RADICAL SCAVENGING ACTIVITY AND PHYTOCHEMICAL INVESTIGATION OF ANTIOXIDATIVE COMPOUNDS FROM STEM AND LEAVES OF *LEEA INDICA* SPECIES

Aiza Harun[1,] Shaari Daud[1], Siti Suhaila Harith[1],Nor Erlena Asmira Ab Rahim[1], Mohd Azfar Abd Jalil[1], Nurul 'Atiqah Muhammad Rosdi[1], Fatin Azieyati[1] , Naila Nabihah [1], Siti Zaiton Mat So'ad[2], Norazian Mohd Hassan[2]*

[1]Faculty of Applied Sciences, Universiti Teknologi MARA Pahang, 26400 Bandar Tun Razak Jengka, Pahang, Malaysia

[2]Kulliyyah of Pharmacy, International Islamic University Malaysia Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

**Corresponding author: aizaharun@pahang.uitm.edu.my*

Abstract

In this paper, the potency of stem and leave extracts of *Leea indica* locally known as Memali from Tasik Chini, Pahang as a potent antioxidant agent had been studied. The antioxidant properties of three types of extracts was quantitatively assessed by 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The isolation of antioxidative compounds was conducted through preparative thin layer chromatography with the use of certain binary solvents. The structure determination was carried out using Gas Chromatography Mass Spectrometer (GCMS). The methanol extracts from both stem and leaf exhibited highest antioxidant activity with lowest IC_{50} of 1.14 *ug*/ml and 81 *ug*/ml respectively compared to other extracts. The stem showed stronger antioxidant activity compared to leaves part. Based on GCMS analysis, the antioxidative compounds isolated from leaves part were proposed as 6-Hyroxy-4,4, 7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one **(1)**, 3,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone **(2),** Methyl stearate **(3),** 9,12-Octadecadienoic acid **(4),** Palmitic acid **(5)** and 9-Oxononanoic acid **(6)**. The antioxidative compounds isolated from stem part were determined as oleic acid **(7)**, 1-(hydroxymethyl)-1,2- ethanediyl ester **(8)** and 9-octadecene **(9)**. This finding can be used as scientific baseline information for the future treatment of diseases caused by harmful free radicals.

Keywords: *Leea indica*, antioxidant, secondary metabolites, radical scavenging

Introduction

Medicinal plants were widely spread in all over the world with rich sources of bioactive compounds that responsible for the treatment of various diseases. Products that derived from the natural sources have become momentous agents as medicinal sources (Reddy et al., 2012) and recently, many species have raised their remarkable potential in pharmacology and cosmetic industry. The conscious of advantage of using natural medicine in daily life give high implication of nearly 80% of earth population consumed it as their main healthcare necessity (Sasidharan et al., 2011). A lot of recent research investigations have been done on many medicinal plants, but not all of them have been studied thoroughly in scientific manner.

Leea indica comes from the family of Vitaceae and also known as Memali. It is characterized as soft wooded parennial shrub with stout that having glabrous stems with 7 to 10 meters height. *L. indica* can be wildly found in the Malaysia and Thailand rainforest, China and

India's tropical and subtropical forest (Reddy et al., 2012). Previous studies reported that both leaves and roots of *L. indica* were used by the locals to cure soreness, dizziness, eczema, fever (Wong et al., 2012). *L. indica* was also reported to act as an antioxidant, anti-cancer, anti-fungal and anti-microbial (Srinivasan et al., 2008). As the plant extracts usually exists as a mixture of bioactive compounds synergistically, it became a huge challenge for the characterization of the bioactive compounds (Amsath, 2013)

The presence of ursolic acid (triterpenes), β-sitosterol (steroids) and lupeol (triterpenes) that were identified from methanol extract of leaves extract of *L. Indica* had been reported[4] . Currently, the bioactive agents such as flavanoids, tannins, terpenoids, steroids and alkaloid from the plant extract of *L. tetramera* was successfully identified (Rahman et al., 2013).

Nowadays, the beneficial interests of oxidative effect on human body and food industry have been spreading widely. Oxidation process can either be advantageous or detrimental to our life. Thus, antioxidant compound is crucial in complementing a well-balanced ecosystem such as to function as neuroprotectants in stroke and health injury as well as lipid peroxidation inhibitor (Antolovich et al., 2002). *Leea* species has become one of the antioxidant medicinal plant that were widely used as alternative remedy to treat disease caused-free radicals as it is compose a high content of non-flavonoid phenolic like gallic acid (Srinivasan et al., 2008). Later, the ethanolic extract of root of *L. rubra* was recorded to exhibit strong anti-oxidant property (Kadchumsang et al., 2014).

Materials and Methods

Sample Preparation and Plant Extraction

Leea indica leaves and stem was first washed and cut into small pieces to encourage the drying process. The plant's pieces were dried for about a five days at room temperature (25±2°C) with the shade of dried air before grounding into powder. The fine powder then was stored for the next procedures.

Extraction of Plant for Crude Extract

The extraction process was performed by utilizing three types of solvents with different polarities and they were petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH). About 500 g of fine powder sample was soaked in pure PE for about three days with occasional stirring at room temperature. The soaking process was repeated three times or until no color was detected. Then, the crude extract was filtered using Whatman No.1 filter paper before being concentrated with rotary evaporator .The extraction process was repeated consecutively using DCM and MeOH in same manner.

Thin Layer Chromatographic (TLC) Analysis and Isolation of Antioxidative Components

The TLC of each extracts was developed in suitable developing solvents until all components were well separated on TLC. The migrated components were then sprayed with DPPH spraying reagent to visualize any yellow spots against purple background. The presence of yellow spots indicated the presence of antioxidative components and the retention factor (R_f) was determined (Azfar, 2016)

For the isolation of antioxidative components, new TLC was freshly prepared and was developed in the same manner. The bands of antioxidative components were visualized under UV light and the desired band was marked with pin. The desired band were then scraped off

from the plate with spatula and placed into a sample tube. The mixture of silica gel and isolated components was washed with suitable solvents and was filtered through cotton wool. The filtrate (isolated compound) was left to dry prior to use. Each of the isolated component was resubjected to TLC analysis for R_f value confirmation prior to structure determination.

Gas Chromatography (GCMS) Analysis

The isolated component was introduced to the instrument by split-less injection method automatically. The operating conditions of the analysis were 220 °C of injection temperature with 290°C of transfer line. Oven temperature programme was set at 60-290 °C with 5 °C min^{-1} ramping. The carrier gas was helium at 1.5 ml min^{-1}. Using electron impact mass spectra, the temperature of ion source was at 250 °C and the analyzed component was identified from Wiley 275.L database matching individually.

DPPH Radical Scavenging Activity

Each of the crude extract was serially diluted to concentration ranging from 3.125-100 µg/ml. About 1 ml of each crude extract and ascorbic acid (reference) at each concentration was mixed with 0.004% of DPPH solution. The samples were incubated in dark place for half an hour at room temperature. The absorbance of all tested samples and ascorbic acid were recorded using Ultra Violet spectrophotometer at wavelength 517 nm [10]. The scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \left[\frac{(Acontrol - Asample)}{Acontrol}\right] \times 100\%$$

Acontrol = absorbance of solvent+DPPH
Asample = absorbance of sample + DPPH

Scavenging ability was expressed as IC_{50} and was obtained from the graph scavenging activity (%) vs concentrations. All antioxidant assays will be conducted in triplicated (Reddy et al., 2012).

Results and Discussion

DPPH radical scavenging effect of leaf and stem of *L. indica*

The free radical scavenging activity (IC_{50}) of leaf and stem was examined by means of scavenging activity with stable DPPH radicals. Lower IC_{50} value indicated stronger ability of the extract as DPPH scavenger. While higher IC_{50} value corresponded to low ability to act as DPPH scavenger as it required more scavengers to achieve the 50% DPPH scavenge reaction [1] . Table 1 shows the IC_{50} values of three types of extracts of leaf and stem of *L. indica.*

Table 1 Scavenging activity of *L. indica* leaf and stem extracts on DPPH radicals (IC_{50} value)

Extract	IC_{50} values (µg/ml)	
	Leaves	Stem
Petroleum ether	> 100	7.37
Dichloromethane (DCM)	> 100	9.60
Methanol	81	1.14
Ascorbic acid*	1.12	1.12

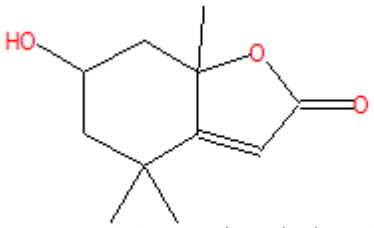
Note * Standard reference

Among the three extracts, methanol extracts of stem part exhibited the strongest scavenging activity (1.14 μg/ml) followed by petroleum ether extract (7.37 μg/ml) and DCM extract (9.60 μg/ml). For leaf part, methanol extract also demonstrated the strongest scavenging activity (81 μg/ml) compared with DCM and petroleum ether extracts. Both DCM and petroleum ether extracts showed higher IC_{50} values (>100 μg/ml) which means they scavenged the radicals of DPPH less effective. However, previous investigation showed that the scavenging activity for methanol extract of *L. indica* leaf was11.55 μg/ml and our present result of scavenging activity of methanol extracts from leaf did not show any consistency with the reported data (Azfar, 2016).

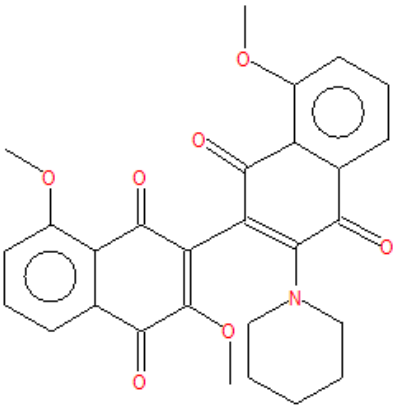
Between leaf and stem part, the methanol extract from stem part had scavenged the DPPH radicals more effectively compared with methanol extracts from leaf part. The difference could be due to the composition of antioxidants in stem methanol extract were greater compared with the leaf part. The present result is consistent with the previous data reported (Azfar, 2016; Nurul 'Atiqah, 2016) which explained that methanol extract was the most active scavenger in semiquantitative dot blot assay, where the yellow colour of antioxidative compounds were still consistent even at low concentration (0.19 mg/ml) for stem methanol extract. The radical scavenging activity of stem methanol extract was also comparable with the radical scavenging activity of reference standard ascorbic acid (1.2 μg/ml).
The result of antioxidant activity could be explained regarding the bleaching of the purple colour of methanol solution of DPPH, and the reduction of absorbance reading at 517 nm indicated the presence of scavenging activity between DPPH and free radical antioxidant in the stem methanol extracts leads to scavenging of the radical that triggered by electron transfer (Reddy et al., 2012; Antolovich et al., 2002). Conceptually, the odd electron in DPPH free radicals resulted strong absorption maximum at 517 nm and is purple in colour. The transformation of purple colour to yellow colour occurred as the odd electrons of DPPH radicals bonded with hydrogen from antioxidant sources (extract) to form the reduced DPPH-H. The more the decolorization occurred (purple to yellow), the more the electrons being caught. Thus, the higher the antioxidant in the extracts, the more DPPH reduction occurred and eventually the antioxidant activity was enhanced. Since methanol extract appeared the highest radical scavenging activity, it means that a large number of antioxidants could be found in methanol extracts compared with DCM and petroleum ether extract.

Strong antioxidants properties of methanol extract compared with DCM and petroleum ether extracts were probably due to high activity of its phenolic content and terpenoid. Phenolic activity of methanol extract relatively due to its high redox ability that enable them to react as hydrogen donating agents, reducing agents and also singlet oxygen quenching agents (Reddy et al., 2012).

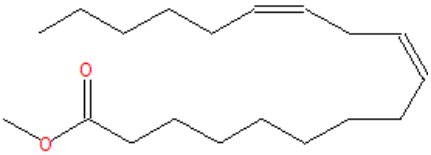
Structure determination of antioxidative compounds from GCMS
Figure 1 and Figure 2 illustrate the antioxidative compounds from the leaf and stem of *L. indica* after identifying through GCMS with the assistance of Wiley 275. L database.



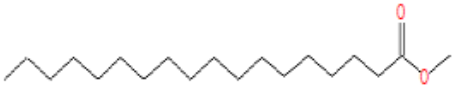
6-Hyroxy-4,4, 7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one **(1)**



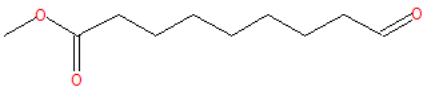
3,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone **(2)**



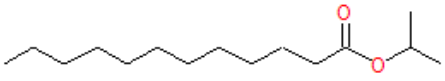
9,12-Octadecadienoic acid **(4)**



Methyl stearate **(3)**

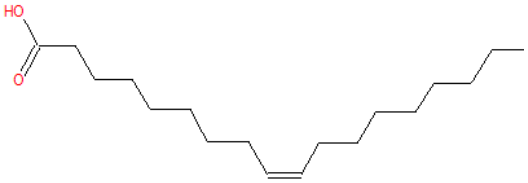


9-Oxononanoic acid **(6)**

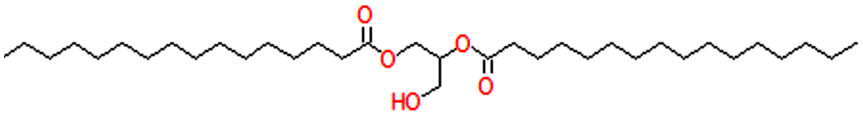


Palmitic acid **(5)**

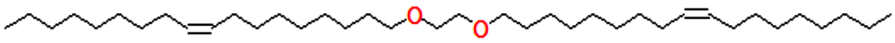
Figure 1 Antioxidative compounds from the leaf of *L. indica*



oleic acid **(7)**



1-(hydroxymethyl)-1,2-ethanediyl ester **(8)**



9-octadecene **(9)**

Figure 2 Antioxidative compounds from the stem of *L. indica*

Previous studies proclaimed that several compounds identified from the methanol extract such as Palmitic Acid and Phthalic Acid were also been identified in petroleum extract of *L. indica* leaf extract (Srinivasan et al., 2008). Thus our reported structural data seems to be in agreement with previous report since one of the structures that responsible for antioxidant activity was also Palmitic acid **(5)**. Compound **(1)** and compound **(2)** which were 6-Hyroxy-4,4, 7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one and ,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone respectively were also recognized as phenolic compound and terpenoid compound respectively. Both structures were also consistent with the data reported (Azfar, 2016; Nurul 'Atiqah, 2016) where, terpenoid and phenolic compounds were the main antioxidative constituents of stem and leaf of *L. indica*. Up until now, all compounds except compound **(5)** have not yet been proposed from other previous studies regarding *L. indica*, and this is the first time reported from the stem and leaf of *L. indica.*

Conclusion

The quantitative DPPH radical scavenging activity revealed stem methanol extract of *L. indica* exhibited strongest antioxidant activity with IC_{50} value as low as 1.14 *u*g/ml. Six types of antioxidative constituents from *L.indica* leaf have been successfully isolated and recognized as 6-Hyroxy-4,4, 7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one **(1)**, 3,8,8-Trimethoxy-3-piperidyl-2,2-binaphthalene-1,1,4,4-tetrone **(2),** Methyl stearate **(3),** 9,12-Octadecadienoic acid **(4),** Palmitic acid **(5)** and 9-Oxononanoic acid **(6)**. Three antioxidative compounds isolated from stem part of *L. indica* were determined as oleic acid **(7)**, 1-(hydroxymethyl)-1,2- ethanediyl ester **(8)** and 9-octadecene **(9).** Only compound **(5)** was a known structure and the rest of them were first time reported herein.

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Conflict of interests

Author hereby declares that there is no conflict of interests with any organization or financial body for supporting this research.

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