The phytochemical content and antimicrobial activities of Malaysian *Calophyllum canum* (stem bark)

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Abstract: Recently there was huge increase in using of 'herbal products'. These can be defined as plants, parts of plants or extracts from plants that are used for curing disease. However, *Calophyllum* species is a tropical plant and it has been used in traditional medicine, the limitation in safety and effectiveness information could lead to serious health problems. Providing information for communities by evaluating the phytochemical contents, antioxidant, antimicrobial and cytotoxic activities will improve the therapeutic values. Three main *Calophyllum canum* fractions (none – high polar) were tested to find out the phenolic, flavonoid, flavonol content, DPPH radical scavenging, reducing power and chelating iron ions. Also were tested against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Psedomonas aeruginosa, Candida albicans,* and *Cryptococcus neoformans*. In addition, cytotoxic activity was assayed against lung cancer *A549* cell line. The methanol fraction showed no bioactivity but achieved the highest amount of phenolic, flavonol and flavonoid contents, also it showed a significant result as antioxidant, reducing power and chelating agent. The n-hexane fraction achieved the minimum inhibitory concentration (MIC) value 12.5 μg. mL^{-1} against *B. cereus* while the MIC value for DCM fraction was 25 μg. mL^{-1}. The DCM fraction was more active against *S. aureus* where the result was 50 μg. mL^{-1} while the n-hexane fraction was 100 μg. mL^{-1}. The three main fractions have shown no activity against gram negative bacterial and fungal. The n-hexane and DCM fractions have shown cytotoxicity against lung cancer cell line; the 50 % inhibition concentration (IC_{50}) was 22 ± 2.64 and 32 ± 3.78 μg. mL^{-1} respectively. The results were statistically significant ($P < 0.05$). Among the results, *C. canum* fractions proved to be effective against gram positive bacterial and anti-proliferation activity. Also it showed antioxidant activity as well. The results provided beneficial information for communities as well as can help to search for alternative drugs, and will contribute to establish safe and effective use of phytomedicines in the treatment of diseases.

Keyword: *Calophyllum canum*; phytochemistry; antioxidant; antimicrobial; antiproliferative (cytotoxicity).

INTRODUCTION

The genus *Calophyllum* belongs to *Clusiaceae / Guttiferae* family which consists of 180 – 200 different species distributed in the warm humid tropics of the world, also it is available in Malaysia. Wide phytochemical studies have reported that *Calophyllum* genus rich in xanthones, coumarins, biflavonoids, chalcones, benzofurans and triterpenes (Pretto *et al*., 2004; Taher *et al*., 2005). A few studies have improved the bioactivity of *Calophyllum* genus. Chen and his co-author have showed that *C. lanigerum Miq*. and *C. inophyllum L* have strong activity against human immunodeficiency virus type 1 (HIV-1) (Chen *et al*., 2008). Guilet and Li have reported that triterpenoids, coumarins and the mammea coumarens which have isolated from *C. inophyllum*, *C. dispar* and *C. brasiliens,* have shown a cytotoxicity effect against human *leukemia HL-60*, *Nasopharynx carcinoma KB* and *K562 Lymphoma, U251 central nervous system* and *PC3 Prostata* cell lines respectively (Guilet *et al*., 2001; Li *et al*., 2010). On the other hand Reyes-Chilpa has reported that *Calophyllum* genus able to inhibit the growth of *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis* (Reyes-Chilpa *et al*., 2004). In addition, the extract from *C. brasiliens* showed activity against yeast (Albernaz *et al*., 2010). Some of *Calophyllum* species are commonly employed in folk medicine in tropics area, however it is used to treat bronchitis, gastric, hepatic disturbances, pain, inflammation, diabetes, hypertension, diarrhea, herpes, rheumatism, varicose, hemorrhoids and chronic ulcer (Pretto *et al*., 2004), also treating toothache, preventing wound infections and lumbago (Cottiglia *et al*., 2004; Zou *et al*., 2005). As mentioned *Calophyllum* species were used traditionally very often for treating many diseases. The *C. canum* species provides no information about its activity and it may be used indiscriminately, and not scientifically

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proven in terms of effectiveness or toxicity. Inadequacy of previous scientific studies on the nature of *C. canum* prompted us to evaluate the biological activity in terms of phytochemical, antioxidant, antimicrobial, and anti-proliferative (cytotoxicity) by using *in vitro* model of relevance.

MATERIALS AND METHODS

Chemicals

All solvents used were of analytical grade, n-hexane, dichloromethane (DCM) and Methanol, also silica gel 60 (230-400 mesh), Sodium acetate, and Aluminium chloride was obtained from MERCK. Trichloroacetic acids, potassium hexacyano ferrate, potassium phosphate, ferrous chloride, ferric chloride, ascorbic acid, BHT (Butylated hydroxytoluene) were purchased from R & M chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid 1-Na x H_2O were purchased from Sigma-Aldrich Chemical. Gallic acid obtained from Alfa Aesar. The media were used in this study for bacteria, Mueller-Hinton broth medium (MHB) used in MIC method, and Mueller-Hinton agar used in disc diffusion method. For fungus the potato dextrose agar was used for disc diffusion method. Chloramphenicol, and Amphotericin B a standard antibiotic used to compare with for bacteria and fungus respectively.

Plant material

The stem bark of *C. canum* was collected from Bukit Pelindung Kuantan, Malaysia in June, 2009, with a herbarium specimen (No MT-01). The specie was identified by Dr Shamsul Khamis, botanist from University Putra Malaysia.

Extraction and fractionation

Air-dried and powdered steam bark (1 kg) was macerated in (2.5 L) 98 % of ethanol (EtOH) for 72 hours. The EtOH extract was filtrated and evaporated under reduce pressure to yield the dark brown gummy (200 g) EtOH crude in order to use for fractionation.

A part of EtOH crude (100 g) was fractionated by using vacuum liquid chromatography (VLC) method (silica gel 230-400 mesh, in the ratio 1:30). Three main fractions n-hexane, dichloromethane (DCM) and methanol (MeOH) were obtained.

Phytochemical screening

Determination the phenolic content

Total phenolic content was carried out regarding to the Folin-Ciocalteau's method (Su *et al*., 2009) with slight modifications. In brief, 0.5 mL of sample (10 mg. mL^{-1}) was mixed with 5.0 ml of Folin-Ciocalteau's reagent (1:10). The mixture was settled in a tube for 5 min and then 4.0 mL of 1 M Na_2CO_3 was added. After incubation at room temperature for 15 min, a 100 µL of the mixture was moved to 96-microwell plat. The absorbance was measured at 765 nm by using multi-detection micro-plate reader (INFINITE M 200 NANOQUANT). The sample was tested in triplicate and the results explained ± standard deviation. Calibration curve with five data points for Galic acid was obtained. The results were compared to a Galic acid calibration curve and the total phenolic content of fractions was expressed as µg of Galic acid equivalents per 10 mg of extract.

($Y= 0.0507 X – 0.011$) and $R^2 = 0.9937$

Determination the total flavonoid

Total flavonoid contents were determined using the method of (Adedapo *et al*., 2008) with slight modification. A volume of 0.1 mL of sample was placed in a 96-microwell plate and mixed with 0.1 mL (2%) $AlCl_3$ ethanol solution. The mixture was incubated at room temperature for 1 hour, after the absorbance was measured at 420 nm using the multi-detection micro-plate reader (INFINITE M 200 NANOQUANT). The yellow colour indicated flavonoids existence. The three main fractions were evaluated at final concentration of 10 mg. mL^{-1} in triplicate and the results explained ± standard diveation. The blank prepared by mix the sample with ethanol. Total flavonoid content was calculated as quercetin equivalent (µg Que. 10 mg^{-1} extract) using the following equation based on calibration curve:

$Y= 0.0174 X – 0.048$, $R^2 = 0.9976$.

Determination the total flavonol

Total flavonols were estimated by using the method of (Adedapo *et al*., 2008) with slight modification, in brief 50 µL of sample or standard was mixed with 50 µL of (2 %) $AlCl_3$ ethanol solution and 75 µL of (50 g. L^{-1}) sodium acetate solution, the mixture was seated in a 96-microwell plate and it was incubated for 2.30 hour at room temperature. The absorption measured at 440 nm by using the multi-detection micro-plate reader (INFINITE M 200 MAMOQUANT). The main fractions were evaluated at a final concentration of 10 mg. mL^{-1}, where the blank was prepared by mixing the sample with ethanol. Total flavonoid content was calculated as quercetin equivalent (µg Que. 10 mg^{-1} extract) using the following equation based on the calibration curve:

$Y = 0.0097 X + 0.055$, $R^2 = 0.9994$.

Antioxidant activity

DPPH assay

The scavenging effect of *C. canum* fractions was assessed by using the method of (Amarowicz *et al*., 2010) with slight modifications. A 100 µL of methanolic solution containing between 0.031-1 mg of the fractions was mixed with (200 µL) methanolic solution of DPPH (1 mM) in a 96-microwell plate. The content was mixed and left in a dark area at room temperature for 20 min, and then the absorbance of the mixture was measured at 517

nm by using multi-detection micro-plate reader (INFINITE M 200 NANOQUANT). The control was prepared by mixing methanol instead of the fractions. The blank was 100 µL of fraction with 200 µL of methanol. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the equation 1.

% Radical scavenging = ((COD – SOD)/COD)*100 (1)

Where COD optical density for control and SOD optical density for sample.

The Required fraction's Concentration (RC_{50}, µg. mL^{-1}) for scavenging of 50% of DPPH radical was determined. All measurements were curried out in triplicate.

Reducing power assay

The reducing power of *C. canum* fractions and two standards ascorbic acid and BHT was determined according to the method of (Öztürk *et al*., 2007) with slight modification in volume of samples and reagents. A 100 µL of three different concentrations 1, 5 and 10 mg. mL^{-1} of each fraction were mixed with 50 µL of (0.2 M) phosphate buffer (pH 6.6) and 250 µL of potassium ferricyanide (1 %). After the mixture was incubated at 50 °C for 20 min, 250 µL of (10 %) trichloroacetic acid were added and the mixture was centrifuged at 3000 rpm for 10 min. 250 µL supernatant was moved to 48-microwells plate and mixed with 250 µL distilled water and 50 µL of ferric chloride (0.1 %). The mixture was measured at 700 nm by using the multi-detection micro-plate reader (INFINITE M 200 NANOQUANT). All the measurements were carried out in triplicates. However, higher absorbance of the reaction mixture indicates greater reducing power.

Iron (II) chelating activity

The chelation of iron (II) ions by different fractions concentrations was curried out by the method described in (Hinneburg *et al*., 2006). 100 µL of four different concentrations 1, 2.5, 5 and 10 mg. mL^{-1} of each fraction, which dissolved in methanol, was mixed with 10 µL of (2.0 mM) aqueous $FeCl_2$. After 5 min incubation at room temperature, the reaction was initiated by 20 µL of (5.0 mM) ferrozine. After 10 min the absorbance was measured at 562 nm by using the multi-detection micro-plate reader (INFINITE M 200 Nanoquant). The control was contained all the reaction reagents except the sample which was replaced by methanol. The iron chelating activities were calculated from the equation 2.

% chelation = ((Ac-As)/Ac)*100 (2)

(Ac) represented the absorbance for control and (As) the absorbance for sample. The values were presented as the means of triplicate analyses ± standard deviation.

Anti microbial assays

Microbial strains

Six reference microbial strains of human pathogens were used for the anti-microbial activity. The two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778), and two Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218) were used for antibacterial test. Also two fungal strains (*Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 90112) were used for antifungal test.

Disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activities of the EtOH crude according to (Qaralleh *et al*., 2010). In brief, inoculums containing 10^6 - 10^7 CFU. mL^{-1} bacteria strain was spread upon Mueller-Hinton agar plate surface and 10^4 CFU. mL^{-1} fungus strain was spread upon potato dextrose agar plate surface. The sterilised forceps was used to place sterile (6 mm diameter) filter papers on the top of agar. The filter papers were loaded with crude extract (10 or 20 µg) and tow standard antibiotics (chloramphenicol 30 µg and amphotericin B 100 µg) for comparing bacteria and fungus respectively as well as negative control (DMSO). The loaded filter papers were laid down on the surface of inoculated agar plate. The plates were incubated at 37 °C for 24 hour for bacteria and at room temperature (18 - 20 °C) for 24 - 48 hour for yeast strains. Each sample was tested in triplicate and the zone of inhibition was measured as millimetre diameter.

Microdilution method (MIC and MBC)

Minimal inhibitory concentration (MIC) was measured by determining the smallest amount of fractions (n-hexane, DCM and MeOH) or standard antibiotic needed to inhibit the growth of a test microorganism. This was done using 96-microwell plates and performed on (Versa MaxTM Tunable) micro-plate reader. In brief, the plates were filled with Mueller-Hinton broth medium containing different concentrations of the n-hexane, DCM or MeOH fractions for testing. Also Chloramphenicol standard and solvent control were applied for comparing the result with. The test micro-organism (10^6 -10^7 CFU. mL^{-1}) were added to all wells. After 24 hour incubation periods at 37°C, the turbidity was measured at 600 nm.

Minimal bactericidal concentration (MBC) was determined by transferring and spreading the treated culture broth of the wells containing the concentrations equal to and higher than the MIC on agar plates. The lowest concentration of the fractions or the standard antibiotic required to completely destroy test microorganisms (no growth on the agar plate) after incubation at 37°C for 24 hour was reported as Minimum bactericidal concentration (MBC).

The cytotoxicity assay (MTT assay)

Anti-proliferative effects of *C. canum* fractions against *A549* cell line was investigated by using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay)] described by (Susanti *et al*., 2007). For this purpose, *A549* cells were cultured in a completed (DMEM) media in a T-flask until the cells were confluent. Then, the cells were seeded in a 96-microwell plate at a density of 0.5×10^5 cells per well and incubated at 37°C in 5% CO_2 humidified incubator. After 24 hour a fresh media was added and the cells were treated with different concentrations of samples obtained by double fold serial dilution. The (95% ethanol) was used as control. After 24 hours incubation, the supernatants were discarded, and the adherent cells were washed twice with phosphate buffer saline (PBS). 20 µL of (5 mg. mL^{-1}) MTT stock solution was added to each well and the plate was further incubated overnight at 37°C. A 100 µL DMSO was added to dissolve the water insoluble purple formazan crystals produced by the viable cells. When formazan completed dissolve a 100 µL was transferred to a new 96-microwell plate and the absorbance was measured at 570 nm and reference at 690 nm, using multi-detection microplate reader (INFINITE M 200 NANOQUANT).

All samples were assayed in triplicate. The percentage of cell viability was calculated and the concentrations required for inhibition of 50% of cell viability (IC_{50}) were determined according to equation 3, where ODT optical density for treated cells, ODC optical density for control.

% of cell viability = (ODT / ODC)*100 (3)

RESULTS

Phytochemical content

The phytochemical analysis was curried out in this study to investigate the total phenolic, flavonoid and flavonol content in three main fractions of *C. canum*. Table 1 shows the contents value for each fraction, it is clearly appearing that MeOH fraction contains the highest amount of polar phytochemicals, where another fractions have less amount. The DCM fraction has a significant amount of flavonoid content.

Antioxidant activity

DPPH assay

The result display in table 2 shows the RC_{50} of free radical scavenging activity, which was curried out by using the DPPH assay. The MeOH fraction shows a good result as an antioxidant, while n-hexane and DCM fractions show a moderate activity. Fig.1 depicts the % of radical scavenging activity versus concentrations. The n-hexane fraction curve turned to steady after 125 µg. mL^{-1}, while the DCM fraction curve turned to steady after 250 µg. mL^{-1}. MeOH fraction became steady after 62.5 µg. mL^{-1} and the RC_{50} is much less than 31.25 µg. mL^{-1}.

Table 2: RC_{50} free radical scavenging activity

Fractions	RC_{50} (µg. mL^{-1})
n-hexane	85 ± 4.35
DCM	91.6 ± 6.65
MeOH	< 31.25

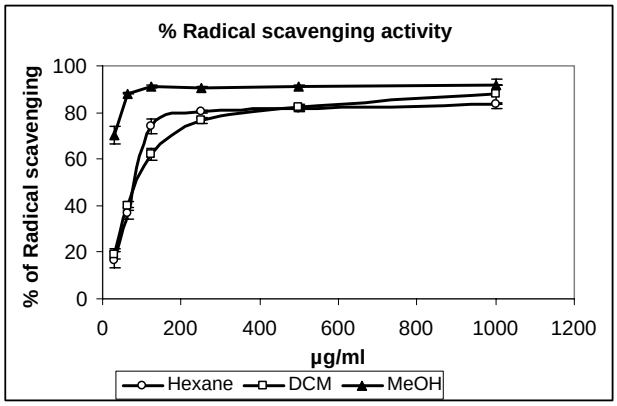

Fig. 1: Free radical scavenging activity of *C. canum* fractions n-hexane, DCM, and MeOH by DPPH assay.

Reducing power

The reducing power test was conducted to evaluate the *C. canum* fractions ability to reduction. Fig. 2 depicts the reducing power value of *C. canum* fractions n-hexane, DCM, MeOH and standards. The n-hexane and DCM fractions show low reducing power activity, where the values are less than BHT the moderate standard. The MeOH fraction shows reducing power value is closer to BHT the moderate standard. The strongest reducing power is ascorbic acid where there are no *C. canum* fractions get closer to.

Table 1: The fractions of *C. canum* used in screening the phytochemistry content

Fractions	Phenolic content*	Flavonoid content◊	Flavonol content◊
n-hexane	1.292 ± 0.001	5.040 ± 0.184	1.202 ± 0.547
DCM	1.992 ± 0.002	11.201 ± 2.446	2.164 ± 0.415
MeOH	3.517 ± 0.017	14.643 ± 1.222	11.639 ± 2.479

*µg Galic Acid/10 mg extract, ◊ µg quercetin/10 mg extract.

Iron (II) chelating activity

The chelation of ferrous ions by the *C. canum* fractions and standard was examined by the method of (Hinneburg *et al*., 2006). Fig. 3 shows two standard Quercetin moderate and EDTA high chelating agent. All fractions were comparing with standard; the n-hexane and DCM show activities less than Quercetin at all concentration, while the MeOH fraction shows dramatically activity, it chelates the iron at 2.5-5 mg. mL^{-1} as the Quercetin does, also it chelates iron at 10 mg. mL^{-1} to reach activity as EDTA. The iron chelating activity result for MeOH fraction was directly proportion with concentration.

Antimicrobial assay

The antibacterial and antifungal activities of EtOH crude using disc diffusion method are summarized in table 3. The *C. canum* crude extract was screened at concentrations of 10 and 20 µg. $disc^{-1}$ against six human pathogens. Both gram negative and fungal strains appear to be resistant to the tested concentration since no inhibition zone was observed while the gram positive bacteria showed moderate sensitivity with no significant difference between the concentrations. Therefore the MIC and MBC were determined using different polarity fractions (n-hexane, DCM and methanol) of *C. canum* against gram positive strains only (table 4). As a result, the MIC values of the tested fractions ranged from 12.5 - 100 µg. mL^{-1} whereas the MBC values were 100 or 250 µg. mL^{-1}. The inhibition growth of the microbes at concentration as low as 12.5 µg. mL^{-1}, was indicated to potential antimicrobial activity of *C. canum* fractions. Furthermore, the highest activity was obtained against *B. cereus* for the n-hexane fraction (MIC and MBC was 12.5 µg. mL^{-1} and 100 µg. mL^{-1}, respectively).

Table 3: Antimicrobial activity of ethanol crude extract using disc diffusion method

	Bacteria				Fungi	
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*C. albicans*	*C. neoformans*
EtOH Crude (10 µg. $disc^{-1}$)	11.5 ± 0.7	9.5± 0.7	0±0.0	0±0.0	0±0.0	0±0.0
EtOH Crude (20 µg. $disc^{-1}$)	12.5± 0.7	10.5 ±0.7	0±0.0	0±0.0	0±0.0	0±0.0
Chloramphenicol (30 µg)	24.5 ± 0.7	26 ± 0.5	33±.03	37 ± 0.4	ND	ND
Amphotericin B (100 µg)	ND	ND	ND	ND	20±0.7	19±0.4
Negative control	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0

Mean diameter of zone of inhibition in mm± standard deviation including the diameter of the disc 6 mm.
Negative control: 100% DMSO, 0: no inhibition zone, ND: Not Determined

Table 4: MIC and MBC for Chloramphenicol, n-hexane, DCM and MeOH fractions

Fraction	*S. aureus* (µg. mL^{-1})		*B. cereus* (µg. mL^{-1})	
	MIC	MBC	MIC	MBC
n-hexane	100	250	12.5	100
DCM	50	250	25	250
MeOH	>1000	ND	>1000	ND
Chloramphenicol	20	ND	20	ND

ND: Not Determined

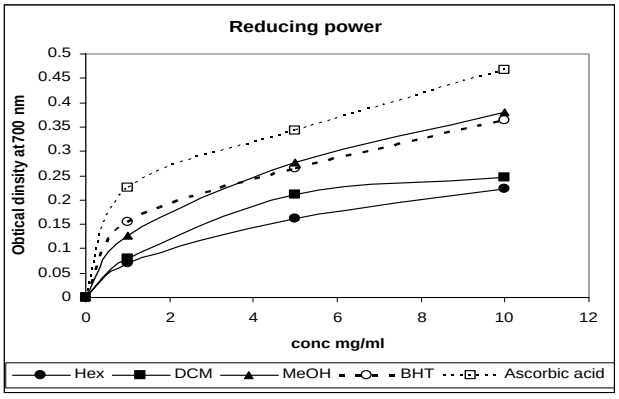

Fig. 2: The reducing power activity of three *C. canum* fractions n-hexane, DCM and MeOH as well as standard BHT and Ascorbic acid at 700 nm.

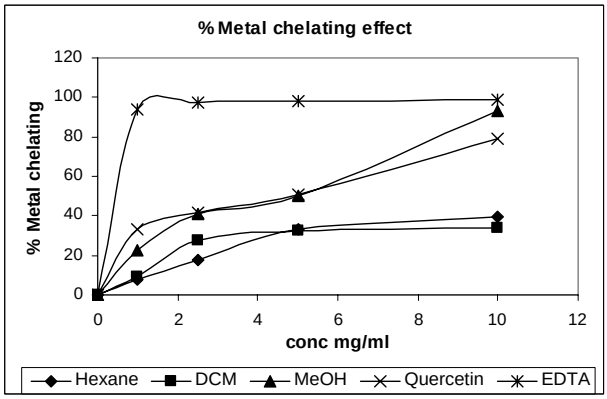

Fig. 3: The % of Iron chelating activity for three *C. canum* fractions at four concentrations (0, 1, 2.5, 5, 10 mg. mL^{-1}).

Cytotoxicity (MTT assay)

The anticancer activity of the *C. canum* fractions was investigated using MTT assay on human lung cancer cell line *A549*. A mitochondrial enzyme in living cells can succinate dehydrogenase and cleaves the tetrazolium ring converting the MTT to an insoluble purple formazan. Fig. 4 shows the % of viability where it display the activity of n-hexane, DCM and MeOH fractions. The n-hexane fraction shows a good activity, where it is inhibiting the cell growth at low concentration. The DCM fraction shows a good activity but it is less than n-hexane. The MeOH fraction shows no activity where the growth is 100 %.

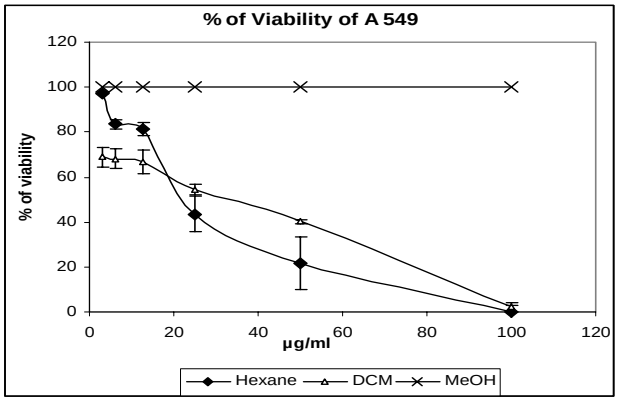

Fig. 4: The % of viability activity for *C. canum* fractions against *A549* cell line.

The criteria of cytotoxicity activity for the crude extract, as established by the American National Cancer Institute (NCI) is an IC_{50} < 30 µg. mL^{-1} in the preliminary assay (Itharat *et al*., 2004).The IC_{50} values for n-hexane and DCM fractions are 22 and 32 µg. mL^{-1} respectively. The % of viable cell at concentration 30 µg. mL^{-1} as recommended by NCI for n-hexane and DCM fractions is 35 and 51 % respectively. These indicate to the efficacy of n-hexane at low concentration as it is illustrating in table 5.

Table 5: The IC_{50} and % of viability of *C.canum* fractions

Fractions	IC_{50} µg. mL^{-1}	% of viability◊
n-hexane	22 ± 2.64	35
DCM	32 ± 3.78	51
MeOH	N.d	N.d

N.d: Not determined, ◊:% of viability at 30 µg. mL^{-1}

DISCUSSION

Phenolic compounds are most abundant secondary metabolites in the plants. Additionally, it has been found in many food and their derivatives (Velioglu *et al*., 1998). Flavonols and flavonoids are a part of phenolic compounds and they are sharing in the physical properties. Phenolic compounds are known as powerful chain breaking antioxidants (Shahidi and Wanasundara, 1992). Also it is very important constituents of plants and their radical scavenging ability is due to their hydroxyl groups (Hatano *et al*., 1989). The phenolic compounds my contribute directly to anti-oxidative action (Duh *et al*., 1999). In various studies, antioxidant activity of the plant extracts which are rich in phenolic compounds was found to be fairly high (Cakir *et al*., 2003). The chemical complexity of extracts often a mixture of dozens of compounds, with different functional groups could lead to various values, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary. There are several methods using for determining of the antioxidant activity. In this study, mainly three methods were curried out, DPPH radical scavenging activity, metal chelation activity and ferric reducing power. The concentrations of total phenolic, flavonol and flavonoids were also calculated for the *C. canum* fractions. The DPPH results show that n-hexane and DCM fractions were statistically not significant ($P \geq 0.05$) in the range of concentration (31.25 - 1000 µg. mL^{-1}) except at concentration (125 and 250 µg. mL^{-1}) the result was statistically significant ($P < 0.05$). In addition, the MeOH fraction shows a significant result even at low concentration. The other fractions were compared with MeOH fraction and the results were statistically extremely significant at ($P < 0.001$), except at concentration 62.5 µg. mL^{-1} for n-hexane and 250 µg. mL^{-1} for DCM was just significant at ($P < 0.05$). The radical scavenging activity increases with increasing the amount of the fractions.

Free radicals have a significant effect on oxidation of unsaturated lipids (Kaur and Perkins, 1991) the DPPH radical scavenging was used as a stable free radical to determine antioxidant activity of natural compounds (Shimada *et al*., 1992). The method is based on the reduction of alcoholic DPPH solution in presence of a hydrogen-donation antioxidant due to the formation of the non-radical DPPH-H. The reducing power assay was curried out as a second antioxidant test, the yellow colour which was observed of the tested solution, was turned to various shades of green and blue colour, which was indicated reducing power of each extract existence. The abundance of reducing substances (i.e. antioxidants) in the fractions will lead to reduction the Fe^{+3} ferricyanide complex to the ferrous form. Therefore, the Fe^{+2} can be examined by measuring the formation of *Perls'* Prussian blue at 700 nm (Chung *et al*., 2002). In other words, the $FeCl_3/K_3Fe(CN)^6$ system offers a sensitive method for the "semi-quantitative" determination of dilution concentration of polyphenolics, which share in redox reaction. The reducing powers of n-hexane and DCM fractions were showed a weak activity, they were statistically significant ($P < 0.05$) comparing with BHT and ascorbic acid. While the MeOH fraction was showed a moderate activity and

statistically was not significant ($P \geq 0.05$) comparing with BHT, this result was predicted where normally methanol fraction contains the high polar components which normally contain (-OH) groups. The high phenolics or polyphenolics content come into view to function as good electron and hydrogen-atom donors and therefore should be able to terminate radical chain reaction by converting free radicals to more stable products. The data for all fractions from this assay, correlated well with the content of total phenolics, and regarding to the chemical roll "like dissolve like", these results are going logically with.

Among the transition metals, iron is known as lipid oxidation pro-oxidant. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton Reaction.

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\bullet}$$

Ferric ion also produces radical from peroxides although, the rate is 10-fold less than ferrous (Halliwell and Gutteridge, 1984; Miller, 1996). The n-hexane and DCM fractions have shown a weak chelating activity comparing with Quercetin. The MeOH fraction has shown activity similar to Quercetin at concentration $\leq$ 5 mg. mL^{-1} and statistically was not significant ($P \geq 0.05$). Also, it has shown a good activity like EDTA when the concentration was raised up to 10 mg. mL^{-1} and statistically was not significant ($P \geq 0.05$) comparing with EDTA. MeOH fraction was showed the highest metal chelating activity among all the fractions have studied, which has comparable results with EDTA.

Nowadays, many biological activities have been evaluated for numerous species of plants. This demonstrates that compounds from medicinal plants are indeed useful as alternative therapy, either directly or as models for new synthetic substances (Houghton, 2000). However, the use of these substances of plant origin is not always monitored by health professionals, which would ensure efficacy and safety procedures, and can lead to absence of biological effects or even to toxic effects (Rates, 2001). In this study, no activity was observed against Gram-negative bacterial and yeasts. This is in agreement with many antimicrobial studies, which studied the antimicrobial activity of some species belong to *Calophyllum* genus. (Ha *et al*., 2009) reported that *C. inophyllum* extracts demonstrated promising antibacterial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* while the gram negative *Pseudomonas aeruginosa* was resistant to this extracts. Pretto and his co-authors (2004) showed that all the parts (roots, stems, leaves, flowers and fruits) of *C. brasiliense* exhibited antimicrobial activity against Gram-positive bacteria, and no activity was observed against Gram-negative bacteria and yeasts tested. On the other hand, this difference in susceptibility between the bacteria is related to the outer membrane of gram-negative bacteria. The outer membrane endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier (Nikaido and Nakae., 1979).

The search for anticancer agents from natural sources has been successful worldwide; active materials have been isolated and are nowadays used to treat human tumours. *Petiveria alliacea* has been reported to be utilized in treating patients with cancer and leukaemia in Cuba where promising results were obtained (Chirinos, 1992). The *paclitaxel* has been isolated from the pacific *yew* plant and it is used nowadays for treating the lung and breast cancer as well. The present study was undertaken to evaluate the cytotoxic activity of Malaysian *C. canum* which is never investigated before. Also it is used in the treatment of a few aliments and cancer related illnesses in the country.(Guilet *et al*., 2001) were isolated *Coumarin* compounds from *C. dispar* species and tested the cytotoxicity against human *Nasopharynx carcinoma KB* cell lines and they find out that *dispar* species inhibit the cell growth at low concentration. Also Zhi and his colleague showed that *C. inophyllum* species from china inhibited the growth of human *leukemia HL-60* cells by isolated triterpenoide (Li *et al*., 2010). The information based on the traditional medicinal use of plants has been one of the common useful ways of the discovery of biological activity compounds from plants (Cordell *et al*., 1991; Cragg and Newman, 1999). In this study, different fractions supposed to contain mainly high concentration of secondary metabolise were used. The result of the present study indicates the presence of cytotoxic activity in n-hexane and DCM fractions whereas the MeOH fraction showed no activity. The statistical analysis was showed a significant ($P < 0.05$) in the range of (3.06 - 12.5 µg. mL^{-1}) between n-hexane and DCM fractions where the result was not significant ($P \geq 0.05$) at the range (25-100 µg. mL^{-1}) for the same fractions. The n-hexane fraction was not significant ($P \geq 0.05$) at the range (3.06 - 12.5 µg. mL^{-1}), which it is mean that the n-hexane fraction has an anticancer properties and it might become a drug for anticancer with farther investigation. Misdiagnosis of cancer by traditional healers might explain the observed lack of correlation between the reported anticancer activities of plant extracts and their cytotoxic activity on the tested cell lines (Rosskopf *et al*., 1992). The promising result obtained from this plant curried us to go forward in progress to investigate the biological activity and provide the communities with scientific information about there traditional medicines.

CONCLUSION

C. canum fractions have showed a significant biological activity, regarding to the previous results it is possible to isolate phytochemicals employ as antioxidant or

antimicrobial. In term of cytotoxicity we can conclude that *C. canum* species contains an anti-proliferative agent, it may improve the therapeutics value. In general the results are provided beneficial information can help to search for alternative drugs. Further investigation is highly recommended in the level of pharmacology.

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