IDENTIFICATION OF TENTATIVE ANTIOXIDANT CONSTITUENTS FROM STEM AND LEAVES OF *LEEA INDICA* SPECIES USING SPECTROSCOPIC ANALYSIS

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Abstract

The tentative antioxidative constituents from stem and leaves extracts of *Leea indica* which traditionally known as Mali-mali had been investigated. Three different polarities of crude extracts of each part was prepared using consecutive soaking with petroleum ether, dichloromethane and methanol. Screening of antioxidants of each extract on TLC was performed with the use of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as spraying reagent and have been previously reported. The isolation of antioxidative compounds was performed through preparative thin layer chromatography (pTLC) with the use of certain binary solvents system. The structure determination was carried out using Fourier Transform Infra-Red (FTIR), Gas Chromatography Mass Spectrometer (GCMS) and Proton Nuclear Magnetic Resonance (NMR) along with comparison with literature. FTIR analysis depicted some important functional groups such as OH, C-H, C=C and C-O stretching in both parts. The C=O and C-N stretching only appeared in leaves extract of *L. indica*. According to proton NMR spectrum of leaves part, the chemical shifts belonged to aliphatic protons, vinylic protons, hydroxyl proton and aromatic protons. The proton NMR spectrum of stem part revealed the existence of proton aliphatic, vinylic proton and hydroxyl proton. Based on GCMS analysis with the assistance of Wiley 275.L database matching individually as well as with proton NMR data, the antioxidative compounds isolated from stem part were proposed as 1-(hydroxymethyl)-1,2-ethanediyl ester (LI-1), 9-Oxonomonoic acid methyl ester (LI-2), 9,12-Octadecadienoic acid (LI-3), 3’,8,8’-Trimethoxy-3-piperidyl-2,2’-binaphthalene-1,1’,4,4’-tetrone (LI-4).

Keyword: *Leea indica*, Antioxidant, Secondary Metabolites, proton NMR

Introduction

*Leea indica* species from Leeaceae family has a vast application in ethnomedicinal purpose and lately the search of various active components is crucial and gaining popularity. Previous research found some important secondary metabolites as an anti-cancer, anti-hyperglycemic activity, hypolipidemic activity and antioxidant agent (Dalu et al., 2014). The leaf part has been used traditionally to treat vertigo and diabetes while the root was used as antidysentric, anthelmintic, spasmolytic and sudorific and to treat cardiac and skin diseases (Chatterjee and Prakashi, 1997; Khare, 2007; Rahman et al., 2007). According to Srinivasan et al. (2008), the presence of gallic acid, ursolic acid (triterpenes), β-sitosterol (steroids) and lupeol (triterpenes) was identified from phytochemical screening test done on methanol extract of leaves extract of *L. Indica* by utilizing co-TLC technique. The flower contains ester of
phthalic acid, di-isobutylphthalate, di-n-butylphthalate, n-butylisophthalate, and 3,5 butylisohexylphthalate (Srinivasan et al., 2009).

Rahman et al. (2013) reported the presence of alkaloids, flavanoids, glycosides, terpenoids, and tannin in *L. indica* leaf extract. Studies conducted by Emran et al. revealed methanol leaf extract exhibited significant DPPH free radical scavenging activity compared with standard antioxidant ascorbic acid. IC$_{50}$ value of ascorbic acid and leaf extract was found 1.468μg/ml and 139.837μg/ml, respectively. Phytochemical analyses have screened the presence of alkaloid, flavonoid, terpenoids and steroid which responsible for antioxidant potency of methanol leaf extract of *L. indica*. This herb shows seasonal and varietal variation in the quantity of secondary metabolites (Emran et al., 2012).

Currently, the investigation of total phenolic content, flavonoid and its antioxidant properties of *L. indica* leaves extracts from n-hexane, ethyl acetate, and methanol was successfully conducted (Sulistyaningsih et al., 2017) and the results revealed that the highest fraction of total phenolic as well as total flavonoid was resulted from methanol extract. It was also demonstrated the strongest antioxidant activity with the IC$_{50}$ of DPPH scavenging effect 1.62 + 0.02 g GAE/ml and the highest percentage of inhibition for hydroxyl radical 57.60 + 2.52 %.

This study was conducted by means to confirm and establish our previous work (Harun et al., 2018) through proton NMR analysis of four proposed structures of antioxidative constituents from stem and leaves of *L. indica*.

**Materials and Methods**

**Sample Preparation and Plant Extraction**

About 500 g of *L. indica* leaves and stem were collected from Hutan Simpan UiTM Pahang. They were washed and cut into small pieces to assist the drying process. The leaves and stem were dried for several days at room temperature and were grinded into fine powder.

**Extraction Process**

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). The soaking process and extract preparation of both parts were followed the same method as reported by Harun et al., 2018.

**Fourier Transform Infra-Red (FTIR) analysis**

The extract was placed on the sampling plate using dropper. Since the instrument is an ATR-FTIR, the sample can be analysed directly in liquid form. The wavenumber was set between 400 – 4000 cm$^{-1}$. A background spectrum was firstly obtained before the sample was analysed. The functional groups were detected and were recognized as absorption peaks at different wave number.

**Isolation of Selected Antioxidative Components of LI-1, LI-2, LI-3 and LI-4 from Preparative Thin layer Chromatography(pTLC)**

The TLC of each extracts were developed in suitable developing solvents such as combination of hexane with dichloromethane, dichloromethane with methanol until all components were well separated on TLC (Harun et al., 2018). Further work on isolation of antioxidative constituents was conducted through pTLC development. The target constituent at certain R$_f$ value was carefully scratched and collected. Based on our previous investigation (Harun et al., 2018) the antioxidative constituents were observed at R$_f$ values of 0.14, 0.66 and 0.85 for leaves part and at R$_f$0.17 for stem part. The constituent was separated the from silica gel and was kept in the vial prior to analysis.
Gas Chromatography (GCMS) Analysis of LI-1, LI-2, LI-3 and LI-4
The procedure of GCMS analysis of four antioxidative constituents was followed the same method as previously reported (Harun et al., 2018)

One Dimensional Nuclear Magnetic Resonance (NMR) Analysis of LI-1, LI-2, LI-3 and LI-4
The isolated sample was subjected to one dimensional 500 MHz ¹H-NMR (JOEL) for structure determination. The sample was mixed with standard tetramethylsilane (TMS) in CDCl₃ prior to analyze. The resolve peaks at different chemical shifts indicated different type of protons present in the isolated compounds. The proton NMR data was used to confirm the types of protons present in the structure which was previously identified from GCMS (Harun et al., 2018)

Results and Discussion

Fourier Transform Infra-Red (FTIR)
Fourier Transform Infrared Spectrometer (FTIR) is a common device used to determine functional group of a compound based on absorption of ultraviolet light at certain wavelength. Since methanol extract was used for analysing purpose, all of the functional groups present in this extract represented the functional groups of L. indica. The FTIR analysis was started by obtaining the background spectrum to eliminate the noises which can interfere the spectrum. The sample was directly analysed on the Spectrum 100 Spectrometer’s crystal plate since we used Attenuated Total Reflectance (ATR) sampling. The FTIR spectrum of both stem and leaf revealed the presence of hydroxyl group, OH absorption peak at range 3208-3325 cm⁻¹, C-H stretching at 2947-2950 cm⁻¹, C=O stretching at 1611 cm⁻¹, C=C stretching at 1646-1539 cm⁻¹, C-N stretching at 1340 cm⁻¹ and C-O stretching at 1014-1016 cm⁻¹. The result of FTIR revealed some basic information to aid structure determination since it depicted some important functional groups present in the compound as well as covalent bond between atoms. In normal cases of the determination of structure of unknown compound, FTIR data was used along with Rule 13 of molecular weight to predict actual molecular formula of unknown compound.

Preparative Thin Layer Chromatographic (pTLC)
Preparative TLC method is the most convenient method for isolating matters especially in isolating active components such as antioxidative constituents. Figure 1 shows an example of the target antioxidative constituent of dichloromethane extract of L. indica stem at Rₜ 0.17 whereby this Rₜ value was obtained from developed TLC sheet in 100% dichloromethane. The greenish yellow colour of antioxidative compound was successfully isolated after careful scrapping process at targeted Rₜ value, dissolving and filtering to get rid of gel silica. The pTLC analysis of petroleum ether extract of L.indica leaves revealed two antioxidative marks at Rₜ 0.14 and 0.85 and one antioxidative marks at Rₜ 0.66 for DCM extract of L. indica leaves. The isolation of respective antioxidative marks was done in the same manner as depicted for compound at Rₜ 0.17 in Figure 1. Previously, all marks constituents revealed yellow color against purple background when sprayed with DPPH reagent (Harun et al., 2018).
All four isolated compounds were subjected to GCMS analysis by means of determination of their structural formula since this instrument was equipped with WILEY database. The structure determined from the WILEY database was further proved by comparing the structure with proton NMR spectrum.

LI-1 was isolated from *L. indica* stem extract and was observed as amorphous powder. The GCMS analysis of LI-1 revealed molecular formula of C_{35}H_{58}O_{5} with molecular mass of 558 (Harun et al., 2018). The Wiley database of GCMS proposed LI-1 compound as 1-(hydroxymethyl)-1,2-ethanediyl ester as illustrated in Figure 2 with index of hydrogen deficiency of 2.

The GCMS analysis of LI-2 indicated molecular formula of C_{10}H_{18}O_{3} (Harun et al., 2018) with molecular mass of 186. The Wiley database of GCMS revealed LI-2 as 9-Oxononanoic acid methyl ester with index of hydrogen deficiency of 2 which consistent with its molecular formula. The value of index of hydrogen deficiency was also in accordance with two double bonds in the structure. Figure 3 illustrates the proposed structure of LI-2.
Figure 3 Proposed structure for antioxidative LI-2 from *L.indica* leaves.

The GCMS analysis of LI-3 showed molecular formula of C_{19}H_{34}O_{2} (Harun et al., 2018) with molecular mass of 294. The Wiley database of GCMS suggested LI-3 as 9,12-Octadecadienoic acid methyl ester with index of hydrogen deficiency of 3. The value of index of hydrogen deficiency was also in accordance with three double bonds in the structure. **Figure 4** demonstrated the proposed structure of LI-3.

Figure 4 Proposed structure for antioxidative LI-3 from *L.indica* leaves 9,12-Octadecadienoic acid methyl ester

The GCMS analysis of LI-4 suggested the molecular formula of C_{28}H_{25}NO_{7} (Harun et al., 2018) with molecular mass of 487. The Wiley database of GCMS identified LI-4 as 3’’,8,8’’-Trimethoxy-3-piperidyl-2,2’-binaphthalene-1,1’’,4,4’’-tetrone which was an alkaloid. The molecular formula gave the index of hydrogen deficiency of 17 which consistent with two aromatic rings (8), three isolated rings and six double bonds. **Figure 5** illustrated the proposed structure of LI-4.

Figure 5 Proposed structure for antioxidative alkaloid of LI-4 from *L.indica* leaves

**One Dimensional Nuclear Magnetic Resonance (NMR)**

One antioxidative constituent had been isolated from preparative thin layer chromatography.
from DCM extract of *L. indica* stem was labelled as LI-1 and three constituents from petroleum extract and DCM extract of *L. indica* leaf was labelled as LI-2, LI-3 and LI-4. All samples have been analyzed with one dimensional proton NMR to determine the type of proton present in the compounds. Previously, these four constituents were reported (Aiza et al., 2018) without proton NMR analysis to establish the structure. LI-1 was observed as yellowish amorphous compound. The proton NMR spectrum of LI-1 depicted the ranging of chemical shift, δ between 1.24 – 1.56 ppm which corresponded to hydrocarbon protons such as methyl proton and methylene proton. The presence of OH group in LI-1 was established by the absorption peak at chemical shift, δ at range 3.64-3.65 ppm with triplet multiplicity. The chemical shift range between 3.89-3.91 ppm was owned by the group of proton that attached to ether group RO-C-H and ester group RO-CO-CH. After comparing the proton NMR data and with GCMS Wiley database the structure of LI-1 is proposed as 1-hydroxymethyl-1,2-ethanediyl ester as illustrated in Figure 2. The structure revealed methyl proton, methylene proton and hydroxyl proton and were consistent with proton NMR data of LI-1.

The proton NMR spectrum of LI-2 suggested the absorption peaks of methyl group at chemical shift ranging from 0.79 – 0.91 ppm. The absorptions of methylene and methine groups were found at chemical shifts ranging from 1.25 -1.28 ppm and 1.55 ppm respectively. One of methylene group has shown splitting pattern of doublet which suggested the interaction with one of neighbouring proton. After interpreting and comparing proton NMR absorption peaks with the structure proposed from WILEY database of GCMS, LI-2 compound was determined as 9-Oxonoranoic acid methyl ester as illustrated in Figure 3.

The proton NMR spectrum of LI-3 shows the absorption peaks of proton methyl in the range of chemical shift 0.86-1.12 ppm. The absorption of methylene proton can be seen at chemical shift ranging from 1.27 -1.34 ppm. The absorption peaks at chemical shift ranging from 1.84-2.01 ppm suggested the absorption peaks for vinylic protons. After interpreting and comparing proton NMR absorption peaks with the structure proposed from WILEY database of GCMS, LI-3 compound was suggested as 9,12-Octadecadienoic acid as illustrated in Figure 4.

The proton NMR spectrum of LI-4 shows absorption peaks of proton methyl in the range of chemical shift 0.79 – 0.88 ppm. The absorption of methylene proton can be seen at chemical shift ranging from 1.25 -1.27 ppm. The absorption peaks at chemical shift ranging from 3.58-3.60 ppm suggested the absorption peaks for amine proton whereas the sharp peak which depicted at chemical shift 7.25 ppm was belonged to aromatic proton. The high intensity of the peak could probably due to the presence of more than one aromatic ring. After interpreting and comparing proton NMR absorption peaks with the structure proposed from WILEY database of GCMS, LI-4 compound was suggested as 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone as shown in Figure 5. Table 1 depicts proton NMR analysis of antioxidative compounds from stem and leaves of *L. indica* by means of chemical shifts for each type of proton.

<table>
<thead>
<tr>
<th>Antioxidative Compounds</th>
<th>Chemical shift</th>
<th>Types of proton</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI-1 (stem part)</td>
<td>0.9 – 1.56</td>
<td>CH₃, CH₂</td>
</tr>
<tr>
<td></td>
<td>3.64-3.65</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td>3.89-3.91</td>
<td>RO-C-H, RO-C-H</td>
</tr>
<tr>
<td>LI-2 (leaf part)</td>
<td>0.79 – 0.91</td>
<td>CH₃</td>
</tr>
<tr>
<td></td>
<td>1.25 -1.55</td>
<td>CH₂</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>H-C=O</td>
</tr>
</tbody>
</table>

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Conclusion
The investigation of potent antioxidative constituents from stem and leaf of *L. indica* with the use of chromatographic and spectroscopic analysis established four active constituents namely as 1-(hydroxymethyl)-1,2-ethanediyl ester (LI-1), 9-Oxononanoic acid methyl ester (LI-2), 9,12-Octadecadienoic acid methyl ester (LI-3), 3’,8,8’-Trimethoxy-3-piperidyl-2,2’-binaphthalene-1,1’,4,4’-tetrone (LI-4).

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Conflict of interests
Author declares no conflict of interest.

References


