

Antioxidant, antimicrobial activity and *in-vitro* cytotoxicity screening study of Pili nut oil

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Abstract

Some vegetable oils contain natural antioxidants such as beta carotene and vitamin E namely alpha tocopherol. The objective of this study was to screening the value of α -tocopherol, β -carotene, antioxidant capacity, antimicrobial activity and toxicological properties of roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO). The result showed that RPNO contained higher amount of vitamin E and less amount of beta carotene compared to UPNO. RPNO and UPNO scavenged DPPH radicals by 24.66% and 9.52% at concentration of 140 μ g/ml. The total phenolic compound (TPC) in UPNO and RPNO were about 19.96 ± 0.52 mg/kg and 12.43 ± 0.69 mg/kg respectively. It was observed that bacteria species exhibited different sensitivities towards RPNO, UPNO, Gentamycin, Ampicillin and Chloramphenicol. *Bacillus cereus* 14570 was the most sensitive bacterium and all strains of *Staphylococcus aureus* tested were resistant against both samples RPNO and UPNO. An *in vitro* toxicological study based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay was also performed. *In vitro* cytotoxicity indicated that both RPNO and UPNO had no effect against HeLa (cervical cancer cell), MCF-7 (breast cancer cell) and HT-29 (human colon adenocarcinoma cell) cell lines tested.

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Introduction

The *Canarium ovatum* (pili) is indigenous to the Philippines (Merrill 1912, 1923; Wester 1921; Brown 1954; Li 1970). Much attention has been recently focused on the utilization of underutilized agricultural products (Yoshida *et al.*, 2006). In Malaysia, the Department of Agriculture Sabah has planted pili for research purposes. According to Coronel (1996), the kernel contains 12 - 16% protein, 69 - 77% fats and 3 - 4% carbohydrates. The pili nut kernel's high oil content may prove to be hindrance to its success in international markets.

The oxidative damage caused by reactive oxygen species on lipids, proteins and nucleic acids may trigger various chronic diseases, such as atherosclerosis, cancer and ageing (Jadhav *et al.*, 1996). According to Rimm *et al.* (1993) and Roberts II *et al.* (2007), epidemiological studies show that various dietary sources of antioxidants such as vitamin C, vitamin E and β -carotene minimize the oxidative damage. The main sources of vitamin E active compounds in the human diet are vegetable fats and oils and products derived from them while tocopherols are

generally present in nuts and common vegetable oils. A large number of research papers published have reported the separation and quantification of the eight vitamins from various sources (Choo *et al.*, 2005). α -Tocopherol has three methyl groups and is the most active form (Ubaldi *et al.*, 2005). Some synthetic antioxidants, such as butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA), need to be replaced with natural antioxidants, as they were found to be toxic and carcinogenic in animal models (Safer and Al-Nughamish, 1999). Thus, it is important to identify new sources of safe and inexpensive antioxidants of natural origin. The development of alternative natural antioxidants such as those found in plants is of great importance for our health and holds considerable commercial potential. They may be replaced by naturally occurring antioxidants (Matsukawa *et al.*, 1997).

Phenolic compounds were also found to have antioxidant activity in terms of their capacity to inhibit LDL oxidation *in vitro* (Fito *et al.*, 2000). According to Hao *et al.* (1998), in the past few years, due to concerns regarding the safety of synthetic antimicrobial agents, there has been an increase in

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consumer demand for naturally derived compound such as plant extracts as antimicrobials in foods. Several studies indicate that medicinal plants contain compounds like peptides, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols and water or ethanol soluble compounds. These compounds are significant in therapeutic application against human and animal pathogen, including bacteria, fungi and viruses (Khan *et al.*, 2003; Pavrez *et al.*, 2005).

The antioxidant properties of the pili nut oil phenolic extract have been poorly investigated and a literature search did not reveal any published report on the antibacterial effect of pili nut oil on strains of microorganisms and the toxicological properties of this oil. Therefore, the main aim of this study were to screening the value of vitamin E, β -carotene, antioxidant capacity, antibacterial activity and in vitro cytotoxicity of roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO).

Materials and Methods

Instruments

High Performance Liquid Chromatography (Waters) was used to determine the vitamin E (α -tocopherol) and spectrophotometer UV/VIS spectrophotometer (Shimadzu, UV 1800) was used for the measurement of β -corotene, the percentage of radical scavenging activity (DPPH) and the total phenolic content (TPC).

Materials

Roasted pili nut and unroasted pili nut were obtained from Department of Agriculture (DoA), Kota Kinabalu Sabah. Both samples were extracted for their oil by using mechanical press machine (cold press method) and stored in sealed glass bottle at -20°C until analyzed.

Determination of concentration of vitamin E (α -tocopherol) by HPLC

The concentration of vitamin E in the RPNO and UPNO was determined by HPLC HPLC (Waters) using AOAC 16th Edi. 967.21 (STP/Chem/A12). HPLC analysis was performed using C18 column (symmetry). The mobile phase used was composed of 95% methanol and 5% deionized water and the flow rate was 1 ml/min. Total runtime for standard and samples was 50 minutes. The injection volume was 10 μl . detection was performed using the florescence detector at excitation 296 nm and emission 330 nm. The determination of α -tocopherol in RPNO and UPNO were done in duplicate.

Determination of concentration of β -carotene by spectrophotometer

Carotenes contents in the samples were analysed by Ultraviolet-Visible (UV-vis) spectrophotometer at 446 nm according to PORIM test methods no. p2.6 (1995). The sample was homogenized and weighed to the nearest ± 0.0001 g, 0.1 g of the sample into a 25 mL volumetric flask. The sample was dissolved with n-hexane and diluted to the mark. The solution was transferred into a 1 cm quartz cuvette and the absorbance was measured at 446 nm against n-hexane. The carotene content of different vegetables oil is defined and calculated as β -carotene in parts per million (ppm). The calculation was as follows:

$$\text{Carotenoids content} = [V \times 383 \times (A_s - A_b)] / (100 \times W)$$

Where:

V = The volume used for analysis

383 = The extinction coefficient for carotenoids

A_S = The absorbance of the sample

A_b = The cuvette error

W = The weight of the sample in g

Antioxidant capacity assays

Antioxidant activity of RPNO and UPNO were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay. The radical scavenging activity assay (RSA) was measured following the methodology described by Brand-Williams *et al.* (1995) with some modifications. To prepare a sample stock solution, (200 $\mu\text{g/ml}$), a 0.02 g sample of oil (RPNO and UPNO) was extracted with 100 ml of hexane. Then 100 ml of methanol: water (60:40,v/v) was added and the mixture was vortex vigorously for 2 min. The stock phenolic fraction of oils with concentration of 200 μg then was used to prepare few different concentration, (20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg). The bleaching rate of stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH \cdot) was monitored in the presence of the sample. A 3.9 ml aliquot of 6×10^{-5} M DPPH \cdot in methanol solution was used and the reaction was started by adding 0.1 ml of phenolic extract with concentration of 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg . The mixtures were well shaken and kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using Shimadzu UV-Vis spectrophotometer. Butylhydroxyl toluene (BHT) was used as positive control. Inhibition (I%) of DPPH radical was calculated using the equation:

$$I\% = (A_o - A_s / A_o) \times 100$$

Where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the test compound.

Percentage of inhibition were plotted against concentration of pili nut oil to calculate the concentration providing 50% inhibition (IC_{50}). The experiment were done in triplicate.

Determination of total phenolic content

Total phenolic content of RPNO and UPNO were determined by Folin-Ciocalteu method (Gutfinger, 1981) with some modification. A 2.5 g sample of oil (RPNO and UPNO) was extracted with 5 ml of hexane. Then 5 ml of methanol:water (60:40, v/v) was added and the mixture was vortexed vigorously for 2 min. 1 ml of the phenolic fraction was added with 5 ml of Folin-Ciocalteu reagent: water (1:10, v/v). Then the mixture was added with 4 ml (7.5%, w/v) of sodium bicarbonate and was incubated for 2 hrs in the dark at room temperature and then the absorbance of the mixture was measured at 765 nm. Phenolic contents are expressed as gallic acid equivalents per gram (GAE/g) of extract.

Antimicrobial assay

Determination on antibacterial effect (Disk diffusion method)

The antimicrobial susceptibility test discs (blank discs, chloramphenicol, ampicillin, gentamicin) were purchased from OXOID, UK. Antibacterial capacity of the roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO) were investigated by the disk diffusion method (Bauer et al., 1996; Alzoreky and Nakahara, 2003). Before experimental use, cultures from solid medium were subcultivated in liquid media and incubated in incubator shaker. A 16 hrs culture was diluted with a sterile physiological saline solution [PS: 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standards to achieve an inoculum size of approximately 10^6 colony forming unit mL^{-1} . A population of each strain of was inoculated on triplicate plates of Mueller Hinton Agar (MHA) by using sterile cotton swabs (Andrews, 2001). Then, the plates were allowed to dry at room temperature for 15 minutes. 10 μ l each of pili nut oil (RPNO and UPNO) was added to paper discs and allowed to dry for 15 min. Similarly, sterile blank paper discs impregnated with sterile water served as negative control and antibiotic discs (6.0 mm dia) of 30 μ g/mL of chloramphenicol, ampicillin and gentamicin were also used as positive control. The discs were then placed aseptically over the bacterial cultures on Mueller Hinton Agar (MHA). All plates were incubated at 37°C for 24 hrs and after

that, the zone of inhibition around the paper discs were measured accurately using a metric ruler to the nearest millimeter. Data were analyzed using the Statistical Analysis Software 9.2 (SAS Institute, Inc., Cary, NC, USA), to determine significant differences ($p < 0.05$) in the antibacterial effects of pili nut oil, chloramphenicol, ampicillin and gentamicin on twenty one strains on microorganisms.

Test microorganisms

The strain of bacteria used in this study were *Klebsiella pneumoniae* 13883 *Yersinia enterocolitica* 9610, *Salmonella typhimurium* 14028, *Salmonella choleraesuis* 14028, *Salmonella choleraesuis* 14024, *Staphylococcus aureus* 29178, *Staphylococcus aureus* 29213, *Staphylococcus aureus* 33591, *Staphylococcus aureus* 3862, *Staphylococcus epidermidis* 12228, *Listeria monocytogenes* 7644, *Serratia marcescens* 8100, *Bacillus subtilis* 6051, *Bacillus cereus* 14579, *Bacillus cereus* 14570, *Escherichia coli* 8739, *Escherichia coli* 25922, *Escherichia coli* 35218, *Enterobacter cloacae* 13047, *Enterobacter aerogenes* 13048 and *Pseudomonas aeruginosa* 10145. All bacterial strains were obtained from the Microbiology Laboratory, Universiti Sultan Zainal Abidin Terengganu.

Cytotoxicity assay

Cell line

The cell lines used were HeLa (cervical cancer), MCF-7 (breast cancer cell) and HT-29 (human colon adenocarcinoma) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The cells were grown and maintained in RPMI 1640 (Sigma, USA) medium; supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA), 100 IU/ml penicillin (PAA, Austria) and 100 g/ml streptomycin at 37°C, 5% CO_2 and 90% humidity.

Sample preparation

Roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO) were dissolved in DMSO to get a stock solution of 10 mg/ml. The working solution of 0.06 mg/ml was prepared by diluting 6 μ l of stock solution into 994 μ l serum-free culture medium. The stock and working solution were stored at 4°C.

MTT cell viability assay

The effects of RPNO and UPNO on cell viability of HeLa, MCF-7 and HT-29 were first determined by using a colorimetric technique, which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Tim Mosmann, 1983).

Briefly, 100 μ l RPMI 1640 media with 10% (v/v) of fetal bovine serum (FBS) was added into all the wells except row A in the 96-well plate (TPP, Switzerland). Then 100 μ l of diluted compounds at 60 μ g/ml was added into row A and B. A series of twofold dilution of compound was carried out down from row B until row G. The row H was left untouched and the excess solution (100 μ l) was discarded, then 100 μ l of cells (HeLa, MCF-7 and HT-29) with cell concentration at 4×10^5 cell/ml was added into all wells in the 96-well plate and incubated at 37°C, 5% CO₂ and 90% humidity for 72 h. After incubation period, 20 μ l of MTT (Sigma, USA) at concentration of 5 mg/ml was added into each well and incubated for 4 h at 37°C, 5% CO₂ and 90% humidity. 170 μ l of medium with MTT was removed from every well and 100 μ l DMSO (Sigma, USA) was added to each well to solubilize the formazan crystal by incubating for 20 min at 37°C, 5% CO₂ incubator. Finally, the plate was read at 570 nm and reference wavelength at 630 nm by using microplate reader Infinite M200 (TECAN, Switzerland). Each compound and control was assayed in triplicate or three times. The cytotoxic dose that kills cells by 50% (IC₅₀) was determined from absorbance (OD) versus concentration curve, and the percentage of proliferation was calculated by the following formula:

$$\text{Percentage (\%)} \text{ of cell proliferation} = \frac{\text{OD sample} \times 100}{\text{OD control}}$$

Colour measurement

The colour of the oil samples and nuts were determined by measuring CIE Lab, L* (lightness), a* (redness) and b* (yellowness) values with Chromameter DR-400 Minolta.

Statistical analysis

The vitamin E analysis were performed in duplicate and other experiments were performed in triplicates. Data obtained were tested for significance using ANOVA and Duncan Multiple Range Test at $p < 0.05$ using SAS version 9.2. and the result were expressed as mean \pm standard deviation.

Results and Discussions

Vitamins E is an important antioxidant that plays an important role in prevention of chronic diseases. The major biological role of vitamin E is to protect unsaturated fatty acids contained in vegetable oils from oxidation by free radicals. Supplementation with vitamin E in humans decrease the susceptibility of LDL to oxidation *in vivo* (Madhavi *et al.*, 2009). A typical HPLC profiles of RPNO were shown

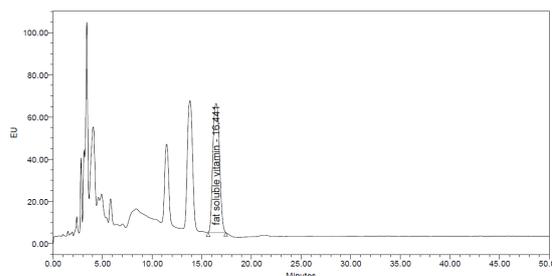


Figure 1. Typical HPLC chromatogram of roasted pili nut oil (RPNO)

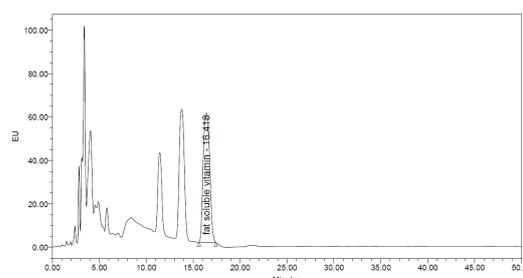


Figure 2. Typical HPLC chromatogram of roasted pili nut oil (RPNO)

in Figure 1 and Figure 2 and UPNO were shown in Figure 3 and Figure 4 respectively. These four figures illustrate chromatograms that identifiable peaks which correspond to α -tocopherol. The value of α -tocopherol in RPNO and UPNO were 37.5450 ± 0.4031 ppm and 10.1850 ± 0.1485 ppm respectively.

Table 1 shows the percentage concentration of Vitamin E (α -tocopherol) in roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO). The concentration of α -tocopherol in RPNO was 0.004% and were quite higher compared to UPNO which was about 0.001%. It was observed that the level of α -tocopherol for both samples were only detected at small amounts if compared with the result of Dauqan *et al.* (2011) for the red palm olein, palm olein and corn oil but the α -tocopherol was not detected in coconut oil. It means that, the pili nut oil were still considered as a good source of vitamin E. Furthermore, Matkowski (2008) stated that the antioxidant is the compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration. This result also is in agreement with the result of Durmaz and Gökmen (2011), where the amount of α -tocopherol was found to be higher in the sample roasted for 40 min than in the sample roasted for 30 min ($p < 0.05$) and it was found that longer roasting times caused fluctuation in total tocopherol content. Meanwhile, the effect of roasting on the level of tocopherol isomers in extracted oil has been previously reported by others for different nuts and seeds. Heat pretreatment was reported to cause no change (Chiou and Tsai, 1989), increase (Kim *et al.*, 2002) or decrease (Anjum *et al.*, 2006) in the tocopherol content of different seeds and nuts. Furthermore, according to Durmaz and Gökmen

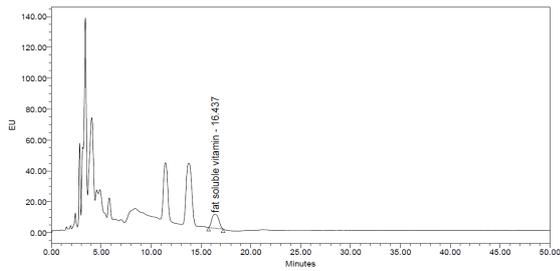


Figure 3. Typical HPLC chromatogram of unroasted pili nut oil (UPNO)

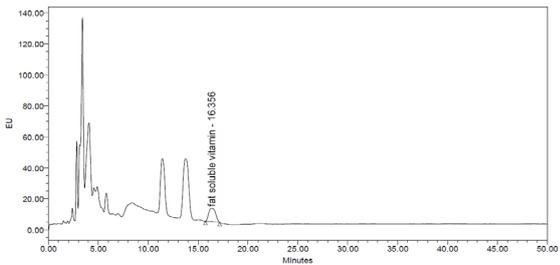


Figure 4. Typical HPLC chromatogram of unroasted pili nut oil (UPNO)

(2011), those different results imply that roasting may affect tocopherol distribution in different ways depending on the seed variety or the type and intensity of heat pretreatment.

Carotenoids also play an important potential role in human health by acting as biological antioxidants protecting cells and tissues from the damaging effects of free radicals and singlet oxygen (Zeb and Mehmood, 2004). According to Hathcock (2004), β -carotene is the most abundant form of provitamin A in fruits and vegetables and is currently incorporated in a wide variety of dietary supplements, including multivitamin, vitamin A and antioxidant formulations (Schierle *et al.*, 2004). The content of β -carotene in roasted pili nut oil and unroasted pili nut oil is summarized in Table 1. Oil from roasted and unroasted sample was found to contain 18.604 ± 3.0319 ppm and 55.8717 ± 0.4462 ppm of β -carotene. There was significant decrease in β -carotene concentration of pili nut oil with roasting. According to Jideani (1992), the loss of carotenoids with roasting was accelerated at high temperatures due to sensitive nature of these pigments.

DPPH radical scavenging activity assay has been extensively used for screening antioxidant activity because it can accommodate many samples in short period and is sensitive enough to detect active ingredients at low concentrations (Sanchez-Moreno, 2002). The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is usually noticeable as a color change from purple to yellow. A lower value of IC_{50} indicates a higher antioxidant activity.

Table 1. Concentration of vitamin E and β -Carotene in roasted pili nut oil and unroasted pili nut oil

Sample	α -T (ppm)	α -T (%)	β -carotene (ppm)
RPNO	$37.5450 \pm 0.4031^*a$	0.004	$18.604 \pm 3.0319^{*b}$
UPNO	$10.1850 \pm 0.1485^{*b}$	0.001	$55.8717 \pm 0.4462^{*a}$
Red palm olein ^a	171	0.017	542.09
Palm olein ^a	218	0.022	0
Corn oil ^a	254	0.025	0.91
Coconut oil ^a	0	0	0

Abbreviations: α -T, α -tocopherol ; RPNO, Roasted pili nut oil ; UPNO, Unroasted pili nut oil. Note: *Different alphabet showed there are significant difference ($p < 0.05$) between samples.

^a Dauqan *et al.* (2011)

Table 2. Total Phenol Content (TPC) and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH[•]) of roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO)

	RPNO	UPNO
	Mean \pm SD	Mean \pm SD
TPC (mg gallic acid equivalent)	12.43 ± 0.69^b	19.96 ± 0.52^a
DPPH (% radical scavenging)	24.49 ± 1.02^a	9.52 ± 0.78^b

Note: *Different alphabet showed there are significant difference ($p < 0.05$) between samples

Table 3. Colour differences between roasted pili nut, unroasted pili nut, roasted pili nut oil and unroasted pili nut oil

Sample	L*	a*	b*
Roasted pili nut	66.87 ± 25.24^a	3.28 ± 0.13^a	22.35 ± 1.16^a
Unroasted pili nut	68.04 ± 1.20^a	1.77 ± 0.19^b	21.52 ± 0.47^a
Roasted pili nut oil	40.82 ± 0.02^b	5.50 ± 0.01^a	-4.71 ± 0.02^b
Unroasted pili nut oil	41.93 ± 0.13^a	4.92 ± 0.01^b	-4.63 ± 0.02^a

Total antioxidant capacity reflects presence of naturally occurring and neo-formed antioxidant constituents in oils obtained from either roasted or raw pili nut seeds. The results are summarized in Table 2. There were a significantly different on the percentage of radical scavenging between RPNO and UPNO. The result shows that roasting caused a clear increase in antioxidant activity that was measured by DPPH. In this study, the DPPH scavenging activity was only observed in the phenolic extract of RPNO and UPNO at concentration of $140 \mu\text{g/ml}$. The IC_{50} values were not determined for the phenolic extract of RPNO and UPNO as none were potent enough to scavenge more than 50% of the DPPH radical. According to Table 2, RPNO and UPNO scavenged DPPH radicals by 24.66% and 9.52% at concentration of $140 \mu\text{g/ml}$ respectively. According to Durmaz *et al.* (2010), the increase of antioxidant activity in methanolic extract of oils with roasting was reported earlier. This increase is mainly related to the relatively polar compounds in oil that were accumulated during roasting (Durmaz and Gökmen, 2011) and might be because of the MRPs that formed during the roasting process (Durmaz *et al.*, 2010).

According to Eichner (1981), one of the main desired outcomes of roasting process is the increase in antioxidant activity that occurs mainly due to the formation of Maillard reaction products (MRPs) and this reaction takes place in oily seeds by the interaction of carbonyls with amines during thermal processing. Meanwhile, Acar *et al.* (2009) stated that beside the formation of MRPs, roasting may change

Table 4. Inhibitory effect of roasted pili nut oil (RPNO) and unroasted pili nut oil (UPNO) on few strains of microorganisms

Strains	ATCC	RPNO	UPNO	Gentamycin	Ampicillin	Chloramphenicol
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
<i>Klebsiella pneumoniae</i>	13883	8.33 \pm 1.53 ^d	8.33 \pm 0.58 ^d	22.67 \pm 2.31 ^b	12.00 \pm 1.73 ^c	26.67 \pm 1.15 ^a
<i>Yersinia enterocolitica</i>	9610	0.00 ^d	0.00 ^d	25.33 \pm 2.52 ^b	22.00 \pm 1.00 ^c	30.00 \pm 2.00 ^a
<i>Salmonella typhimurium</i>	14028	9.00 \pm 1.00 ^d	9.00 \pm 1.00 ^d	20.33 \pm 1.53 ^c	26.67 \pm 1.53 ^b	29.33 \pm 1.15 ^a
<i>Salmonella choleraesuis</i>	14028	9.33 \pm 0.58 ^d	8.67 \pm 1.15 ^d	19.67 \pm 0.58 ^c	26.00 \pm 1.00 ^b	28.67 \pm 1.15 ^a
<i>Salmonella choleraesuis</i>	14024	9.33 \pm 1.15 ^d	9.00 \pm 1.00 ^d	21.67 \pm 1.15 ^c	25.33 \pm 2.52 ^b	28.67 \pm 1.53 ^a
<i>Staphylococcus aureus</i>	29178	0.00 ^d	0.00 ^d	23.33 \pm 0.58 ^a	21.67 \pm 0.58 ^b	14.67 \pm 0.58 ^c
<i>Staphylococcus aureus</i>	29213	0.00 ^d	0.00 ^d	25.33 \pm 0.58 ^b	23.33 \pm 0.58 ^c	30.33 \pm 0.58 ^a
<i>Staphylococcus aureus</i>	33591	0.00 ^d	0.00 ^d	26.33 \pm 1.53 ^a	12.00 \pm 1.00 ^c	16.00 \pm 3.00 ^b
<i>Staphylococcus aureus</i>	3862	0.00 ^d	0.00 ^d	26.00 \pm 1.00 ^c	36.33 \pm 1.53 ^a	29.33 \pm 0.58 ^b
<i>Staphylococcus epidermidis</i>	12228	9.00 \pm 0.00 ^c	8.67 \pm 0.58 ^c	30.67 \pm 1.15 ^a	22.67 \pm 0.58 ^b	0.00 ^d
<i>Listeria monocytogenes</i>	7644	0.00 ^b	0.00 ^b	21.00 \pm 2.65 ^a	0.00 ^b	0.00 ^b
<i>Serratia marcescens</i>	8100	9.00 \pm 0.00 ^b	8.00 \pm 0.00 ^c	23.33 \pm 0.58 ^a	7.67 \pm 0.58 ^c	23.33 \pm 0.58 ^a
<i>Bacillus subtilis</i>	6051	0.00 ^c	0.00 ^c	33.67 \pm 1.53 ^a	31.67 \pm 1.53 ^{ab}	31.00 \pm 1.00 ^b
<i>Bacillus cereus</i>	14579	8.67 \pm 1.53 ^c	8.33 \pm 0.58 ^c	23.67 \pm 1.53 ^b	8.00 \pm 1.00 ^c	26.67 \pm 2.31 ^a
<i>Bacillus cereus</i>	14570	11.00 \pm 1.00 ^c	9.67 \pm 0.58 ^c	25.00 \pm 0.00 ^b	10.33 \pm 0.58 ^c	28.00 \pm 1.00 ^a
<i>Escherichia coli</i>	8739	9.67 \pm 0.58 ^d	9.67 \pm 0.58 ^d	23.33 \pm 2.08 ^b	21.00 \pm 1.00 ^c	27.00 \pm 1.73 ^a
<i>Escherichia coli</i>	25922	8.00 \pm 0.00 ^d	8.33 \pm 0.58 ^d	22.33 \pm 2.08 ^b	18.67 \pm 1.15 ^c	29.33 \pm 1.15 ^a
<i>Escherichia coli</i>	35218	8.33 \pm 0.58 ^b	8.67 \pm 0.58 ^b	20.67 \pm 1.15 ^a	0.00 ^c	0.00 ^c
<i>Enterobacter cloacae</i>	13047	9.00 \pm 0.00 ^c	9.00 \pm 0.00 ^c	19.33 \pm 0.58 ^b	0.00 ^d	28.67 \pm 1.15 ^a
<i>Enterobacter aerogenes</i>	13048	9.67 \pm 0.58 ^d	9.00 \pm 0.00 ^d	23.33 \pm 1.53 ^b	15.67 \pm 0.58 ^c	27.00 \pm 1.00 ^a
<i>Pseudomonas aeruginosa</i>	10145	8.00 \pm 0.00 ^c	8.00 \pm 0.00 ^c	20.00 \pm 0.00 ^a	0.00 ^d	13.33 ^b

^{abcd} Means with same letter are not significantly different.

No antimicrobial activity, inhibition zone <1. Weakly antimicrobial activity, inhibition zone 1mm. Slight antimicrobial activity, inhibition zone 2 - 3 mm. Moderate antimicrobial activity, inhibition zone 4 - 5 mm. High antimicrobial activity, inhibition zone 6 - 9 mm. Strong antimicrobial activity, inhibition zone > 9 mm. (Rodríguez Vaquero *et al.*, 2007)

Table 5. IC₅₀ values (as %,v/v) for roasted pili nut oil (RPNO), unroasted pili nut oil (UPNO) and baseline standards following an incubation period of 72 hours using HeLa, MCF-7 and HT-29 cell lines

Sample	*IC ₅₀ value (μ g/ml)			
	Cell lines	HeLa	MCF-7	HT-29
Roasted pili nut oil		>30	>30	>30
Unroasted pili nut oil		>30	>30	>30

*Indicates estimated IC₅₀ values extrapolated graphically due to absorbance values below test limit.

the total antioxidant capacity of oils by increasing or decreasing the passage of naturally occurring antioxidant phytochemicals to the oil. The net effect of roasting on the total antioxidant capacity of the oil seeds depends on the balance between the thermal degradation of naturally occurring antioxidant compounds and the formation of new MRPs having antioxidant capacity. Furthermore, according to Chiou and Tsai (1989), higher antioxidant concentration in oils extracted from roasted seeds compared to unroasted ones is generally attributed to the cellular deformations which take place during roasting process which facilitates extractability of those phytochemicals along with the oil. In addition, Wijesundera *et al.* (2008) and Kim *et al.* (2002) also reported that phenolic compounds and tocopherols are extracted well if the oil is obtained from a roasted material. From the result in Table 2, the total phenolic compound (TPC) in RPNO and UPNO were about 12.43 \pm 0.69 mg/kg and 19.96 \pm 0.52 mg/kg respectively. The total phenol content of UPNO was higher than RPNO. This result was in agreement with the findings of Durmaz and Gökmen (2011), where

further roasting caused a fluctuation and no more increase was observed in TPC.

According to Table 3, there was a clear decrease of L values in roasted pili nut and roasted pili nut oil. While the L values of unroasted pili nut and unroasted pili nut oil were 68.04 \pm 1.20 and 41.93 \pm 0.13 respectively. On the other hand, a value was significantly changed of roasting both for the nut and the extracted oil. In this study, pili nut oil that extracted from roasted pili nut was reported to have a darker colour compared to the oil from unroasted pili nut. This is most probably because of the passage of the MRPs formed during roasting (Kim *et al.*, 2002).

The antimicrobial and toxicological properties of RPNO and UPNO were also investigated. It was observed that bacteria species exhibited different sensitivities towards RPNO, UPNO, Gentamycin, Ampicillin and Chloramphenicol (Table 4). *Bacillus cereus* 14570 was the most sensitive bacterium and all strains of *Staphylococcus aureus* tested were resistant against both samples RPNO and UPNO. An *in vitro* toxicological study based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay was also performed. *In vitro* cytotoxicity indicated that both RPNO and UPNO had no effect against HeLa (cervical cancer cell), MCF-7 (breast cancer cell) and HT-29 (human colon adenocarcinoma cell) cell lines tested (Table 5). The cytotoxicity IC₅₀ (50% inhibitory concentration) values of both samples towards these three cell lines tested were more than 30 (μ g/ml) after 72 hrs of treatment.

Conclusion

As a conclusion, the result showed that RPNO contained high amount of vitamin E (α -tocopherol), high percentage of radical scavenging and less amount of beta-carotene compared to UPNO. The result also revealed that heat pretreatment of pili nut may be significantly modify the composition of oil extracted from this seed and both samples can be considered as a good sources of natural antioxidant. RPNO and UPNO failed to exhibit any antibacterial against all strains of *Staphylococcus aureus* tested. RPNO and UPNO were also found did not inhibit or no effect towards HeLa, MCF-7 and HT-29 cell lines tested, respectively, with the IC_{50} value more than 30 μ g/ml after 72 h treatment.

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