Bioassay-Guided Isolation and Antioxidant Evaluation of Rutin from Leaf of *Polyalthia longifolia*

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ABSTRACT— ***Medicinal plants are sources of antioxidant compounds that have been reported to protect the human body from the adverse effects of free radicals. Therefore, the present study was intended to identify the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free-radical scavenging constituent from the methanol extract of Polyalthia longifolia leaf using bioassay-guided fractionation. On the basis of DPPH radical scavenging assay-guided isolation, the leaf extract of P. longifolia was separated by employing a solvent partition of methanol leaf extract followed by Medium Pressure Liquid Chromatography (MPLC) and High-Performance Liquid Chromatography (HPLC) fractionation. The ethyl acetate fraction (EtOAc) revealed a strong antioxidant activity, compared to other fractions through in vitro DPPH radical-scavenging assay. The repeated fractionation of active EtOAc by MPLC separation and elution procedure yielded a sub-fraction EtOAc_F007 with strong antioxidant potential. The results indicated that the ethyl acetate sub-fraction EtOAc_F007 has noticeable effects on DPPH radical compared with other sub fractions. The sub fraction EtOAc_F007 was further purified using a preparative HPLC system. Subsequent Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis of the purified constituent from sub-fraction EtOAc-F007 led to the identification of rutin as the antioxidant agent in P. longifolia leaf extract. The results obtained suggested that extracts from P. longifolia leaf have potential use as a bioactive source of natural antioxidants by contributing valuable health effects.***

Keywords— natural antioxidants; Bioassay-Guided Isolation; *P. longifolia*; DPPH radical

1. INTRODUCTION

Plant based products have been used as an important ingredient in traditional medicine systems and also serve as the main source of inspiration for several major pharmaceutical drugs. Natural products play a dominant role in the development of novel drug leads for the treatment and prevention of various diseases [1, 2]. The chemical composition of medicinal plants, especially secondary metabolites is a dynamic research field worldwide, and is the foundation for drug discovery programs [3]. However, today the demand for isolation and characterization of novel metabolites with various biological and pharmaceutical properties from plant extracts has increased among the chemists. Hence, the need to screen plants for pharmaceuticals is particularly urgent in the light of rapid deforestation and the concurrent loss of biodiversity throughout the world.

Bioassay guided fractionation is a process to isolate and identify the active compound from plant extracts that is responsible for certain biological activity. Separation of components in a mixture is commonly done by using chromatography techniques, such as Thin Layer Chromatography (TLC), Gas Chromatography (GC), Medium Pressure Liquid Chromatography (MPLC) and High Performance Liquid Chromatography (HPLC). The characterization of

purified compounds can be described by spectroscopic methods such as mass spectrometry and nuclear magnetic resonance spectrometry. Generally, the isolation of bioactive compounds from natural products generally, combines various separation techniques, which depend on the solubility, volatility and stability of compounds to be separated. Furthermore, the choice of different separation methods for an analytical-scale optimization of the separation parameter is worthwhile [4].

The present study focuses on the isolation of the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free-radical scavenging constituent from the methanol extract of Polyalthia longifolia var. angustifolia Thw. (Annonaceae) leaf since we predicted that the observed genoprotective and hepatoprotective activities of *P. longifolia* leaf extract in our previous studies were solely contributed by its antioxidant properties [5, 6]. *P. longifolia* is a small medium-sized tree with linear-lanceolate leaves, 1 to 1 1.5 cm broad, occurring in Sri Lanka and now grown in tropical parts of India along roadsides and in gardens for their beautiful appearance [7]. *P. longifolia* is one of the most important indigenous medicinal plants and is found throughout Malaysia where it is widely used in traditional medicine as a febrifuge and tonic [8]. The bioassay-directed fractionation was used to isolate the antioxidant compound(s) from methanol the crude extract of *P. longifolia*. In this study, solvent-solvent extraction was undertaken to obtain the partitions followed by MPLC fractionation. The fractions were further subjected for HPLC and LC-MS which are an essential analytical chemistry technique. The antioxidant activity of each fraction was identified using DPPH assay. Hence, the present study was undertaken to isolate the active compounds responsible for the antioxidant property of methanol extract of *P. longifolia* leaf through bioassay-guided fractionation using *in vitro* DPPH assay.

2. MATERIALS AND METHODS

2.1 Plant sample collection

The leaves of *P. longifolia* were collected from various areas in Universiti Sains Malaysia, Penang, in January 2012, and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample has been deposited (Voucher specimen: USM/HERBARIUM/11306). The leaves were separated and cut into small pieces, which were first washed with tap water and then with distilled water. The leaves were then dried in an oven at 60°C for 7 days, after which the dried leaves were ground into a fine powder using a grinder and stored in clean, labelled airtight bottles. The flavonoid compounds are not easily oxidized at 60ºC and this temperature does not degrade the flavonoid compounds [9], which was targeted in this study. Therefore, in this study the leaves were dried in an oven at 60°C for 7 days.

2.2 Solvent extraction

The leaf sample was sequentially extracted with methanol by adding approximately 100 g of the dried sample into 400 mL methanol. The extraction was carried out at room temperature by soaking for 7 days with intermittent stirring during the first day. The extracts were filtered through clean muslin cloth and the extraction process was repeated again for a second time by adding another 400 mL of methanol to the sample residue. The extraction process was repeated a total of three times. The filtrate from each extraction was combined and concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40°C to 50°C in order to evaporate the excess methanol solvent and until a dark green methanol extract was produced and stored at room temperature (RT).

2.3DPPH radical-scavenging assay

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used to determine the free radical scavenging activity of the extract [10]. The reaction mixture contained 50 µL of different concentrations of the extracts and 5 mL of 0.04% (w/v) solution of DPPH in 80% methanol. After 30 min incubation at room temperature, the absorbance was recorded at 517 nm using a spectrophotometer (HITACHI U-1900 spectrophotometer 200V). The experiment was performed in triplicate. The percentage of the DPPH free radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A0 - A1)/A0] \times 100$$

where A0 was the absorbance of the control and A1 was the absorbance in the presence of the extract or positive control. The IC50 (concentration providing 50% inhibition) values were calculated using the dose inhibition curve in the linear range by plotting the extract concentrations versus the corresponding scavenging effect.

2.4 Bioassay guided isolation of MPLC fractions from the solvent partitions

The *P. longifolia* leaf extract was dissolved in methanol: water (90:10, v/v) before being further partitioned in hexane:methanol:water (100:90:10, v/v/v) and yielded 0.198 g (0.198%) of hexane fraction. Subsequently the aqueous

layer formed was further partitioned in ethyl acetate (100 mL) to yield about 0.303 g (0.303%) of the ethyl acetate fraction. Consequently, the aqueous layer formed was further partitioned in butanol (100 mL) and yielded 1.143 g (1.143%) of butanol fraction [11]. The remaining aqueous layer was collected as a water fraction and yielded 0.094 g (0.094%). The entire fractions were evaporated to dryness in a rotary evaporator (BUCHI). The antioxidant activity of each fraction against DPPH free radical was measured by the DPPH scavenging activity [10]. The hexane (0.198 g), ethyl acetate (0.303 g) and butanol (1.143 g) partitions were then further purified using Medium Pressure Liquid Chromatography (MPLC) (Combi Flash Companion (Isco) Lincoln, Nebraska, USA). The column [4g RediSep flash column, (CV 4.8 mL- 18 mL/min flow range), max pressure 600 psi, USA], was packed with Silica gel (Merck, silica gel 60). The column was loaded with the active hexane, ethyl acetate and butanol partition and the fractions were eluted with hexane-acetone with gradient concentrations [10-100 v/v (%)]. A total of 20, 22 and 90 MPLC-fractions were collected for hexane, ethyl acetate and butanol, respectively, through the separation and elution procedure. The peaks were detected by UV at 254 nm by a DAD detector. All fractions of the same compound were combined, and evaporated. Eight sub-fractions (F1-8) were obtained for all three partitions hexane, ethyl acetate and butanol, respectively. All the sub-fractions were evaporated to dryness under reduced pressure using rotary evaporator (BUCHI) before being subjected to DPPH assay**.**

All the active sub-fractions were further analyzed using liquid chromatography/mass spectrometry (LC/MS) with a quadrupole ion trap MS (Bruker Esquire LC/MS, Billerica, MA, USA). The column used was a Symmetry (Waters) C column (250 × 4.6 mm). A 25 µL sample volume was injected using the system's auto sampler. Solvent A contained 5% formic acid in water, and solvent B consisted of HPLC-grade methanol. The UV response during LC/MS was monitored at 360 nm, the highest absorbance wavelength for each set of components as determined from prior HPLC studies. The antioxidant activity of each fraction was determined using DPPH assay.

2.5 Purification of sub-fraction EtOAc_F007 using preparative HPLC

Based on the DPPH antioxidant activity studies, the sub-fraction EtOAc_F007 showed significant activity. Therefore, the antioxidant active sub-fraction EtOAc_F007 was further purified using preparative HPLC (Waters 600 controller) with a Senshu Pak, PEGASIL ODS C18 column (20ø x 250 mm). The major active compound of the fraction EtOAc_F007 was obtained by preparative HPLC. The solvent systems were optimized using acetonitrile with 0.05% aqueous formic acid (solvent A) and acetonitrile with water (solvent B) at different concentration gradients (10-90%) over time and the best separation condition was chosen to draw a defined single peak. The injection volume was 100 µl (10 mg/mL). The fractions were eluted at a flow rate of 10 ml per min over 40 min and monitoring at 200 to 450 nm. The gradient then changed from 10% to 60% (A) over 5 minutes, followed by 60–100% (A) for 50 minutes, and, finally, an isocratic elution of 100% (A) from 20 to 60 min. The separation on preparative-HPLC was repeated three times to procure the compound in sufficient quantity for identification of the antioxidant compound and antioxidant testing. The purity of the purified compound was further confirmed by running under similar HPLC conditions.

2.6 Identification of antioxidant compound using mass spectrometry analysis

Identification of the prominent peak antioxidant compound with 11.862 retention time was performed using the Agilent 1200 series Rapid Resolution liquid chromatography (RRLC) system tandem with an Agilent 6520 Accurate-Mass quadrupole time of flight mass spectrometer (QTOF-MS) (Agilent Technologies, USA). The RRLC system consisted of a vacuum solvent degassing unit, a capillary pump and an automatic sample injector. The MS was equipped with an electrospray ionization (ESI) interface and operated in positive mode with a mass range from 50-1600. ESI conditions were as follows: fragmentor voltage 175 V; nebulizer pressure 30 psi; capillary voltage 3500 V; gas temperature 325ºC, gas flow 5 L/min and skimmer 65 V.

The chromatography was performed using ZORBAX SB-C18 column (0.5 x 150 mm, 5.0 µm) (Agilent Technologies, USA). The auto-sampler compartment was maintained at 4ºC and the mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The following multi-step linear gradient was applied: 0 min, 3% B; 3.75 min, 20% B; 8.75 min, 40% B; 15 min, 90% B; 17.5 min, 90% B; 22.5 min, 3% B. The initial condition was held for 10 min before the next analysis. The injection volume was 10 µL and the flow rate was 20 µL/min. For MS/MS analysis, collision energies of 5 eV intervals from 5 to 20 eV were used. Other conditions were the same as the MS analysis. Autotune and mass calibration was performed using a proprietary mixture of compounds (Agilent Technologies, USA).

Agilent Mass Hunter Workstation software (version B.04.00) was used for data acquisition and processing. The results of MS analysis were then searched through the Chemspider databases; while the MS/MS spectra were matched using the Mass Frontier™ 6.0 (Thermo Fisher Scientific Inc, USA) in order to compare the fragment spectra for verification purposes.

2.7 Statistical Analysis

Statistical analysis involved the use of the statistical package for social sciences (SPSS). Data are given as the Mean ± SD (n=3). For statistical analysis, one-way ANOVA with Duncan's variance (SPSS 15) was used to compare the groups. In all the cases a difference was considered significant when $P<0.05$.

3. RESULTS

3.1 Bioassay guided fractionations of P. longifolia crude extract

The crude *P. longifolia* extract was partitioned with various solvent systems, namely, hexane, ethyl acetate and butanol and yielded hexane, ethyl acetate and butanol partition which were evaporated and weighed. The yield was 0.198 g, 0.030 g, 1.143 g and 0.094 g respectively. Then each partition (except water) was further chromatographically fractionated using the MPLC separation and elution procedure. Hexane, ethyl acetate and butanol fractions yielded eight sub-fractions after the combination of MPLC-fractions according to the peaks detected by UV at 254 nm. The MPLC subfractions were collected into tubes then evaporated and weighed.

3.2 Evaluation of antioxidant activity of sub-fractions

The antioxidant activity of each sub-fraction from all three solvent systems (namely hexane, ethyl acetate and butanol sub-fractions) were tested for its DPPH free radical scavenging activity by using DPPH assay (Figure 1). The results indicated that the ethyl acetate sub-fractions EtOAc_F007 and EtOAc_F008 and butanol sub-fractions BuOH_F003-BuOH_F007 have noticeable effects on the DPPH radical as compared with the hexane sub-fractions. However, the percentage of DPPH free radical scavenging activity among the sub-fractions compared with crude extract was further increased ($P < 0.05$). The DPPH free radical scavenging activity of hexane sub-fraction 1-8 (Hex_F001-F008) was lower than the *P. longifolia* crude extract (Figure 1a).

In addition, among the ethyl acetate sub-fractions, EtOAc_F007 and EtOAc_F008 exhibited significant ($P< 0.05$) radical scavenging activity against DPPH free radical and was the highest when compared with the *P. longifolia* crude extract and other fractions (Figure 1). Meanwhile, the DPPH free radical scavenging activity for butanol sub-fractions BuOH_F003 to BuOH_F007 have showed the higher scavenging activity than sub fractions BuOH_F001, BuOH_F008 and *P. longifolia* crude extract (Figure 1c). Moreover, among the ethyl acetate sub-fractions, EtOAc_F007 was exhibited least number of peaks (two peaks) in the HPLC analysis (data not shown) when compared with *P. longifolia* crude extract and other fractions. Therefore, sub-fraction EtOAc_F007 was used in this study to isolate the antioxidant compound.

3.3 Purification of sub fraction EtOAc_F007

The sub-fraction EtOAc_F007 was further purified using the preparative HPLC system described in Section 2.4 to identify the presence of the free-radical scavenging constituent in the active sub-fraction using LC-MS/MS analysis. The sub-fraction EtOAc_F007 yielded 3.1 mg of yellow color compound, which gave a prominent peak at 11.862 retention time (Figure 2).

3.4 Identification of antioxidant compound using mass spectrometry analysis

The mass spectra obtained in positive full scan mode revealed the dominant protonated ion $[M+H]+$ at m/z 611.1596 with the abundance of ~ 8.5 x 105 (Figure 3). In order to provide a full characterization of this isolated antioxidant compound, a MS/MS product ion scan in positive mode at different collision energies (5-40 eV) was carried out. The collision energy of 10 eV was found to be the best voltage that maximizes the sensitivity for the study of different fragmentation degrees from the precursor ion. The experimental MS/MS fragments were then matched with the predicted MS/MS fragment ions from the Mass Frontier to establish the characterization of the compound including the structural information and their fragmentation pathways.

Figure 3 shows the MS/MS spectra and proposed fragmentation pathways for the isolated antioxidant compound at the collision energy of 10 eV. Ion spectra, with the values of 611.1580, 465.1019 and 303.0479, were identified as rutin (Figure 4). The m/z value of 611.1580 was consistent with the parent ions $[M+H]+$ of rutin with monoisotopic mass of 610.153381 Da. Its fragmentation pattern produced ions at m/z 465.1019 and 303.0479, corresponding to $[M+H-C6H10O4]+$ and $[M+H-C12H20O9]+$ by losing monosaccharide (146 MW) and disaccharide (308 MW), respectively. Both the ions of m/z 465.1019 and 303.0479 are the products of α, with the β-charge-site rearrangement of the parent ion m/z 611.1580.

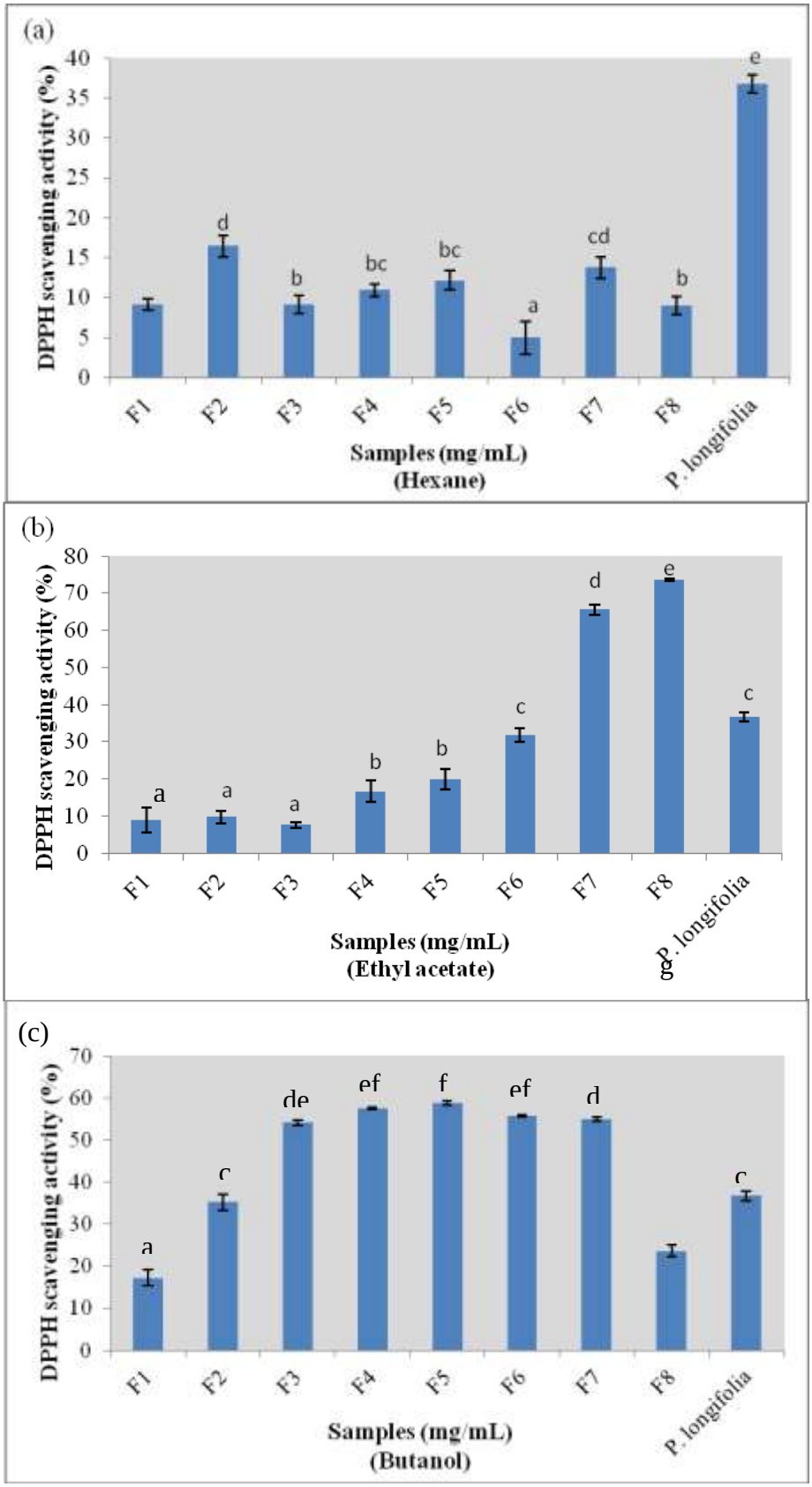


Figure 1: DPPH radical scavenging activity of (a) hexane, (b) ethyl acetate and (c) butanol sub fractions of *Polyalthia longifolia* obtained from MPLC

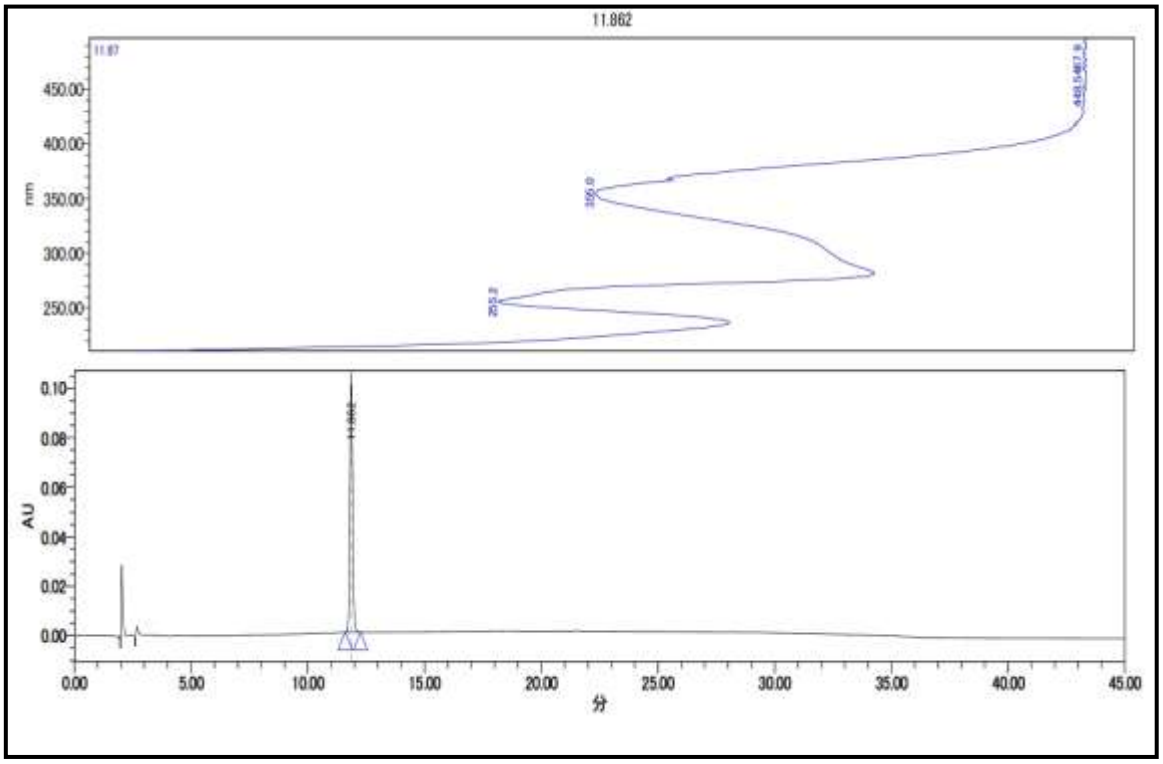


Figure 2: HPLC chromatograms and UV spectrum of antioxidant compound isolated from EtOAc_F007 fraction of *Polyalthia longifolia* leaf at 11.862 retention time

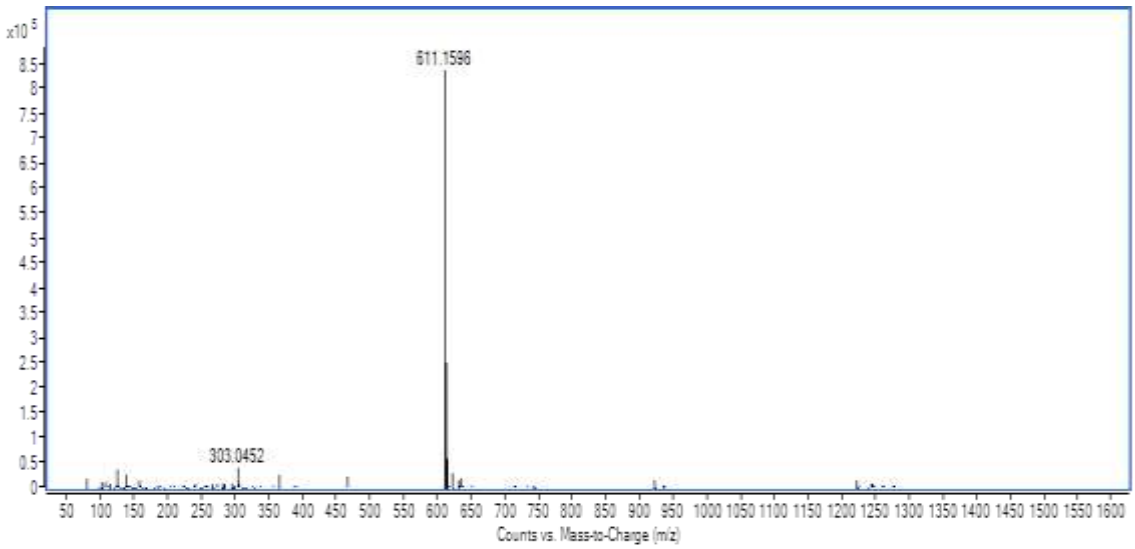


Figure 3: Positive full scan of EtOAc_F007 isolated antioxidant compound from *P. longifolia* leaf using LC-QTOF-MS

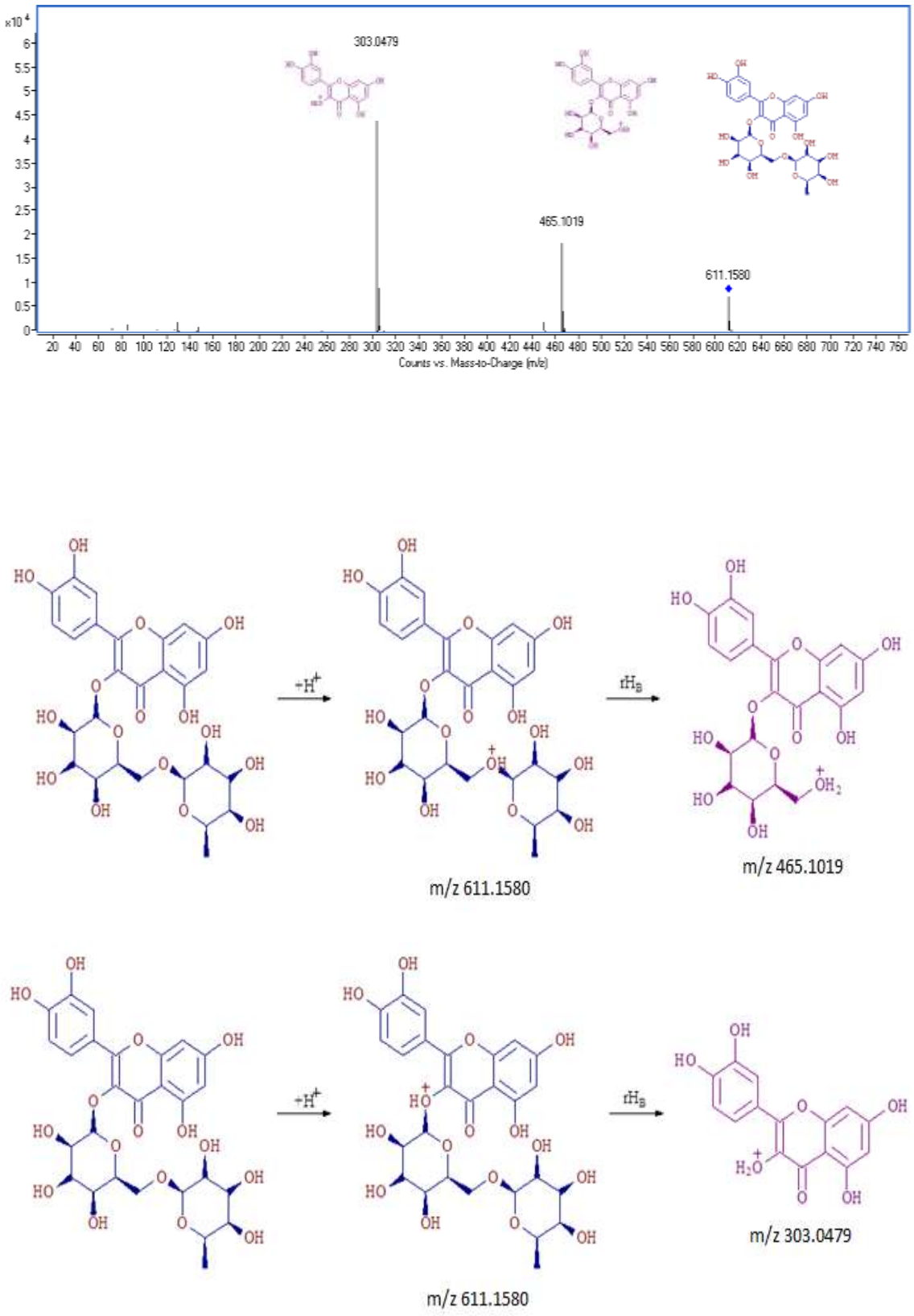


Figure 4: MS/MS spectra and proposed fragmentation pathways for the EtOAc_F007 isolated antioxidant compound from *P. longifolia* leaf at the collision energy of 10 eV.

Figure 5 shows the typical patterns of rutin at a higher collision energy of 20 and 40 eV. When the collision energy increased to 20 or 40 eV, the spectrum of parent ion [M+H]+ disappeared and the ion at m/z 303.0524 became the most dominant ion with 100% abundance. At 20 eV, a smaller peak of 465.1019 (3% abundance) was displayed but a higher peak of 85.0294 (13% abundance) was found at 40 eV. The m/z of 85.0294 is the result of inductive cleavage and the charge-remote rearrangement from m/z 611.1580 (Figure 5). The characteristic spectra at a collision energy of 20 and 40 eV verified that rutin (Molecular Formula: C27H30O16 and Average mass: 610.518 Da) is one of the flavonoids present in the EtOAc_F007 sub-fraction of P. longifolia leaf. The chemical structure of rutin is depicted in Figure 6.

4. DISCUSSION

The isolation of bioactive compounds in a pure state from natural resources, such as *P. longifolia* leaf extract, is essential, yet can be a complicated and time-consuming step in the search of natural product based therapeutic agents. Every attempt to isolate bioactive compounds from natural resources is unique and so the isolation process is not really suited to a practical manual that gives detailed recipe-style methods [12]. However, in this study, an attempt was made to identify the presence of phytochemicals with antioxidant activity using the bioassay-guided isolation procedure. Bioassay-guided isolation is an easy procedure whereby medicinal plant extracts are fractionated and refractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system and only active fractions are further fractionated. The bioassay-guided fractionation method is regularly employed in drug discovery programs due to its efficacy to directly link the analyzed extract and targeted bioactive compounds using the fractionation procedure that followed with certain biological activity [13]. Apart from the available chromatographic separation techniques, the solvent partitioning method was successfully applied to separate the different classes of phytochemical presence in the *P. longifolia* leaf extract in this study.

In this study, the antioxidant activity exhibited by *P. longifolia* leaf extract may be responsible for the observed genoprotective and hepatoprotective activities by *P. longifolia* leaf extract by the scavenging of free radicals [5, 6]. Hence, the antioxidant compound was isolated from the extract using the bioassay-guided isolation technique since most bioassay-guided isolation is a procedure in which the extract is chromatographically fractionated until a pure and active compound is isolated. The *P. longifolia* methanol extract was partitioned in sequence with different solvents in increasing polarity ranging from hexane, ethyl acetate, butanol and water. A crude *P. longifolia* methanol extract is literally a cocktail of compounds. It is difficult to apply a single separation method to isolate individual compounds from this crude mixture. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes by using the solvent partitioning method. The separation technique using solvent partitioning primarily involves the use of two immiscible solvents in a separating funnel, and the compounds are distributed in two solvents according to their different partition coefficients. This method is relatively easy to perform and highly effective as the first step of the fairly large-scale separation of compounds from crude natural product extracts [14].

Medium Pressure Liquid Chromatography (MPLC) is frequently used to enrich biologically active secondary metabolites before further purification by HPLC because it requires less time and has relatively low cost and high sample loading [15]. Therefore, the *P. longifolia* partitions, namely, hexane, ethyl acetate and butanol were further fractionated using MPLC to separate the partitions into its active sub-fractions. The separation of individual compounds from the complex *P. longifolia* leaf extract mixture using MPLC is based on the difference between the adsorption strength of the material in the stationary phase to the solute molecules in the mobile phase. The stationary phase consists of silica gel, which is regarded as a typical polar sorbent, while the mobile phase is usually composed of two solvents. The elution ability can be altered by adjusting the concentration gradients of the solvent system to manipulate the interactions between the isolated compounds and the stationary phase. The concentration of separated compounds reflects the each fractions shade drawn into the glass tubes. The various available hyphenated techniques, such as Liquid chromatography-Mass spectrometry (LC-MS) have made possible the pre-isolation analyses of crude extracts or fractions from *P. longifolia* leaf extract. Hence, in this study, the LC-MS method was used for the preisolation analysis of the active fraction (Data not shown) and sub-fraction obtained from the *P. longifolia* leaf extract to isolate the antioxidant fraction or compound using the bioassay guided isolation technique.

Bioassays can be defined as the use of a biological system to detect the properties (e.g., antioxidant activity) of a crude extract, chromatographic fraction, mixture, or a pure compound. This is particularly important for bioassay-guided isolation, in which the breakdown of active compounds often leads to the loss or reduction of biological activity [16]. Moreover, in the bioassay-guided isolation, the compound is monitored by bioassay at each stage [16]. Therefore, in this study the bioassay-guided isolation technique was employed by using in vitro antioxidant activity to detect the antioxidant properties of a crude extract, partition and chromatographic sub-fraction. In this study, the bioassay guided isolation of *P. longifolia* leaf extract led us to the identification of the potent yellow compound rutin, which is a flavonoid. Rutin (quercetin-3-rhamnosyl glucoside), a natural flavone derivative, was first discovered in buckwheat in the 19th century. It is a low molecular weight polyphenolic compound that is widely distributed in medicinal plants [17]. Rutin has been widely used in treating disease, its several pharmacological activities include antiallergic, anti-

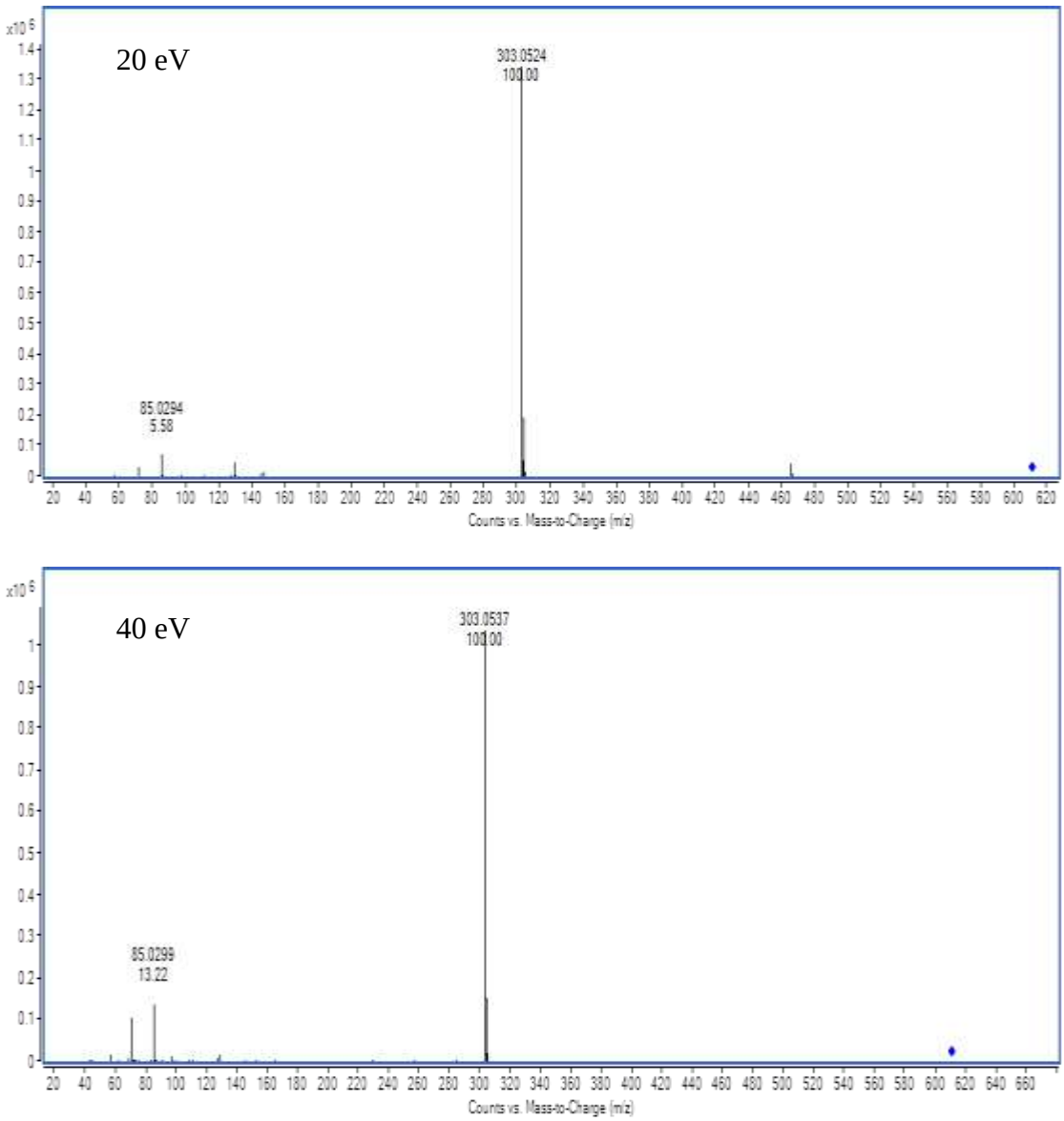
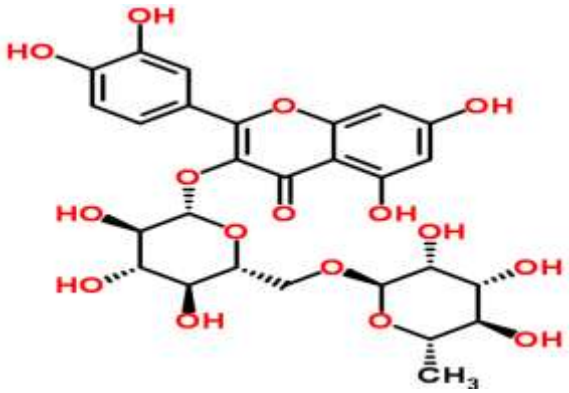


Figure 5: Typical patterns and the percentage of abundance of rutin at higher collision energy of 20 and 40 eV in ESI positive mode.



Rutin

Figure 6: Chemical structure of rutin
Source: http://www.chemspider.com

inflammatory and vasoactive, antitumor, antibacterial, antiviral and antiprotozoal properties [18, 19]. It has also been found to prevent gastric mucosal ulceration in animal models including restraint stress [20]. Interestingly, recent reports reported that rutin exhibits antioxidant and radioprotective activities [21, 22]. The results of the present work also propose that the numerous pharmacological properties exerted by *P. longifolia* leaf extract may be partly due to the presence of antioxidant flavonoid compounds [5, 6].

5. CONCLUSION

In the present study, bioassay-guided isolation has led to the identification of ethyl acetate sub-fractions, EtOAc_F007 with a potent yellow compound rutin as an antioxidant compound in *P. longifolia* leaf extract. Therefore, the results suggest that *P. longifolia* leaf extract with a potent yellow antioxidant compound could be a good candidate for developing antioxidant agents. As *P. longifolia* is used as a traditional medicine and is freely available in Malaysia, it is worthwhile to conducting detailed studies in order to explore the full potential of *P. longifolia* in humans by in vivo evaluation of the compound against free radicals in future studies.

6. CONFLICT OF INTERESTS

The authors declare no conflict of interests.

7. ACKNOWLEDGEMENT

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8. REFERENCES

[1] Newman, D.J., Cragg, G.M., Snadder, K.M., Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod. 66, 1022-1037, 2006.
[2] Gilani, A.H., Rahman, A.U., Trends in ethnopharmacology. J. Ethnopharmacol. 100, 43-49, 2005.
[3] Pitchai, D., Manikkam, R., Rajendran, S.R., Pitchai, G., Database on pharmacophore analysis of active principles, from medicinal plants. Bioinformatic. 5, 43-45, 2010.
[4] Sticher, O., Natural product isolation. Nat. Prod. Rep. 25, 517-554, 2008.
[5] Jothy, S.L., Chen, Y., Kanwar, J.R., Sasidharan, S., 2013. Evaluation of the genotoxic potential against H_2O_2-radical-mediated DNA damage and acute oral toxicity of standardized extract of *Polyalthia longifolia* leaf. Evid. Based Complement Alternat. MedArticle ID 925380, 13 pages, 2013.
[6] Jothy, S.L., Aziz, A., Chen, Y., Sasidharan, S., 2012. Antioxidant activity and hepatoprotective potential of *Polyalthia longifolia* and *Cassia spectabilis* leaves against paracetamol-induced liver injury. Evid. Based Complement Alternat. Med. Article ID 561284, 10 pages, 2012.
[7] Bose, T.K., Das, P., Maiti, G.G., Trees of the world, Vol. 1. Regional Plant Resource Centre, Bhubaneswar, India, 1998.
[8] The Wealth of India, Publication and information directorate, vol. 3, 1st ed. CSIR, New Delhi, India, 1969.
[9] Palma, M., Pineiro, Z., Barroso, C.G, Stability of phenolic compounds during extraction with superheated solvents. J, Chromatogr. A. 921, 169-174, 2001.
[10] Sasidharan, S., Darah, I., Mohd Jain Noordin, M.K., Free radical scavenging activity and total phenolic compounds of Gracilaria changii. Int, J. Nat. Eng. Sci. 1, 115-117, 2007.
[11] Duan, X.J., Zhang, W.W., Li, X.M., Wang, B.G., Evaluation of antioxidant property of extract and fractions obtained from red alga *Polysiphonia urceolata*. Food Chem. 95, 37-43, 2006.
[12] Sarker, S.D., Latif, Z., Gray, A.I., Natural products isolation: an overview. in: Sarker, S.D., Latif, Z., Gray, A.I. (Eds), Natural products isolation. 2nd ed. Humana Press, New Jersey, 2005.
[13] Atta-ur-Rehman, Choudhary M.I., William, J.T., Manual of Bioassay Techniques for Natural Product Research. Harward Academic Press, Amsterdam, pp. 82-84., 1999.
[14] Otsuka, H. Purification by solvent extraction using partition coefficient, in: Sarker, S., Latif, Z., Gray, A.L. (Eds), Natural products isolation. Humana Press, New Jersey, 2nd ed., pp. 269-273, 2006.
[15] Bucar, F., Wube, A., Schmid, M., Natural product isolation- how to get from biological material to pure compounds. Nat. Prod. Rep. 30, 525-545, 2013.

[16] Sarker, S.D., Zahid, L., Alexander, I.G., Natural Products Isolation. in: Sarker, S.D., Zahid, L., Alexander, I.G. (Eds), Methods in Biotechnology. 2nd edn. Humana Press, Totowa, NJ, 2006.
[17] Yang, J., Guo, J., Yuan, J., In vitro antioxidant properties of rutin. LWT- Food Sci. Technol. 41, 1060–1066, 2008.
[18] Rosane, W.I., Oliveira, Z.D., Fernandes, S.C., Vieira, I.C., Development of a biosensor based on gilo peroxidase immobilized on chitosan chemically crosslinked with epichlorohydrin for determination of rutin. J. Pharm. Biomed. Anal. 41, 366–372, 2006.
[19] Calabro`, M.L., Tommasini, S., Donato, P., Stancanelli, R., Raneri, D., Catania, S., Costab, C., Villarid, V., Ficarraa, P., Ficarraa, R., The rutin/b-cyclodextrin interactions in fully aqueous solution: spectroscopic studies and biological assays. J. Pharm. Biomed. Anal. 36, 1019–1027, 2005.
[20] Barnaulow, O.D., Machineva, O.A., Kamissarenk,o, N.F., Comparative evaluation of the effect of some flavonoids on change in the gastric wall of reserpine treated or inmobilized mice. Khim, Farm. Zh. 17, 946–951, 1983.
[21] Patil, S.L., Mallaiah, S.H., Patil, R.K., Antioxidative and radioprotective potential of rutin and quercetin in Swiss albino mice exposed to gamma radiation. J. Med. Phys. 38, 87–92, 2013.
[22] Zielińska, D., Szawara-Nowak, D., Zieliński, H., Determination of the antioxidant activity of rutin and its contribution to the antioxidant capacity of diversifed buckwheat origin material by updated analytical strategies. Pol. J. Food Nutr. Sci. 60, 315-321, 2010.