Chemical Characterization and Biological Activities of Methanol Extract From *Castanopsis megacarpa* Seeds of Sarawak

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ABSTRACT *Castanopsis* genus belongs to Fagaceae family consists of 120 species distributed throughout temperate and tropical forest of Asia. *Castanopsis megacarpa* can be found along river and hillside are widely distributed in Peninsular Malaysia and Borneo Island. *C. megacarpa* seeds were initially defatted using petroleum ether and then extracted using methanol. Non-polar and polar fractions of methanol extract were analysed on Gas Chromatography-Mass Spectrometry (GC-MS). Non-polar combined fractions (CF_a to CF_c) contained hydrocarbons and several siloxanes. GC-MS analysis on derivatised polar combined fractions (CF_d to CF_g) revealed several peaks which correspond to carbohydrates, alcohols and fatty acids. Methanol extract and CF_g sample showed antioxidant activities are high with low EC_{50} values of 205.10 and 158.97 mg/L, respectively. Brine shrimp lethality test showed methanol extract and polar combined fractions (CF_d-CF_g) are toxic with LC_{50} < 100 mg/L.

KEYWORDS: *Castanopsis megacarpa*; Gas Chromatography-Mass Spectrometry (GC-MS); derivatisation; antioxidant; toxicity.

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INTRODUCTION

Castanopsis megacarpa can be found along river and hillside areas and nuts were widely distributed at Peninsular Malaysia and Borneo with the characteristics of the nut is brown in colour and the shell is hard (Gamble, 1914). This species is known locally as Berangan can be eaten after it boiled or roasted. Seeds if consume can induce mild dizziness. Resin and leaf of *Castanopsis* can be used as traditional remedies to treat headache, stomach disorder and skin diseases (Dolai *et al.,* 2012). Several *Castanopsis* species were claimed to have anti-allergic, anti-ulcer and anti-virus properties (Pan *et al.,* 2014). Methanol extract of *C. indica* leaf possesed antitumor properties against Ehrlich ascites carcinoma cell (Dolai *et al.,* 2012). There are several studies on other *Castanopsis* species such as *C. indica* extract but information on *C. megacarpa* extracts is still scare. The objectives of this study were to assess the chemical constituents of *C. megacarpa* extracts and their fractions using gas chromatograph-mass spectrometry (GC-MS), and to evaluate the antioxidant properties and toxicity against brine shrimp of methanol crude extract and combined fractions from *C. megarcarpa* seeds. Polar combined fractions from methanol crude extract of *C. megacarpa* seeds were derivatized using N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). Both derivatised and underivatised combined fraction samples were analysed on GC-MS.

METHODOLOGY

Sample Collection

C. megacarpa seeds (Figure 1) were collected from Kampung Palas, Limbang of Sarawak. The seed kernels were ground into fine powder using a grinder mill Model FGR-350.

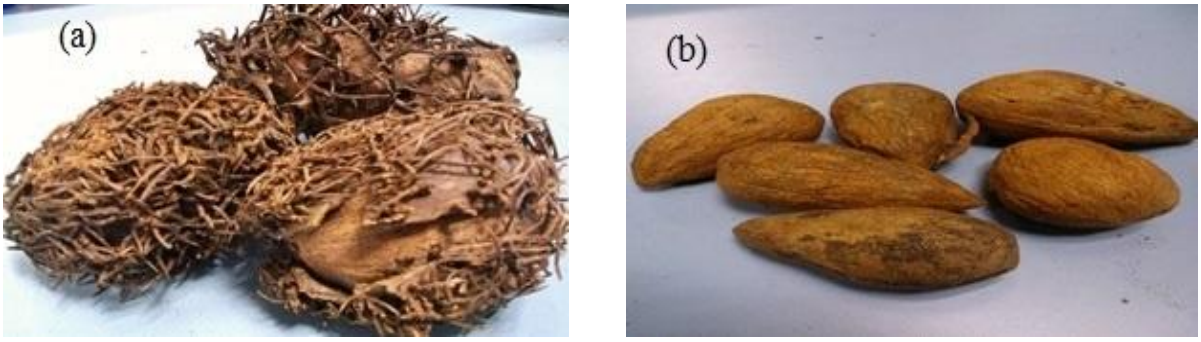

Figure 1: The seeds of *C. megacarpa* show (a) shells covered with cupules, and (b) hard shells.

Extraction and Fractionation of C. megacarpa Seeds

Extraction was carried out according to procedure outlined by Niranjan *et al.* (2013). Briefly, 440 g of powdered kernel was defatted using 2 L petroleum ether and filtered through a filter paper. Defatted sample was soaked for 3 days in 2 L methanol and then evaporated under reduced pressure in a rotary evaporator. Exactly 4.000 g of crude extract was fractionated on silica gel column chromatography. The column was successively eluted using 200 mL of hexane, dichloromethane, chloroform, ethyl acetate and methanol. Approximately 20 mL of each fractions were collected in the test tube. All fractions were analysed on TLC plate and fractions which showed similar TLC profile were then combined and evaporated.

Derivatisation of Combined Fractions

Derivatisation was performed on the polar combined fractions according to procedure as outlined by Bastidas (2013). Exactly 4.000 mg of sample was weighed and dissolved in 100 µL acetonitrile. Exactly 100 µL BSTFA was added to the sample and left at 60°C in an oven for 1 hour. The sample was then diluted with 1 mL dichloromethane before GC-MS analysis.

GC-MS Analysis

A Shimadzu gas chromatograph-mass spectrometer (GC-MS) QP-2010 Plus was used to analyse the combined fractions obtained from methanol crude extract. Separation was performed using a capillary BPX-5 column (30 m length x 0.25 mm internal diameter x 0.25 µm film thickness). The initial temperature of a column was programmed at 50°C for two minutes and then ramped at linear rate of 6°C/min to 300°C. The final temperature was held for ten minutes. Temperature of injector and detector were programmed at 280°C and 320°C, respectively. Exactly 1 µL of sample was injected in a split mode (1:20). The mass spectra of detected components in the samples were compared with mass spectral library of National Institute Standard and Technology (NIST) incorporated in GC-MS data system. The name, molecular weight and structure of the detected components in the sample was then ascertained.

1,1-Diphenyl-2-picryl hydrzyl (DPPH) Radical Scavenging Assay

The free radical scavenging capacity was determined as described by Mohan *et al.* (2013). Briefly, 1 mL of 2 mM DPPH solution was added to 1 mL of samples with different concentrations. Ascorbic acid was used as a control. Absorbance was recorded at 517 nm after the samples were

incubated for 30 minutes. The antioxidant activity of the samples were expresed by EC_{50} value (mg/mL).

Brine Shrimps Lethality Bioassay

Brine shrimps lethality testing was performed according to procedure used Kabubii *et al.* (2015). Brine shrimp eggs, *Artemia salina* were hatched for 48 hours in sea water in a 2 L beaker with the aided of oxygen pump and light source. A stock solution of 1000 mg/L was prepared by dissolving the extract with methanol. Exactly 5 µL, 50 µL, 250 µL and 500 µL of stock solutions were transferred into a petri dish and allowed to dry. Approximately 5 mL of seawater and 0.2 mL of dimethyl sulphoxide (DMSO) in was then added to four samples with different concentration in petri dishes. A total of 10 brine shrimps were transferred into each petri dish and the surviving brine shrimps were counted after 24 hours. Samples containing mixture of sea water and DMSO were used as control. Percentage of mortality was calculated using Equation 2. The concentration killing 50% of the larvae (LC_{50}) was calculated from the linear equation by taking antilogarithm.

$$\text{Mortality (\%)} = S_c - S_t \quad (1)$$

where S_c is % nauplii survived in control, while S_t is % nauplii survived the treatment.

RESULTS AND DISCUSSION

Methanol Extract

Methanol extract yield 9.37% based on dry weight. Fractionation of 4.00 g of methanol extract on column chromatography for methanol extract from seeds of *C. megacarpa* obtained 138 fractions. A total of 7 combined fractions obtained were CF_{1-16} (CF_a), CF_{17-78} (CF_b), CF_{79-95} CF_c), CF_{96-100} (CF_d) $CF_{101-108}$ (CF_e), $CF_{109-110}$ (CF_f) and $CF_{111-138}$ (CF_g) with percentage yields 0.67%, 2.67%, 2.00%, 2.67%, 20.33%, 7.00% and 7.67%, respectively. Most of the combined fractions were in brown color solid at room temperature.

Combined Fractions of CF_a to CF_c

These samples were analysed on GC-MS without derivatization. These combined fractions were expected to possess sufficient volatility because eluted using non-polar solvents. GC-MS chromatograms showed a total of 23 peaks in these samples which correspond to non-polar compounds such as alkanes, alkenes and others. Non-polar compounds are not undergone substitution of an active functional group with TMS silylation reaction (Khoomrung *et al.*, 2015).

Combined Fractions of CF_d to CF_g

The combine fractions CF_d to CF_g were derivatised in order to increase the volatility and detectability of the polar compounds (Schummer *et al.*, 2009). GC-MS chromatograms identified 60 components in all samples. Among compounds identified were carboxylic acids, carbohydrates and alcohols. These compounds were detected as TMS derivatives where compounds with active hydrogen were replaced by a silyl group to reduce its polarity and hydrogen bonding (Khoomrung *et al.*, 2015). For example, arabitol was identified as pentakis(trimethylsilyl)-arabitol. Figure 2 shows GC-MS chromatograms of derivatized CF_d, CF_e, CF_f and CF_g combined fractions. Table 2 list five major compounds identified in each of CF_d to CF_g samples and α-D-xylopyranose has been identified in all combined fractions analyzed. Carbohydrates represent 67.64%, 43.34%, 15.22% and 8.07 % of chemical composition detected in CF_d, CF_e, CF_f and CF_g samples, respectively. Carbohydrates are polar compounds with complex structure and unsufficient volatility to be detected in GC-MS and need to be derivatised prior to GC-MS analysis (Schummer *et al.*, 2009). High amount of carbohydrate in all samples is correlated with proximate data for *C. megacarpa* seed. Sugar alcohols

such as arabitol, pentitol, glucitol, xylitol and galactitol were also identified all polar samples analysed with percentage composition between 0.93-71.95%. Xylitol is known as nutritive sweeteners and also iniate sensation of cooling in mouth (Livesey, 2003). Sugar alcohol sweetness was less than sugar but excessive intake of these sugar alcohol can cause laxative effect led to discomfort of stomach (Grembecka, 2015). Sugar alcohol was generally found in fruits and vegetables. It is beneficial to human health due to the low calorie and non cariogenic properties compared to sugar (Akinterinwa *et al.*, 2008).

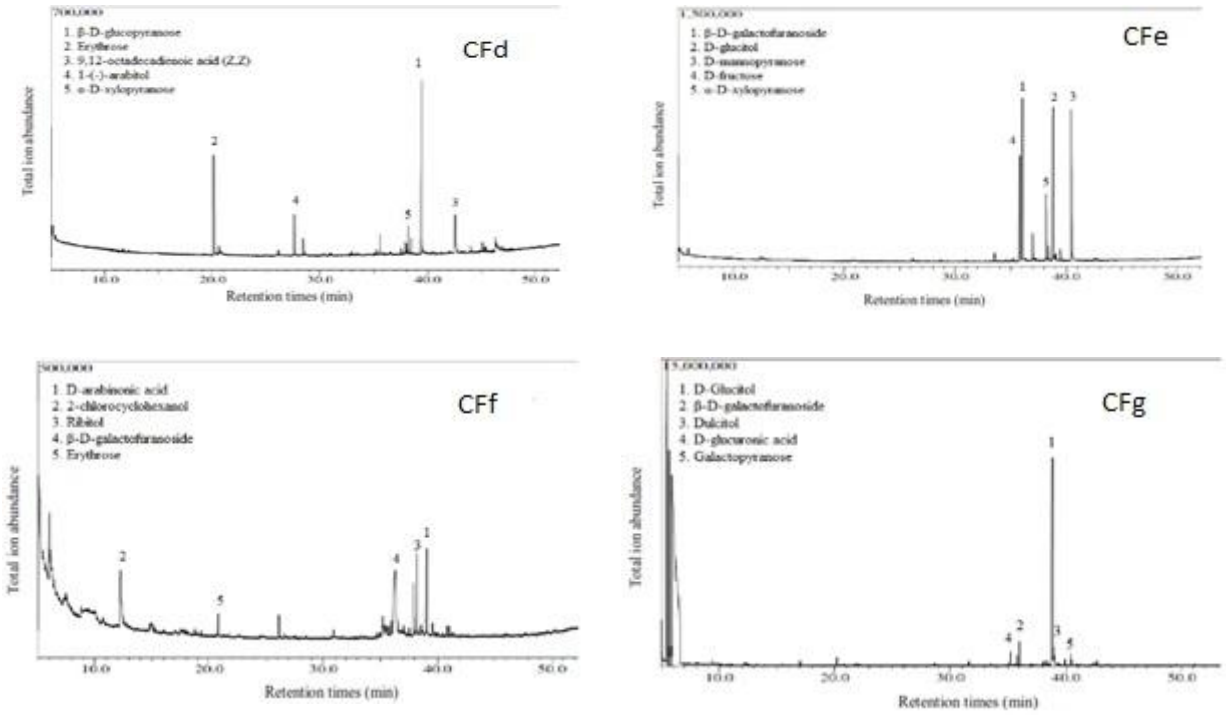

Figure 2. Profile of components identified in derivatised polar combined fractions as traced by GC-MS.

Table 2. Five major compounds identified in four combined fractions of methanol extract.

Combined fractions	Compounds identified (% composition)
CF_d	β-D-glucopyranose (36.49%), erythrose (19.51%), 9,12-octadecadienoic (9.73%), 1-(-)-arabitol (8.03%) and α-D-xylopyranose (5.45%).
CF_e	β-D-galactofuranoside (20.55%), D-glucitol (19.59%), D-manopyranose (18.67%), D-fructose (12.53%) and propanoic acid (8.72%).
CF_f	D-arabinonic acid (31.71%), 2-chlorocyclohexanol (14.60%), ribitol (9.19%), β-D-galactofurnaoside (9.00%) and erythrose (8.67%).
CF_g	D-glucitol (71.95%), β-D-galactofuranoside (5.41%), galactitol (3.86%), D-manopyranose (2.34%) and D-fructose (2.10%).

Antioxidant Activities of Crude Extract and Combined Fractions

Methanol crude extract and CF_g sample showed low EC_{50} value with 205.10 and 158.97 mg/L, respectively (see Table 3). This indicates the ability of methanol extract and CF_g sample to scavenge 50% of DPPH radical at a concentration below 1000 mg/L which may due to the presence of phenolic compounds such as 2-chlorocyclohexanol and sugar alcohol in *C. megacarpa* seed. Phenolic compounds can undergo redox reaction to neutralize free radical activity that led to better antioxidant properties (Seal, 2011). Lower radical scavenging activities with EC_{50} 115.27 mg/L was reported for extracts from *C. indica* seeds compared to *S. arvensis* (Seal, 2011). All non-polar samples (CF_a to CF_c) have not shown antioxidant properties. This aligns with the compounds detected in these samples were composed high percentage of hydrocarbons.

Table 3. EC_{50} value for antioxidant activites of ascorbic acid, methanol crude extract and all combined fractions from *C. megacarpa* seeds.

Samples	EC_{50} (mg/L)
Ascorbic acid (control)	51.23
Methanol crude extract	205.10
CF_a to CF_c	>1000
CF_d	291.74
CF_e	259.49
CF_f	256.45
CF_g	158.97

Brine Shrimps Lethality Test

Table 4 summarised the LC_{50} values for methanol extract and combined fractions. Toxicity test against brine shrimp showed that methanol extract and all combined fractions were toxic, except CF_a to CF_C. The LC_{50} value for methanol extract and combined fractions of CF_d to CF_g are low (<100 mg/L). Toxic fractions composed glycoside derivative, pyrazine and carboxylic acid. Glycoside compounds could react with the glycosidase enzyme and release cyanogenic glycoside which is toxic constituent of the plants (Gupta & Dhingra, 2015). However, CF_a to CF_C were found not toxic due to high content of hydrocarbons.

Table 4. LC_{50} for brine shrimp lethality test against *C. megacarpa* methanol seeds extract and combined fractions.

Samples	LC_{50}
Methanol extract	19.88
CF_a to CF_c	>100
CF_d	28.93
CF_e	15.80
CF_f	26.92
CF_g	19.38

CONCLUSION

GC-MS analysis revealed hydrocarbons, fatty acids and several hydroxyl compounds as constituents of non-polar samples (CF_a to CF_c). Polar samples need to be derivatised prior to GC-MS analysis in order to enhance the volatility and detectability of the polar compounds in the samples. GC-MS chromatograms of derivatised polar fractions (CF_d to CF_g) showed several peaks correspond to carbohydrates, alcohols and carboxylic acids. Antioxidant testing on methanol extract and CF_g showed high anti-oxidant activities with low EC_{50} values of 205.10 mg/L and 158.97 mg/L, respectively. Brine shrimp lethality test showed methanol extract and all polar fractions (CF_d to CF_g) from *C. megacarpa* seeds are toxic with LC_{50} < 100 mg/L. However, non-polar fractions (CF_a to CF_c) are not toxic with LC_{50} > 100 mg/L.

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