Phenolic contents, antioxidant and cytotoxic activities of *Elaeocarpus floribundus* Blume

Rahayu Utami[1], Nurhasniza Khalid[1], Mohd Aspollah Sukari[1]*, Mawardi Rahmani[1], Ahmad Bustaman Abdul[2] and Dachriyanus[3]

[1]Department of Chemistry, Faculty of Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[2]UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[3]Faculty of Pharmacy, Andalas University, Padang, West Sumatera, Indonesia

Abstract: *Elaeocarpus floribundus* is higher plant that has been used as traditional medicine for treating several diseases. There is no previous report on phytochemicals and bioactivity studies of this species. In this investigation, triterpenoids friedelin, epifriedelanol and *β*-sitosterol were isolated from its leaves and stem bark. Determination of total phenolic content of methanolic extract of leaves and stem bark was carried out using Folin-Ciocalteu reagent. All extracts and isolated compounds were subjected to screening of antioxidant activity using DPPH free radical scavenging method and cytotoxic activities by MTT assay towards human T4 lymphoblastoid (CEM-SS) and human cervical (HeLa) cancer cells. In the total phenolic content determination, methanolic extract of leaves gave higher value of 503.08±16.71 mg GAE/g DW than stem bark with value of 161.5±24.81 mg GAE/g DW. Polar extracts of leaves and stem bark possessed promising antioxidant activity with methanol extract of stem bark exhibited strongest activity with IC_{50} value of 7.36±0.01 μg/ml. In the cytotoxic activity assay, only chloroform extract of leaves showed significant activity with IC_{50} value of 25.6±0.06 μg/ml against CEM-SS cancer cell, while friedelin and epifriedelanol were found to be active against the two cancer cells with IC_{50} values ranging from 3.54 to 11.45 μg/ml.

Keywords: *Elaeocarpus floribundus,* triterpenoids, phenolic content, antioxidant, cytotoxic.

INTRODUCTION

Plant still remains as major source of secondary metabolites and traditional medicines for thousands of years. Phytochemicals and biological activity studies revealed that natural products derived from plants have been successfully shown some important role particularly in medicinal area as anticancer and anti infective agents. Phenolic compounds are one of secondary metabolites in plants which are reported to possess wide biological activities including antioxidant, anti-mutagenic, anti-carcinogenic and ability in modifying the gene expression (Tapiero *et al*., 2002).

Elaeocarpus is a genus of 350 plants species with a wide distribution in Madagascar, India, Southeast Asia, Malaysia, southern China and Japan as well as Australia and New Zealand, Fiji and Hawaii in the east (Burkill *et al*., 1966). Biological activities and phytochemical studies of some species from genus Elaeocarpus have been reported. Two new ellagic acid derivatives, 4'-O-methylellagic acid 3-(2",3"-di-O-acetyl)-*α*-L-rhamnoside and 4,4'-O-methylellagic acid 3-(2",3"-di-O-acetyl)-*α*-L-rhamnoside along with cytotoxic cucurbitacins, cucurbitacins D and F were yielded from isolation works on bark of *Elaeocarpus mastersii*. A potential antioxidant, 1,2,3,4,6-penta-O-galloyl-*β*-D-glucose isolated from *Elaeocarpus sylvestris* was found to exhibit DPPH free radical activity and intracellular reactive oxygen species, while some indolizidine alkaloids, such as elaeocarpenine, isoelaeocarpine, isoelaeocarpicine and elaeocarpine were successfully isolated from leaves of *Elaeocarpus fuscoides* (Ito *et al*., 2002; Katavic *et al*., 2007; Piao *et al*., 2009). In addition, three flavonoids isolated from leaves of *Elaeocarpus lanceofolius* were identified as 4'-methylmyricetin, myricetin and its 3-O-rhamnoside (Ray *et al*., 1976). Hence, there are potential in investigating the antioxidant activity of the plant species against free radical agents as well as cytotoxic properties of the extracts and isolated compounds towards various cancerous cell lines.

Elaeocarpus floribundus is a species belongs to genus Elaeocarpus that commonly grows in the lowland hills and mountains in India, Burma, Thailand, Vietnam, Malaysia and Indonesia. This plant also known as medang teja (in Malay) and infusion of bark and leaves is used as a mouthwash for inflamed gums (Wiart, 2006). There is no report on phytochemical study of leaves and stem bark of this plant and its bioactivity investigation has not been explored as well as yet. Thus, here we report the chemical constituents, antioxidant and cytotoxic activities and also total phenolic content of leaves and stem bark of *Elaeocarpus floribundus*.

MATERIALS AND METHODS

Plant material

Stem bark and leaves of *Elaeocarpus floribundus* were collected from Siak Sri Inderapura, Riau Province,

**Corresponding author:* e-mail: aspollah@science.upm.edu.my

Indonesia in 2008 and were identified by Mrs. Latipah Zainal Abidin, Faculty of Forestry, Universiti Putra Malaysia. Voucher specimen is deposited in the Herbarium of the Faculty.

Extraction procedures

The finely ground of dried leaves of *Elaeocarpus floribundus* (2 kg) was soaked at room temperature three times with hexane, chloroform, ethyl acetate and methanol sequentially for seventy two hours for each extract. The extracts were evaporated in vacuo to afford hexane (34.4 g), chloroform (32.3 g), ethyl acetate (7.6 g) and methanol (140.2 g) extracts. Twenty grams of chloroform and five grams of ethyl acetate extracts were column-chromatographed eluted with mixtures of hexane, ethyl acetate and methanol as eluents. The fractionation of chloroform extract gave 39 fractions. Purification of fractions 11-15 gave friedelin (**1**), while purification work on fractions 20-27 gave epifriedelanol **(2)**. Isolation work on ethyl acetate extract yielded 42 fractions. Epifriedelanol **(2)** was also isolated from this extract after purification of fractions 8-14.

While 2.7 kg of finely ground dried stem bark of *Elaeocarpus floribundus* was treated with the same procedures to give 4.6 g of hexane, 5.6 g of chloroform, 9.2 g of ethyl acetate and 109.3 g of methanol extracts. Isolation work on three grams of chloroform extract using column chromatography and mixtures of hexane, ethyl acetate and methanol as eluents, has yielded 0.91 g of *β*-sitosterol **(3).** Work-up procedures on other leaves and stem bark extracts did not yield any identified compounds. Its leaves and stem bark extracts were then subjected to antioxidant and anticancer test using DPPH free radical scavenging and MTT assay, respectively.

Friedelin (1) – white needle-shaped crystal, m.p 249 – 251 ^{0}C (lit. 261-264 ^{0}C, (Courtney *et al*., 1956)), $C_{30}H_{50}O$, IR ν_{max} : 2927, 1714. MS m/z (%): 426 (M^+, 25), 411 (5), 123 (55), 109 (62), 95 (86), 69 (100), 55 (90). ^{1}H NMR ($CDCl_3$): δ 1.20 – 2.40 (satd. CH-CH_2), 1.18 (3H, *s*, H-28), 1.05 (3H, *s*, H-26), 1.00 (6H, s, H-27 and H-29), 0.95 (3H, *s*, H-30), 0.89 (3H, *d*, *J* = 6.4Hz, H-23), 0.87 (3H, *s*, H-25), 0.72 (3H, *s*, H-24). ^{13}C NMR ($CDCl_3$) : δ 22.3 (C-1), 41.5 (C-2), 213.2 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.4 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 35.9 (C-16), 29.9 (C-17), 42.8 (C-18), 35.3 (C-19), 28.1 (C-20), 32.7 (C-21), 39.2 (C-22), 6.8 (C-23), 14.6 (C-24), 17.9 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 34.9 (C-29), 31.8 (C-30).

Epifriedelanol (2) – white needle-shaped crystal, m.p : 274 – 276 ^{0}C (lit. 280-282 ^{0}C (Queiroga *et al*., 2000)), $C_{30}H_{52}O$, IR ν_{max} : 3475, 2933. MS m/z (%): 428 (M^+, 20), 413 (18), 165 (76), 95 (100), 69 (88), 55 (92). ^{1}H NMR ($CDCl_3$) : δ 3.73 (1H, *bd*, *J* = 3.0 Hz, H-3), 1.90 (1H, *dt*, *J* = 10.1, 3.0 Hz, H-2a), 1.73 (1H, *dt*, *J* = 12.8, 3.0 Hz, H-6a), 1.57 (1H, *m*, H-2b), 0.88 – 1.55 (satd CH-CH_2), 1.16 (3H, *s*, H-28), 0.99 (3H, *s*, H-29), 0.98 (6H, *s*, H-26 and H-27), 0.95 (3H, *s*, H-30), 0.94 (3H, *d*, *J* = 7.0 Hz, H-23), 0.93 (3H, *s*, H-24), 0.84 (3H, *s*, H-25). ^{13}C NMR ($CDCl_3$): δ 15.8 (C-1), 35.3 (C-2), 72.8 (C-3), 49.2 (C-4), 37.1 (C-5), 41.7 (C-6), 17.5 (C-7), 53.2 (C-8), 37.8 (C-9), 61.3 (C-10), 35.2 (C-11), 30.6 (C-12), 38.4 (C-13), 39.3 (C-14), 32.8 (C-15), 36.1 (C-16), 30.0 (C-17), 42.1 (C-18), 35.6 (C-19), 28.2 (C-20), 32.1 (C-21), 39.3 (C-22), 11.6 (C-23), 16.4 (C-24), 18.6 (C-25), 18.6 (C-26), 20.1 (C-27), 32.3 (C-28), 35.0 (C-29), 31.8 (C-30).

β-sitosterol (3) – white needles crystal, m.p 137-138 ^{0}C (137 – 138 ^{0}C, (Ahmad *et al*., 2010)). IR ν_{max}: 3341, 2934, 2865, 1657, 1460, $C_{29}H_{50}O$, MS m/z (%): 414 (M^+, 77), 396 (49), 381 (34), 329 (67), 255 (70), 213 (69), 145 (100), 105 (91), 81 (90). ^{1}H and ^{13}C NMR spectral data were compared with previously published data (Ahmad *et al*., 2010).

Total phenolic content determination

A colorimetric method with Folin Ciocalteu's as reagent was used for total phenolic content determination (Velioglu *et al*., 1998). Plant sample was dried at room temperature and ground to get finely-air dried sample. Two hundred milligrams of sample was then soaked for two hours in mixture of two ml of 80% methanol and 1% hydrochloric acid as solvents. This extraction work was carried out at room temperature on an orbital shaker set at 200 rpm. The obtained mixture was centrifuged at 1000 gram for 15 minutes and its supernatant was then decanted to give extract solution. The pellets were treated with same conditions. Supernatans were combined and subjected to the assay.

One hundred millilitres of extract solution was added with 0.75 ml of Folin-Ciocalteu's reagent. This reagent was previously diluted 10-fold with distilled water. The mixture was allowed to stand at room temperature for 5 minutes and then 0.75 ml of sodium bicarbonate (60 g/l) solution was put into the mixture. It was shaken thoroughly and left at room temperature for 90 minutes. The absorbance was measured at 725 nm by UV-Vis spectrometer. This procedure was repeated in triplicate for each sample. Result was calculated as gallic acid equivalents in mg per gram dried weight sample from the calibration curve of gallic acid standard solution. The equation formula of gallic acid's calibration curve obtained was $y = 0.0026x + 0.0402$; $R^2 = 0.9989$.

Antioxidant assay

DPPH free radical scavenging activity method was perfomed for screening antioxidant activity of extracts of leaves and stem bark of *Elaeocarpus floribundus* (Saha *et al*., 2004). This antioxidant assay was implemented with some modification. Stock solution of extracts was dilute in methanol with concentration 10 mg/ml. The DPPH

reagent was prepared as 2.5 mg/ml in methanol. Test solutions with different concentrations in a 96-well microtiter plates (500, 250, 125, 62.5, 31.25, 15.63 and 7.8 μg ml^{-1}) was prepared from stock solution using methanol as solvent. Then each well was added with 5μl DPPH reagent. The plate was shook slowly and left in the dark for 45 minutes at room temperature. The absorbance of mixture was determined at 517 nm using UV-Vis spectrometer. The antioxidant activity was estimated as follow:

$$\% \text{ inhibition} = \frac{\text{Abs (DPPH)} \;\; \text{Abs (DPPH + sample)}}{\text{Abs (DPPH)}} \times 100\,\%$$

The antioxidant activity of sample was calculated as IC_{50} values (the inhibition concentration of sample reduce 50 % the absorbance of DPPH). Ascorbic acid was used as standard that was run with the same concentration as applied to sample. All of assays were repeated in triplicates and the readings were averaged.

Cytotoxic assay

MTT Assay was conducted to examine cytotoxic activity of crude extracts towards human T4 lymphoblastoid (CEM-SS) and human cervical (HeLa) cancer cell lines (Mosmann, 1983). These cancer cell lines were obtained from American Type Culture Collection (ATCC), Maryland, United State of America. The medium RPMI 1640 (PAA, Germany) was used to dilute the cells to a concentration of $5x10^3$cells/ml. From this cell suspension, 100 ml of various concentrations of the extracts was pipetted into a 96-well microtiter plate and incubated in 37 ^{0}C, 5 % CO_2 for 72 hours. Various concentrations being used was 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 μg/ml. The assays of each concentration of extracts were performed in triplicates and the control well of untreated population were also included. After 72 hours, 20 μl of MTT (3-[4, 5-dimethylthiozol 1-2yl]-2,5-diphenyltetrazolium bromide) solution with concentration 5 mg/ml in PBS (freshly prepared) was added into each well. The plate was then incubated for 4 hours at 37^{o}C, 5 % CO_2. Supernatant from each well was discarded afterward. Then, 100 μl of DMSO was put into each well and then the plate was shaked thoroughly to dissolve the formazan crystal before reading the absorbance. The absorbance was measured using the ELISA reader with test wavelength of 570 nm and references wavelength of 630 nm. The cytotoxicity was expressed as IC_{50}. The IC_{50} value was defined as the concentration of the test crude extract resulting in a 50% reduction of absorbance.

RESULTS

Two compounds were isolated from non polar extracts of leaves of *Elaeocarpus floribundus* and were identified as friedeline **(1)** and epifriedelanol **(2)**, while isolation work on stem bark has yielded β-sitosterol **(3)**. The structure of compounds were elucidated using several spectroscopic methods including Infrared Spectroscopy, Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) and by comparison data with previous reported data (Mahato *et al*., 1994; Hisham *et al*., 1995; Kundu *et al*., 2000; Li *et al*., 2006; Ahmad *et al*., 2010). However, the fractionation works on polar extracts using sephadex LH-20 in column chromatography did not give any pure compounds.

Fig. 2 showed total phenolic contents of methanolic extract of leaves and stem bark of *Elaeocarpus floribundus*. The values showed that the methanolic extract of leaves contained higher total phenolics content (503.08±16.71 mg GAE/g DW) than the stem bark (161.5±24.81 mg GAE/g DW). Meanwhile, Table 1 presented antioxidant and anticancer activities of extracts and isolated compounds of leaves and stem bark of *Elaeocarpus floribundus*.

As for antioxidant activity, methanol extracts of stem bark and ethyl acetate extract of leaves gave strongest activity with IC_{50} values of 7.36±0.01 and 9.37±0.06 μg/ml, respectively. In cytotoxic activity assay, chloroform extract of its leaves was the only active extract with IC_{50} values of 25.6±0.06 μg/ml against CEM-SS cancer cell line. While, both isolated compounds were found to be active against CEM-SS and Hela cancer cell lines with IC_{50} values of less than 30 μg/ml.

DISCUSSIONS

Isolation and characterization on chemical constituents

Friedelin **(1)** was isolated from fractions 11-15 by the column chromatography separation of the hexane extract using mixtures of hexane, ethyl acetate and methanol as eluents to give an orange solid, which was then washed with hexane to afford white needles-shaped crystal. The MS spectrum displayed a molecular ion peak at m/z 426, which are in agreement with the molecular formula $C_{30}H_{50}O$. The IR spectrum displayed C-H stretching at 2927 cm^{-1} whereas peak at 1714 cm^{-1} indicating the existence of carbonyl group. The presence of carbonyl group was futher confirmed by ^{13}C NMR, which indicated the presence of carbonyl carbon peak at 213.1 ppm. The integration of ^{1}H NMR spectrum corresponded to eight methyl protons, while methylene saturated protons showed at δ 1.2–2.4 are characteristic protons signal for triterpenoid.

Epifriedelanol **(2)** was isolated from mini column chromatography of fractions 20-27 of chloroform crude extract and also from purification work on fractions 8-14 of ethyl acetate extract by using mixtures of hexane, ethyl acetate and methanol as eluents. Epifriedelanol was obtained as white needles crystal with melting point 274–276 ^{0}C. The mass spectrum exhibited the molecular ion peak at m/z 428 corresponded to molecular formula

$C_{30}H_{52}O$. The IR spectrum signal for OH group was observed at 3475 cm^{-1} and absorption peak at 2933 cm^{-1} was due to C-H stretching bond. ^1H NMR spectrum showed characteristic triterpenoid protons signal at δ 0.88–1.55. A broad signal appeared at δ 3.73 (J = 3.0 Hz) was assigned to H-3. The presence of double triplet peaks at δ 1.90 (*dt*, J = 10.1, 3.0 Hz) and 1.73 (*dt*, J = 12.8, 3.0 Hz) were due to H-2a and H-6a respectively, while multiplet signal at δ 1.57 was assigned to H-2b. The presence of singlet peaks at upfield was due to eight methyl protons. ^{13}C NMR spectrum indicated 30 carbon atoms consisting of 6 quarternary, 5 methine, 11 methylene and 8 methyl carbons. A quaternary carbon peak which appeared at downfield δ 72.7 was assigned to C-3 that attached to OH group.

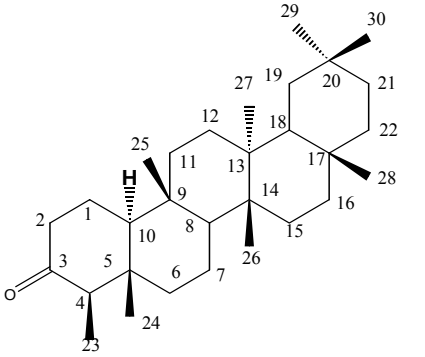
β-sitosterol **(3)**, a common plant sterol was obtained from isolation work on chloroform extract of stem bark. This compound was isolated as white needles crystal with melting point 137-138°C. The molecular ion peak appeared at m/z 414 fits molecular formula of $C_{29}H_{50}O$. The compound was assigned based on the similarity of its physical and spectral data with published data (Ahmad *et al*., 2010). This present work is the first report on the isolation of these three triterpenoid from Elaeocarpus floribundus.

Total phenolics content of leaves and stem bark of Elaeocarpus floribundus

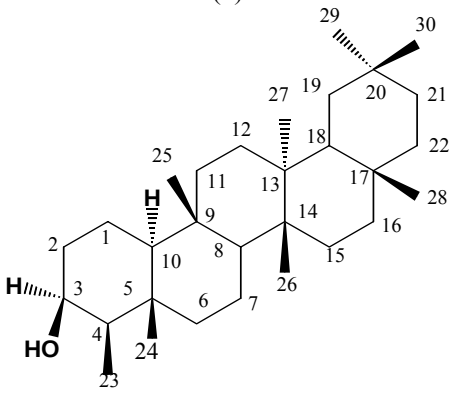
Total phenolics content of methanolic extract of leaves and stem bark of *Elaeocarpus floribundus* was examined by colorimetric assay using Folin-Ciocalteu reagent. Folin-Ciocalteu reagent is a mixture of phosphomolybdic and phosphotungstic heteropoly acids. A complex molybdenum-tungsten blue was resulted from the oxidation of phenolates by this reagent. The absorbance of complex was read by UV-Vis spectrophotometer at wavelength 725 nm (Singleton *et al*., 1965).

The total phenolic content of methanolic extract of leaves and stem bark of *Elaeocarpus floribundus* was determined by extrapolating the absorbance of samples to the calibration curve prepared from gallic acid concentrations and calculated as gallic acid equivalents. The result indicated the presence of phenolic substances in these two methanolic extracts. Methanolic extract of its leaves showed the higher value than its stem bark (fig. 2).

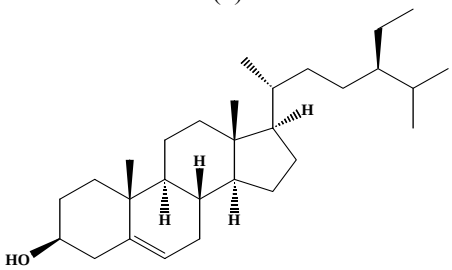
It has been reported that the amount of phenolic compounds in plants are varies. Furthermore, different parts of plant also contain various total phenolic contents due to the physiological function of phenolic compound itself which related to defence mechanism of plants against microorganisms and herbivores attack (Hada *et al*., 2001, Jing *et al*., 2010). Some previous researches revealed that extracting solvent significantly affected the yield of phenolic content in extract of studied plant. Methanol was reported as the most effecient solvent in the extraction of phenolic compounds due to its polarity, ease of evaporation as well as its ability to inhibit the reaction of polyphenol oxidase (Yao *et al*., 2004, Azlim Almey *et al*., 2010).



(1)



(2)



(3)

Fig. 1: Isolated compounds from *Elaeocarpus floribundus*; **(1)** friedelin, **(2)** epifriedelanol, **(3)** β-sitosterol.

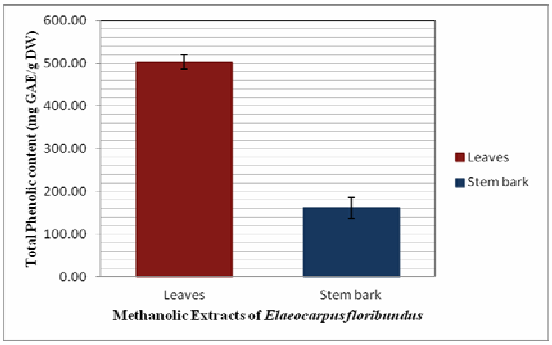


Fig. 2: Total phenolic content of methanolic extract of leaves and stem bark of *Elaeocarpus floribundus*.

Table 1: Antioxidant and anticancer properties of extracts and the isolates

Part of Plant	Extract	IC_{50} (µg/ml)		
		DPPH Free Radical Scavenging Activity	Cytotoxic Activity	
			CEM-SS	HeLa
Leaves	Hexane	>500	>100	>100
	Chloroform	>500	25.6±0.06	>100
	Ethyl Acetate	9.37±0.06	>100	60.4±0.09
	Methanol	58.23±0.04	>100	50.5±0.11
Stem bark	Hexane	>500	49.0±0.02	>100
	Chloroform	>500	>100	>100
	Ethyl Acetate	26.10±0.11	39.5±0.03	60.4±0.06
	Methanol	7.36±0.01	>100	48.9±0.07
Friedelin (1)		>500	8.53±0.11	3.54±0.30
Epifriedelanol (2)		>500	11.45±0.12	8.32±1.92
Vitamin C		6.69±0.02		

Values were the means ± standard deviations of three replicate analyses.

Antioxidant and cytotoxic activities

In DPPH free radical scavenging assay, polar extracts of leaves and stem bark of *Elaeocarpus floribundus* showed potential antioxidant activity. Methanol extract of stem bark exhibited strongest activity with IC_{50} value of 7.36±0.01µg/ml, while ethyl acetate extract of leaves also showed potential activity with IC_{50} value of 9.37±0.06 µg/ml. In contrary, non polar extracts and its isolated compounds, friedelin **(1)** and epifriedelinol **(2)** did not give any antioxidant activity with IC_{50} values more than 500 µg/ml.

As for cytotoxic activity, all of extracts and isolated compounds were tested using MTT assay against human T4 lymphoblastoid (CEM-SS) and human cervical (HeLa) cancer cells. MTT assay is a rapid and high precision colorimetric assay that can be utilized to study the anticancer properties of the active constituents. It is also one of several assays under consideration by American National Cancer Institute for potential application to be a large-scale anti tumour drug-screening programme (Gerlier *et al*., 1986; Alley *et al*., 1988).

Screening of cytotoxic activity on crude extracts of leaves and stem bark of Elaeocarpus floribundus suggested that only chloroform extract of the leaves demonstrated potential activity against CEM-SS cancer cell with IC_{50} values of 25.6±0.06 µg/ml. The other extracts showed weak cytotoxic activities against these two cancer cells with IC_{50} values ranging from 39.5 to 60.4 µg/ml, whilst the remaining extracts were found to be inactive with IC_{50} values >100 µg/ml.

Friedelin **(1)** and epifriedelanol **(2)** showed promising cytotoxic activities against the two cancer cells with IC_{50} values ranging from 3.54 to 11.45 µg/ml. Friedelin **(1)** exhibited strongest inhibitory effect against HeLa cancer cell with IC_{50} value of 3.54±0.30 µg/ml. This finding was in the agreement with a previous report which revealed that friedelin **(1)** displayed strong cytotoxic activities on the proliferation of four human cancer cells namely, A375, L292, HeLa and THP-1 (Lu *et al*., 2010), thus suggesting this compound to be the most important anti-tumour derived from the plant. The cytotoxic activities of these two compounds also have been reported on previous investigation (Wang *et al*., 2006). However, this is the first report of cytotoxic activity of epifriedelanol towards CEM-SS cancer cell line.

CONCLUSIONS

Three terpenoids were successfully isolated from leaves and stem bark of *Elaeocarpus floribundus* and identified as friedelin **(1)**, epifriedelanol **(2)** and β-sitosterol **(3)**. Determination of total phenolics content on methanolic extracts using Folin-Ciocalteu as reagent exhibited that leaves possess higher amount of phenolic substances than the stem bark. Antioxidant assay on plant extracts using DPPH free radical scavenging method demonstrated that polar extracts of its leaves and stem bark gave potential activity. In the cytotoxic assay, only chloroform extract of the leaves showed significant activity against CEM-SS cancer cell. While, the isolated compounds friedelin **(1)** and epifriedelanol **(2)** showed promising activity towards CEM-SS and HeLa cancer cells.

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