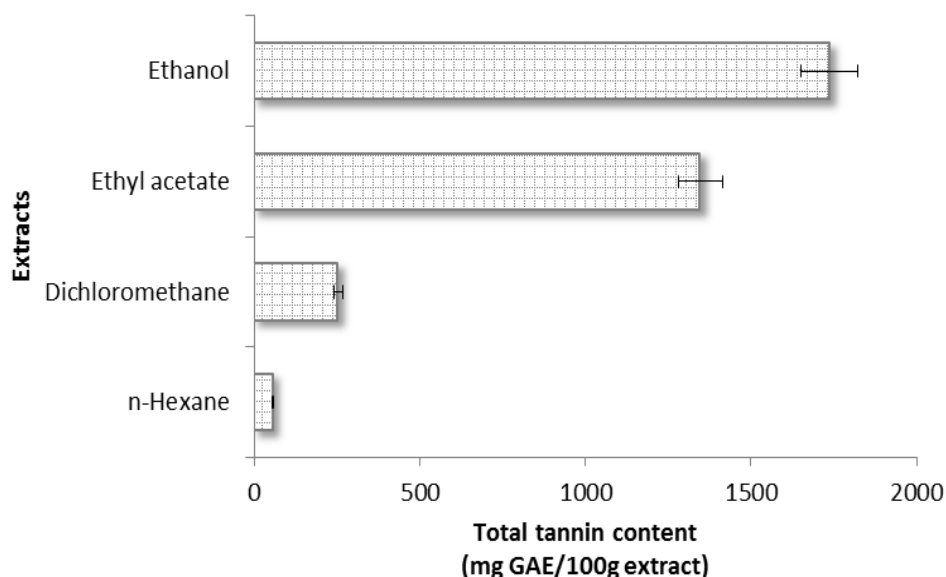


Figure 2. Total tannin content (TTC) of *C. nutans* leaves from four different solvent systems. Error bars show the variations of three determinations in terms of standard deviation ($p < 0.05$).

Nelsonia canescens) had also been reported with higher of phenolic content (Sawadogo et al., 2006). Flavonoids are widely distributed in fruits and vegetables. The antioxidant capacities of flavonoids are related to their degree of unsaturation and oxidation of the three-carbon segment (Chang et al., 2001; Robards et al., 1999).

Figure 3 summarized TFC of *C. nutans* from four different solvents. In this study, TFC of *C. nutans* extracts was determined through a linear rutin standard curve: ($y = 15.32x - 0.0014$; $R^2 = 0.9977$). The values range from 16.6 to 306 mg RE/100 g extract. The highest was recorded in ethanol (306 mg RE/100 g extract) followed

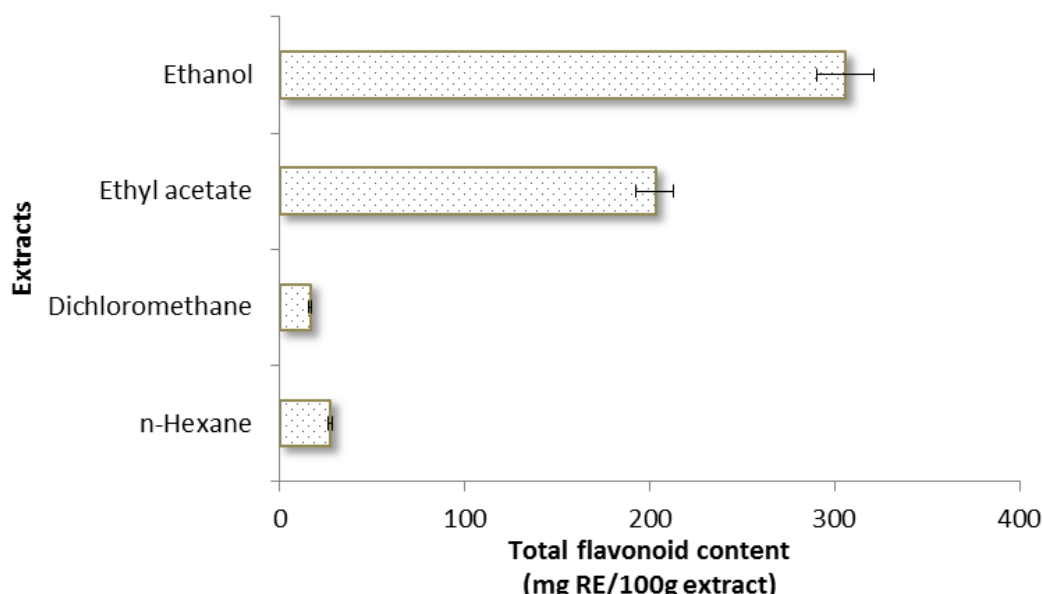


Figure 3. Total flavonoid content (TFC) of *C. nutans* leaves from four different solvent systems. Error bars show the variations of three determinations in terms of standard deviation ($p < 0.05$).

by ethyl acetate (203 mg RE/100 g extract), *n*-hexane (27.3 mg RE/100 g extract), and dichloromethane extracts (16.6 mg RE/100 g extract). Flavonoid content in polar solvents correspond with work reported by Teshima et al. (1997) which isolated six C-glucosyl flavones (vitexin, isovitexin, shaftoside, isomollupentin-7-*O*- β -glucopyranoside, orientin and isoorientin) from methanol extracts of *C. nutans* stems and leaves.

Antioxidant assays

DPPH scavenging activity, ORAC and β -carotene bleaching inhibitory activity

Antioxidant assays from DPPH, ORAC and β -carotene bleaching (BCB) assay can be based on two main reactions: single electron transfer (SET) and hydrogen atom transfer (HAT). Antioxidants in the single reaction system may involve multiple mechanisms or different single mechanisms that respond various ways to different radical sources (Prior et al., 2005). The results obtained for DPPH and BCB are shown in Figure 4. DPPH assay is a decoloration assay using stable organic nitrogen radical 1, 1-diphenyl-1-picrylhydrazyl (DPPH) that changes color from purple to yellow. The assay is commonly used in antioxidant screenings (Sharma and Bhat, 2009). Its antioxidant activity is measured spectrophotometrically through the ability of antioxidant compounds to reduce the DPPH radical by decreasing

the absorbance of the reaction mixture. DPPH assay involving multiple mechanisms monitoring DPPH color loss can be attributed to either SET or HAT as well as unrelated reactions and steric accessibility. In this study, DPPH activity for CN extracts was highest in the ethyl acetate and dichloromethane extracts (semi polar extracts) followed by ethanol and *n*-hexane extracts. Solubility of extracts in different testing systems and stereoselectivity of the radicals may contribute to different antioxidant activities (Prior et al., 2005). A recent report by Yong et al. (2013) also documented that a chloroform extract (semi-polar) of *C. nutans* leaves exhibited highest antioxidant activity in a DPPH assay.

The ability of *C. nutans* extracts to inhibit the discoloration of β -carotene was spectrophotometrically measured by comparing the results to the standard reference of alpha-tocopherol equivalent (Teq) ($y = 0.9733x + 2.9587$; $R^2 = 0.9801$). The presence of antioxidants from extracts may hinder β -carotene bleaching by neutralizing the linoleic free radical (Perumal et al., 2012). In general, the antioxidant activity of *C. nutans* extracts ranged from 120 to 720 mg Teq/100 g extract. *n*-Hexane extract exhibited the highest antioxidant activity (720 mg Teq/100 g extract) through β -carotene bleaching (BCB) assay, whilst ethanol extract showed the least antioxidant activity (120 mg Teq/100 g extract) in the bleaching of β -carotene. Although phenolic, flavonoid and tannin contents are lower in nonpolar extracts (*n*-hexane), the oxidation of linoleic acid may cause by other secondary metabolites such as

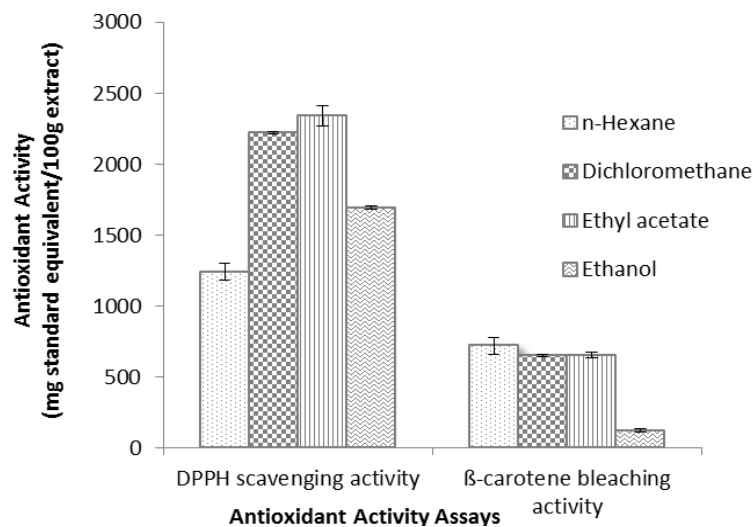


Figure 4. DPPH scavenging activity and BCB activity of *C. nutans* from four different solvent systems. Error bars show the variations of three determinations in terms of standard deviation ($p < 0.05$).

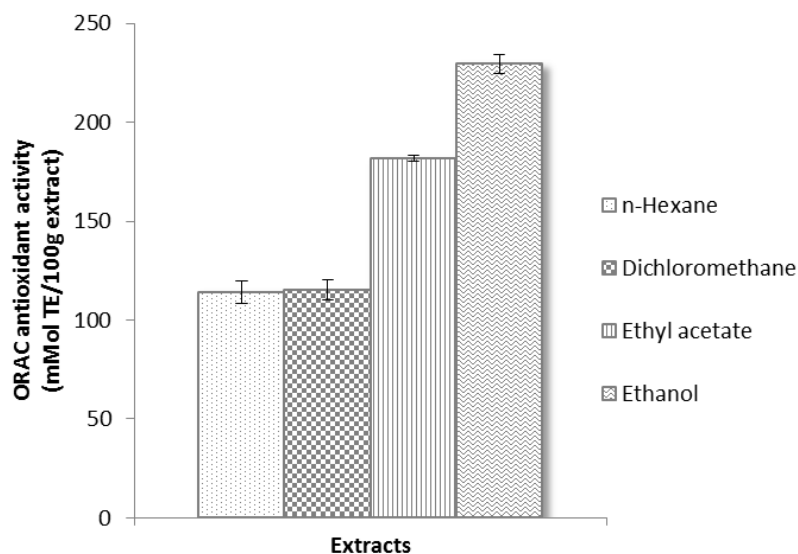


Figure 5. ORAC antioxidant activity of *C. nutans* from four different solvent systems. Error bars show the variations of three determinations in terms of standard deviation ($p < 0.05$).

carotenoids, vitamins and oils (Perumal et al., 2012, 2013).

ORAC assay based on HAT is a considerable antioxidant capacity assay that is the most appropriate to measure *in vitro* and *in vivo* action (Prior et al., 2005; Mariod et al., 2010). In this study, a variation in antioxidant capacity with ORAC assays ranging from

114.3 to 229.5 mMol TE/100 g extract was observed (Figure 5). The ethanol extract of *C. nutans* had highest antioxidant capacity (229.5 mMol TE/100 g extract) followed by ethyl acetate (181.6 mMol TE/100 g extract), dichloromethane (115.5 mMol TE/100 g extract) and *n*-hexane (114.3 mMol TE/100 g extract). The results showed that, TPC, TFC and TTC of *C. nutans* extracts

Table 2. Quantitative analysis major phenolic and fatty acids compounds identified in ethanol and ethyl acetate extracts of *C. nutans*.

Standard references	Retention time (min)	Sample extracts (mg/100 g extract)	
		Ethanol	Ethyl acetate
Alpha tocopherol	14.02	439.7±30.0	1166.0±81.1
Kaempferol	6.75	0.1±0.01	N.D
Sinapic acid	4.38	38.8±2.5	154.2±12.3
Vanillin	4.06	7.6±0.3	N.D
Quercetin	6.06	N.D	65.5±9.0
Rutin trihydrate	4.69	23.5±6.9	21.2±1.7
Syringic acid	3.68	24.7±0.7	24.1±2.2
Protocatechuic acid	1.38	56.0±9.0	66.0±11.2
4-Hydrophenylacetic acid	1.53	48.0±9.2	326.0±23.0
Gentisic acid	1.70	18.0±4.3	70.0±13.0
Cinnamic acid	2.88	18.0±2.7	84.4±12.7
Caffeic acid	1.53	N.D	21.2±0.6
4-Hydroxybenzoic acid	1.64	85.0±13.0	169.4±23.0
Coumalic acid	1.88	79.0±8.8	270.0±25.0
<i>p</i> -Coumaric acid	5.79	110.0±18.4	2.0±0.3
3,4-Dimethoxybenzoic acid	7.08	22.0±3.3	46.0±7.5
Catechin hydrate	2.06	N.D	50.0±6.9
Quercetin hydrate	9.09	54.0±6.8	202.0±21.0
Epicatechin gallate	5.18	N.D	16.0±2.7
Myricitrin	7.99	N.D	10.0±0.2
<i>trans</i> -Ferulic acid	6.52	70.0±13.0	14.0±0.3
Vanilic acid	3.68	118.0±17.0	28.0±3.2
Chlorogenic acid	2.51	52.0±9.1	N.D
Linoleic acid	2.75	65.0±10.0	510.0±17.3
Stearic acid	4.4	585.0±50.0	2535.0±40.0
Oleic acid	3.36	67.5±5.0	387.5±15.0
Palmitic acid	3.44	370.0±20.0	1297.5±25.0
Myristic acid	2.82	10.2±0.6	120.2±20.6

Values are ±SD (n=3), N.D: Not detected

correspond to their ORAC antioxidant capacity, which indicates that the main compounds that contribute to antioxidant activity may come from these groups. The relationship between Folin-ciocalteu and ORAC are in good agreement with what was obtained by Prior et al. (2005) and Bedaway et al. (2010). Since one antioxidant capacity assay would not be comprehensive, various methods are required to measure total antioxidant capacity and none of them are an ideal reference method (Erel, 2004; Prior et al., 2005).

LC-MS analysis of phenolic and fatty acid compounds

Several phenolic and fatty acid compounds were identified in ethanol and ethyl acetate extracts of *C.*

nutans. Phenolic are antioxidant compounds which act as agents to neutralize harmful free radicals (ROS). Table 2 summarizes the analysis of phenolic and fatty acid quantitative analysis of ethanol and ethyl acetate extracts of *C. nutans* through LC-ESI/MS. Saturated, monounsaturated and polyunsaturated fatty acids are good for the health (Piorkowski and McClements, 2014). Long chain omega-3 or -6 polyunsaturated fatty acids (PUFAs) can lower the production of reactive oxygen species (ROS), thus decreasing the risk of various diseases and improves. The effect of omega-3 long chain PUFAs on ROS is stronger than saturates, monounsaturates and polyunsaturates of the omega-6 series. These two types of fatty acids, also known as essential fatty acids (EFA) are important in human diet because they cannot be produced by the body and must

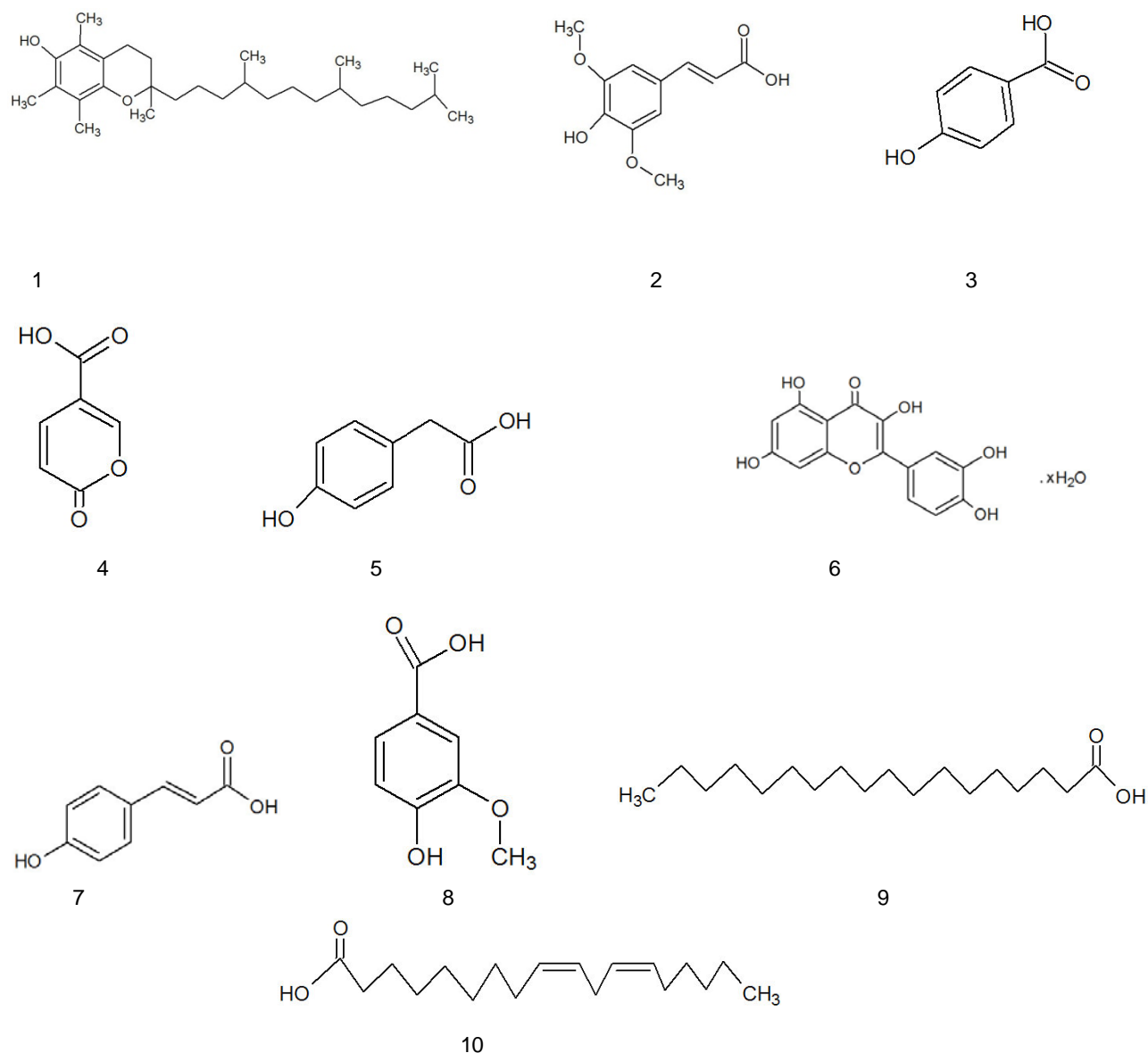


Figure 6. Structure of major phenolic and fatty acid compounds identified in ethyl acetate and ethanol extracts of *C. nutans*.

be obtained from food (Richard et al., 2008; Simopoulos, 1991).

Among of 23 tested phenolic and fatty acid compounds (Figure 6), alpha tocopherol (1) was the most abundant compound in both extracts, 439.7 mg/100 g extract (ethanol) and 1166.0 mg/100 g extract (ethyl acetate). Ethyl acetate extract was quantified to have high content of sinapic acid (2) (154.2 mg/100 g), 4-hydroxyphenylacetic acid (3) (326.0 mg/100 g), 4-hydroxybenzoic acid (4) (169.4 mg/100 g), coumalic acid (5) (270 mg/100 g) and quercetin hydrate (6) (202.0 mg/100 g). Meanwhile,

ethanol extract discovered to have intense of *p*-coumaric acid (7) (110 mg/100 g) and vanilic acid (8) (118.0 mg/100 g).

Mustapa et al. (2015) have reported that polar extract of *C. nutans* with microwave-assisted extraction (MAE) and soxhlet extraction have discovered to have plenty of phytol compound, compared to supercritical fluid extraction (SFE) which have major of palmitic acid. Palmitic acid is one of the fatty acid quantified in the present study in both ethyl acetate and ethanol extracts. The fatty acid profile of both extract contain of linoleic

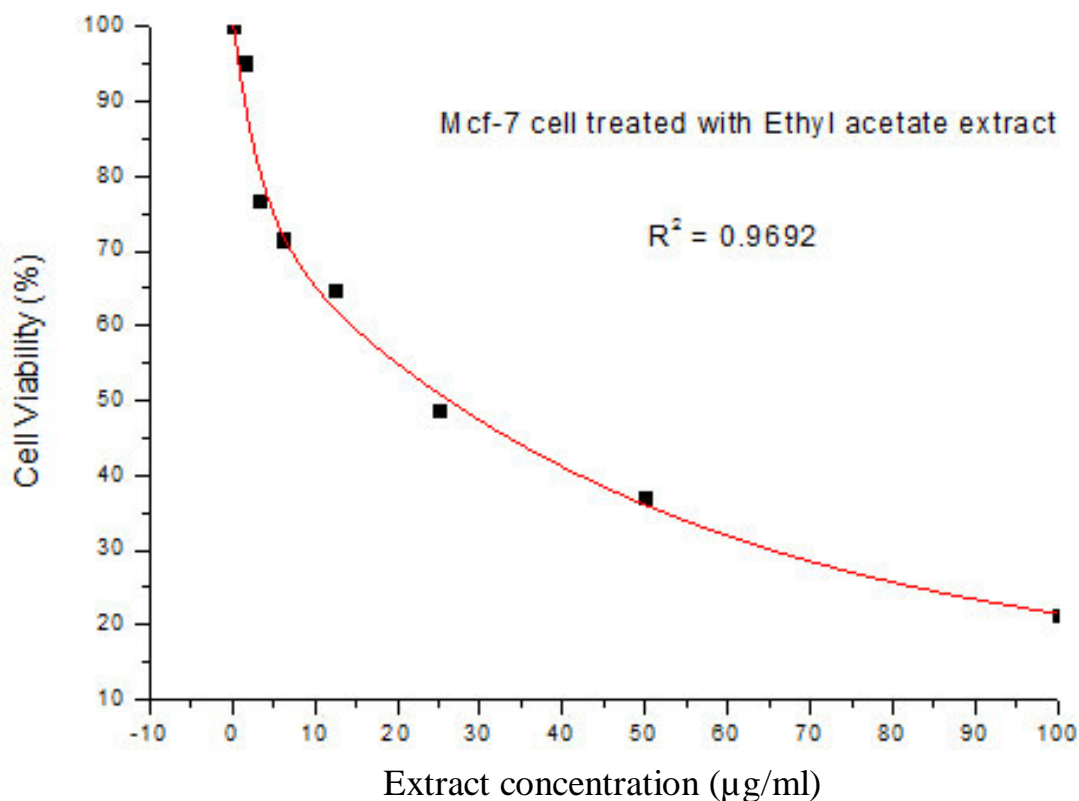


Figure 7. Toxicity effect of ethyl acetate extracts of *C. nutans* at various concentrations (3.125 to 100 µg/ml) on tumorigenic cell MCF-7. Data represented in percentage (%) of cell viability as mean \pm standard deviation (n=3) ($p < 0.05$).

acid (polyunsaturated), stearic acid, oleic acid (monounsaturated), palmitic acid and myristic acid. Both extracts discovered to have an abundance of stearic acid (9) that is, 585.0 mg/100 g extract and 2535.0 mg/100 g extract respectively in ethanol and ethyl acetate extracts. Long chain polyunsaturated fatty acid (LC-PUFAs) linoleic acid (10) was detected in both extracts; 65.0 mg/g extract and 510.0 mg/g extract ethanol and ethyl acetate extracts, respectively. Linoleic acid is an omega-6 fatty acid, required for proper skin function. Increasing levels of EFAs (omega-3 and omega-6) can increase cell membrane fluidity, enhance barrier function and repair, decrease trans-epidermal water loss, moisturize, improve cell immunity, and acts as an anti-inflammatory. In pharmaceutical applications, EFAs can enhance the absorption of bioactive and can also be used as carrier oils (Prottey et al., 1975; Simopoulos, 1991).

Cytotoxicity assay

According to the standards of National Cancer Institute

(NCI), a crude extract may be considered as active for an $IC_{50} < 30$ µg/ml. Both tested extracts demonstrated cytotoxic on the breast cancer estrogen positive (MCF-7) cell. As shown in Figures 7 and 8, the IC_{50} value for ethyl acetate extracts was 24.04 ± 1.7 µg/ml, whereas ethanol extract of CN was 28.90 ± 2.1 µg/ml (Figure 8). This finding is in agreement with Yong et al. (2013) suggested that semi polar extract (chloroform) of *C. nutans* demonstrated higher antiproliferative activity than polar extract (methanol). 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 ethyl acetate compare to ethanol extract. In agreement with Ramadan et al. (2003) antioxidant activity also was affected by the level of PUFA content. This may be explained by the cytotoxicity activity of ethyl acetate extract which is stronger than ethanol extract on tumorigenic cell MCF-7. Furthermore, squalene and several long fatty acids have been reported in this plant previously (Mustapa et al., 2015). Squalene compound, which is good in antioxidant

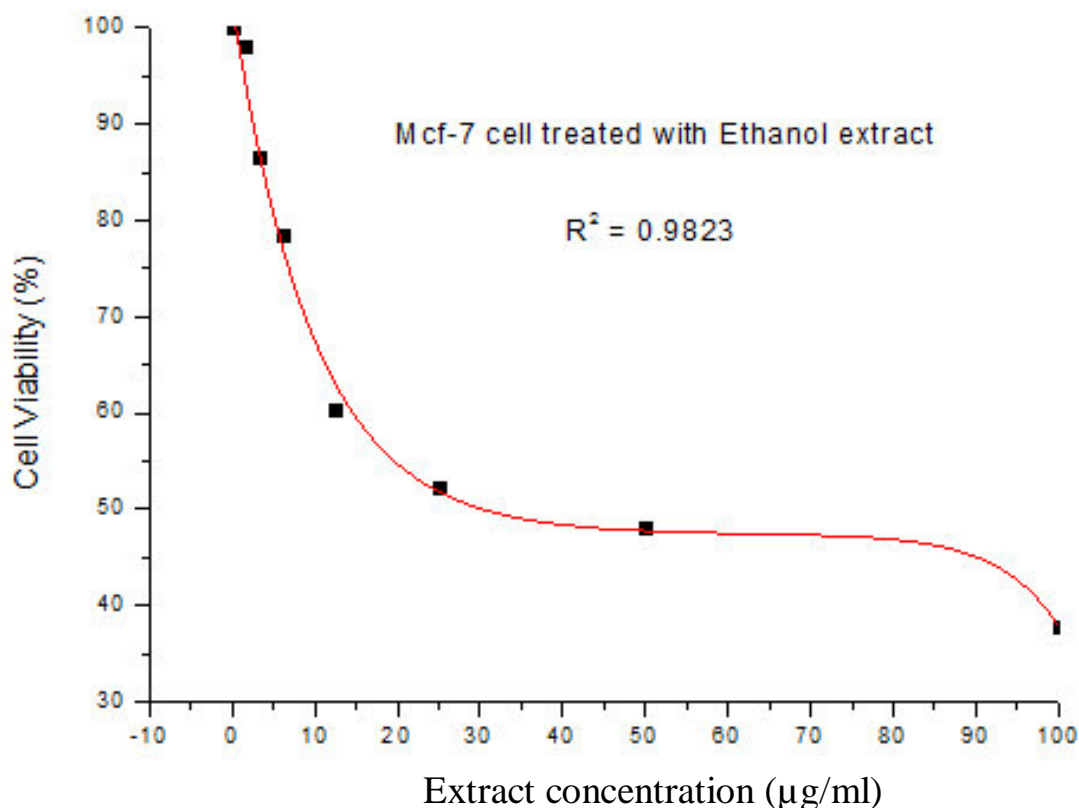


Figure 8. Toxicity effect of ethanol extracts of *C. nutans* at various concentrations (3.125 to 100 µg/ml) on tumorigenic cell MCF-7. Data represented in percentage (%) of cell viability as mean \pm standard deviation (n=3) ($p < 0.05$).

activity, also claimed to have anti-cancer, anti-tumor and chemo-preventive properties (Reddy and Couvreur, 2009; Ezhilan and Neelamegam, 2012).

Highest antioxidant activity of ethyl acetate and ethanol extracts in present study may significantly contribute to its cytotoxic effect. This is because extract with antioxidant can act by scavenging reactive oxygen species (ROS) in human body. ROS are highly reactive and can be categorized into two groups: free oxygen radicals and non-radical. ROS participate in the two stage of carcinogenesis, inducing cancer and carcinogens. Among ROS, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$) are the most found in cancer (Feng et al., 2015). Therefore by reducing the level of the ROS can effectively prevent and suppress the occurrence of cancer. Moreover both extract showed not active against normal fibroblasts cell 3T3 with IC_{50} values 53.06 ± 3.8 µg/ml (Figure 9) and 73.84 ± 5.4 µg/ml (Figure 10) for ethyl acetate and ethanol extracts, respectively. Thus, both extract showed a good selectivity effect on cancer cell (MCF-7) and can potentially be used as a good source of anticancer and antioxidant.

Conclusion

Ethanol and ethyl acetate extracts of *C. nutans* exhibited the highest antioxidant activity amongst all the *C. nutans* extracts. Higher content of phenolic in ethyl acetate and ethanol extracts than other extracts have contributed to its stronger antioxidant activity and cytotoxicity against tumor cell. In addition, antioxidant activity of extract is also affected by higher level of PUFA. Phenolic, flavonoid and tannin are very soluble in the high polarity solvents. Further studies are recommended to be conducted on potential used of these extracts as anticancer developed and pharmaceutical applications.

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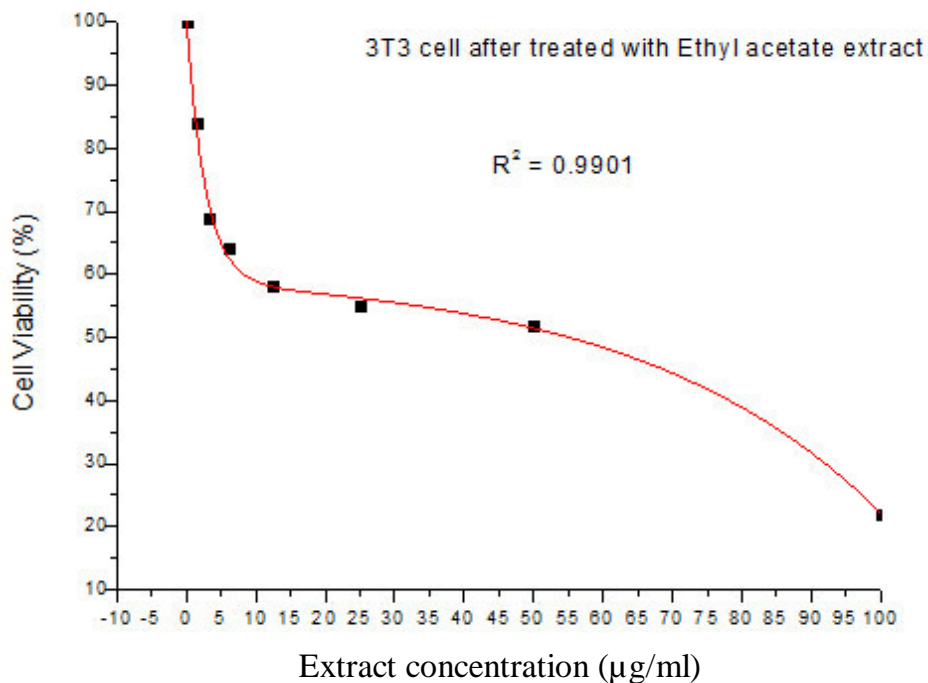


Figure 9. Toxicity effect of ethyl acetate extracts of *C. nutans* at various concentrations (3.125 to 100 µg/ml) on normal fibroblasts cell 3T3. Data represented in percentage (%) of cell viability as mean ± standard deviation (n=3) ($p < 0.05$).

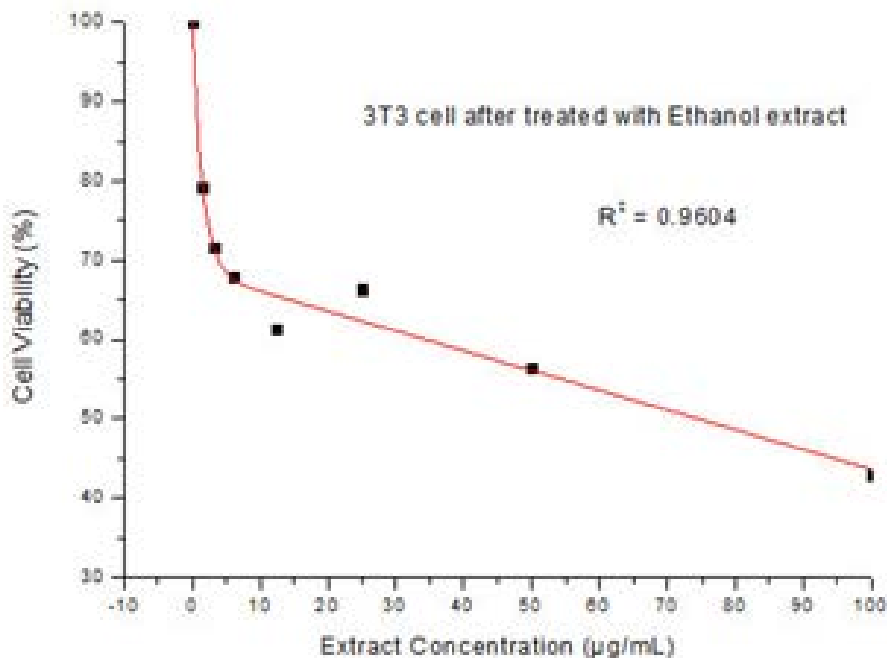


Figure 10. Toxicity effect of ethanol extracts of CN at various concentrations (3.125-100 µg/mL) on normal fibroblasts cell 3T3. Data represented in percentage (%) of cell viability as mean ± standard deviation (n=3). $P < 0.05$.

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Conflicts of interest

Authors have none to declare.

REFERENCES

- Azam S, Hadi N, Khan NU, Hadi SM (2004). Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol. In Vitro.* 18(5):555-61.
- Bedawey A, Mansour E, Zaky M, Hassan A (2010). Characteristics of Antioxidant Isolated from Some Plant Sources. *Food Nutr. Sci.* 1:5-12.
- Cai Y, Luo Q, Sun M, Corke H (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* 74:2157-2184.
- Chang S, Wu J, Wang S, Kang P, Yang N, Shyr L (2001). Antioxidant activity of extracts from *Acacia confusa* bark and heartwood. *J. Agric. Food Chem.* 49(7):3420-3424.
- EI-Ghorab A, EI-Massry K, Takayuki S (2007). Chemical composition of the volatile extract and antioxidant activities of the volatile and nonvolatile extracts of Egyptian corn silk (*Zea mays* L.). *J. Agric. Food Chem.* 55(22):9124-7.
- Erel O (2004). A novel automated method to measure total antioxidant response against potent free radical reactions. *Clin. Biochem.* 37:112-119.
- Ezhilan BP, Neelamegam R (2012). GC-MS analysis of phytochemicals in the ethanol extract of *Polygonum chinense* L. *Pharmacogn. Res.* 4:11-14.
- Farooqui M, Hassali MA, Shatar AKA, Farooqui MA, Saleem F, Haq NU, Othman CN (2015). Use of complementary and alternative medicines among Malaysian cancer patients: A descriptive study. *J. Tradit. Complement. Med.* 8-13.
- Feng XH, Wang ZH, Meng DL, Li X (2015). Cytotoxic and antioxidant constituents from the leaves of *Psidium guajava*. *Bioorg. Med. Chem. Lett.* 25:2193-2198.
- Gomes C, Cruz TG, Andrade J, Milhazes N, Borges F, Marques M (2003). Anticancer activity of phenolic acids of natural or synthetic origin: a structure-activity study. *J. Med. Chem.* 46:5395-5401.
- Huang D, Ou B (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* 50:4437-4444.
- Iqbal S, Bhangar MI, Anwar F (2005). Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. *Food Chem.* 93:265-272.
- Ismail H, Chan K, Mariod A, Ismail M (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chem.* 119:643-647.
- Kunsorn P, Ruangrunsi N, Lilipun V, Khanboon A, Rungsirunrat K (2013). The identities and anti-herpes simplex virus activity of *Clinacanthus nutans* and *Clinacanthus siamensis*. *Asian Pac. J. Trop. Biomed.* 3:284-290.
- Lee J, Kim H, Kim J, Jang Y (2002). Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. *saboten*. *J. Agric. Food Chem.* 50:6490-6496.
- Maestri DM, Nepote V, Lamarque AL, Zygadlo JA (2006). Natural products as antioxidants. *Res. Signpost.* 1:105-135.
- Makkar H (2003). Quantification of tannins in tree and shrub foliage: a laboratory manual. Springer Science & Business Media.
- Mariod A, Fathy S, Ismail M (2010). Preparation and characterisation of protein concentrates from defatted kenaf seed. *Food Chem.* 123:747-752.
- Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-63.
- Mustapa AN, Martin A, Mato RB, Cocero MJ (2015). Extraction of phytochemicals from the medicinal plant *Clinacanthus nutans* Lindau by microwave-assisted extraction and supercritical carbon dioxide extraction. *Ind. Crops Prod.* 74:83-94.
- Negi PS (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *Int. J. Food Microbiol.* 156:7-17.
- P'ng X, Akowuah G, Chin J (2012). Acute oral toxicity study of *Clinacanthus nutans* in mice. *Int. J. Pharm. Sci. Res.* 3:4202-4204.
- Perumal S, Mahmud R, Piaru SP, Cai LW, Ramanathan S (2012). Potential Antiradical Activity and Cytotoxicity Assessment of *Ziziphus mauritiana* and *Syzygium polyanthum*. *Int. J. Pharmacol.* 8:535-541.
- Perumal S, Mahmud R, Piaru SP, Lee WC, Ramanathan S (2013). Antiradical and Cytotoxic Activities of Varying Solvent Polarity Extracts of the Aerial Part of *Euphorbia hirta* L. *J. Chem.* 2013:1-6.
- Piorkowski DT, McClements DJ (2014). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocoll.* 42:5-41.
- Prior RL, Wu X, Schaich K (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53:4290-4302.
- Prottey C, Hartop P, Press M (1975). Correction of the cutaneous manifestations of essential fatty acid deficiency in man by application of sunflower-seed oil to the skin. *J. Invest. Dermatol.* 64:228-234.
- Ramadan M, Kroh L, Mörsel J (2003). Radical scavenging activity of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.), and niger (*Guizotia abyssinica* Cass.) crude seed oils and oil. *J. Agric. Food Chem.* 51:6961-6969.
- Reddy LH, Couvreur P (2009). Squalene: A natural triterpene for use in disease management and therapy. *Adv. Drug Deliv. Rev.* 61:1412-1426.
- Richard D, Kefi K, Barbe U (2008). Polyunsaturated fatty acids as antioxidants. *Pharmacol. Res.* 57:451-455.
- Robards K, Prenzler P, Tucker G (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* 66:401-436.
- Rodríguez J, Olea AC, Cavieres C, Norambuena E, Delgado CT, Soto DJ, Araya MR (2007). Antioxidant properties and free radical-scavenging reactivity of a family of hydroxynaphthalenones and dihydroxyanthracenones. *Bioorg. Med. Chem.* 15:7058-7065.
- Sakdarat S, Shuypprom A, Pientong C, Ekalaksananan TS (2009). Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau. *Bioorg. Med. Chem.* 17:1857-1860.
- Sawadogo W, Meda A, Lamien C (2006). Phenolic content and antioxidant activity of six acanthaceae from Burkina Faso. *J. Biol. Sci.* 6:249-252.
- Sharma O, Bhat T (2009). DPPH antioxidant assay revisited. *Food Chem.* 113:1202-1205.
- Siew Y, Zareisedehizadeh S, Seetoh WG, Neo SY, Tan CH, Koh HL (2014). Ethnobotanical survey of usage of fresh medicinal plants in Singapore. *J. Ethnopharmacol.* 155:1450-1466.
- Simopoulos A (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* 54:438-463.
- Teshima K, Kaneko T, Ohtani K, Kasai R, Lhieochaiphant S, Picheansoonthon C, Yamasaki K (1997). C-Glycosyl flavones from *Clinacanthus nutans*. *Nat. Med.* 51:557.
- Uawonggul N, Chaveerach A, Thammasirirak S, Arkaravichien T, Chuachan C, Daduang S (2006). Screening of plants acting against *Heterometrus laoticus* scorpion venom activity on fibroblast cell lysis. *J. Ethnopharmacol.* 103:201-207.
- Wanikiat P, Panthong A, Sujayanon P, Yoosook C, Rossi AG, Reutrakul V (2008). The anti-inflammatory effects and the inhibition of neutrophil responsiveness by *Barleria lupulina* and *Clinacanthus nutans* extracts. *J. Ethnopharmacol.* 116:234-44.

Wettasinghe M, Shahidi F (1999). Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (*Borago officinalis* L.) seeds. *Food Chem.* 67:399-414.

Yong Y, Tan J, Teh S, Mah S (2013). *Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines. *Evid. Based Complement. Altern. Med.* 2013:1-8.

Yuann JMP, Wang JS, Jian HL, Lin CC, Liang JY (2012). Effects of *Clinacanthus nutans* (Burm. f) Lindau leaf extracts on protection of plasmid DNA from riboflavin photoreaction. *MC-Trans. Biotechnol.* 4:45-58.