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[Research article]

Chromatographical fractionation guided by antioxidant activity of *Morinda citrifolia*

Rasha Saad,Muhammad Aqil, Eddy Yusuf, and FadliAsmani.

Pharmaceutical chemistry unit, School of Pharmacy, Management & Science University, 40100 Shah Alam, Selangor DarulEhsan, Malaysia.

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ABSTRACT

Morinda citrifolia Linn. belongs to family of Rubiaceae. Characteristically, the plant is ascribable by its elongated trunk with 8-10 inches long bright green leaves. The leaves are opposite, pinnately veined and glossy. Initially, the subject plant specimen was further authenticated and identified taxonomically by Forest Research Institute Malaysia (FRIM) and have been granted with the specimen number of PID140314-10 for future research. The dried leaves were then extracted with methanol by maceration-ultrasonication extraction method. Methanolic crude extracts were fractionated on open column chromatography with ethyl acetate-hexane (50:50) as eluate. Ten fractions were collected and analyzed for antioxidative activity assay using in-vitro model of 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radical. The results showed that fraction number three exhibited considerably high antioxidative activity comparing with the standard dose of marketed tocopherol. The IC_{30}value of fraction three was 100mg/ml. This fraction together with fraction two, four, eight and nine were subjected to quantitative and qualitative analysis of phytochemical screening. Results for fraction three shows that five of seven phytochemical screened for were present. They are: alkaloid, flavonoid, glycoside, tannin and anthraquinone. Quercetin, tannic acid and emodin reagent were used as standard for calibration of total flavonoids, tannins and anthraquinone content respectively. The quantification of total flavonoid, tannin and anthraquinone content showed 2.74mg/g quercetin, 47.62µg/g tannic acid and 1.142mg/g emodin equivalent respectively, the study indicates that the leaves of *Morinda citrifolia* Linn. serves as a potent source of natural antioxidant activity and exhibit the highest flavonoid, tannin and anthraquinone content.

Keywords: *M.citrifolia*, DPPH, phytochemical, quercetin, tannic aicd and emodin.

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INTRODUCTION

An edible tropical medicinal plant-*Morinda citrifolia* (Noni) is a traditional medicinal plant belonging to the family Rubiaceae (Samuel et al., 2012). It is ascribable by its elongated trunk with 8-10 inches long bright green leaves (Rivera et al, 2012). It bears peculiar "grenade-like" unripe green to ripe yellowish gawky fruit which softens to lucid an overly ripen form. Due to its lousy taste and odour, it is not preferred to eat by the people (Su et al, 2005). The ancestors of Polynesians, who are believed to have brought Noni from south Asia, have utilized the whole Noni plant in various combinations (Brown, 2012). The roots, leaves,

*** Corresponding author: Rasha Saad.**
E-mail address: rshoo70@yahoo.com

bark and the fruit are widely used for its medicinal values to treat various ailments including breast cancer and eye problems (Su et al, 2005).

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Zima et al., 2001).

Free radicals rise to oxidative stress may contribute to more than one hundred disorders in human beings, including atherosclerosis, arthritis, ischemia, central nervous system disorders, gastritis, cancer and AIDS (Chatterjee et al., 2013). Free radicals due to environmental pollutants, radiations, Chemicals, toxins, deep fried and spicy foods, as well as physical stress, cause depletion of immune system, change in gene expression and induce abnormal proteins. Due to depletion of immune system, natural antioxidants in different maladies consuming antioxidants as free radical scavengers may be necessary (Chatterjee et al., 2013). Antioxidants are the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance (Praveen et al., 2007).

Due to well-known healthy effect to human, increase in the intake of foods rich in antioxidant compounds are generally recommended to the consumers. As a result of these beneficial evidences, the search for antioxidants principles had starting to be accelerated, which led to the identification of natural resources and isolation of active antioxidant molecules.

Following in response to the increased popularity and greater demand for medicinal plants, a number of conservation groups are recommending that wild medicinal plants be brought into cultivation (Noomanet al, 2008).

MATERIALS AND METHODS

The Study Area

The research was carried out at research laboratory of Management and Science University within the month of February 2014 until end of May 2014.

Chemical Reagents

The marketed product of α-tocopherol (Natopherol®), standard Quercetin, Tannic Acid, Emodin and 2,2- diphenyl-1-picrylhydrazyl radical (DPPH) were purchased from Sigma- Aldrich. The solvents used were of analytical grade.

Plant Material

Fresh plant materials were collected from Subang, Selangor, Malaysia. The authentication and taxonomic identification was carried out by Forest Research Institute Malaysia (FRIM) and have been granted with the sample number of PID140314-10 for reference.

Figure 1: *Morinda citrifolia* (Family of Rubiaceae)

Extract Preparation

The plant extracts were prepared as described by Aqil& Ahmad (2007) with modification. The leaves of *Morinda citrifolia* L.was collected and dried in an oven (35 ± 2^{o}C) for 3 days. The dried materials was coarsely powdered and 30g of the same was soaked in a 200ml of methanol for 48 hours with occasional ultrasonication by setting a duration of every 5 minutes for period of 30 minutes to prevent heating in the ultrasonicator bath with the continuous operation. The extract was passed through Whatman filter paper No. 1. It was concentrated by rotary evaporator setting 40^{o}C for 40 minutes. 500mg of the crude extract was weighed in a dry clean vial and was used for further works.

(a) (b) (c)

Figure 2: (a) Maceration of the leaves, (b) Filtration process, and (c) Drying process of the filtrate using rotary evaporator

Fractionation of Column Chromatography

Five gram of silica gel was prepared by dissolving with ethyl acetate-hexane (50:50) as eluate until slurry is formed. With the continuous stirring of the slurry solution, directly poured into the open column following with the addition of a dissolved of crude extract. Ten fractions of 10ml each were collected. Repetitions for another two times with a new set up of column chromatographical fractionation were conducted .

Figure 3: Fractionation process of Morindacitifolia leaves extract

Figure 4: Collection of ten fractios from chromatographical fractionation of the crude extract

Estimation of Radical Scavenging Activity using DPPH Assay

Each fraction collected was prepared from 5 different concentrations (1mg/ml, 2mg/ml, 3mg/ml, 5mg/ml and 10mg/ml) using methanol as a solvent for dilution. 1 ml of every fraction was taken and pours into separate test tube. Each test tube was labelled as 1 to 10 according to the fraction number. Each of the test tubes was added of 2 ml of 0.1 mMDPPH. After 30 min of incubation at room temperature, absorbance was measured at 517 nm using UV/VIS Lambda 45, against methanol as the blank. One control containing 1 ml of marketed tocopherol and 2 ml of 0.1 mM of DPPHwas used as reference compound. The experiment was carried out in triplicate. The scavenging activity (%) on DPPH radical was calculated according to the following equation:

(% of DPPH Radical Scavenging Activity) = A0-A1/A0 x 100).

Where A0 is the absorbance of DPPH radical with methanol; A1 is the absorbance of DPPH radical with plant extract. Measurements were performed in triplicate.

Phytoconstituent Screening

Every fraction was subjected to phytochemical studies to find out the presence of compounds such as alkaloids, tannin, flavonoids, glycosides, phlobatannins, saponin and anthraquinone.35 test tubes were prepared for 3 active and 2 least active fractions and each test will be conducted in one test tube for one fraction.

Test for Alkaloids

3ml of fraction will be stirred with 3 ml of 1% HCl on a steam bath. Mayer's reagents will then be added to the mixture. Turbidity of the resulting precipitate will be taken as evidence for the presence of alkaloids.

Test for flavonoids:

2 ml of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

Test for Glycosides

2 ml of fraction will be dissolved in 2 ml of chloroform. 2 ml of sulphuric acid will be added carefully and shaken gently. A reddish brown colour indicates the presence of a steroidal ring (i.e., a glycone portion of glycoside).

Test for Tannins

2 ml of distilled water will be added into 2 ml of the active fraction. A few drops of feric chloride will be added and observed for brownish green coloration.

Test for phlobatannins

About 2ml of fraction will be added to 2ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate will be taken as an evidence for the presence of phlobatannins.

Test for Saponins

2 ml of active fraction will be shaken vigorously with 2 ml of distilled water in a test tube and warmed. The formation of stable foam will be taken as an indication for presence of saponin.

Test for Anthraquinone

3 ml of the fraction was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Quantification Analysis of Phytochemical Constituents

The most active fraction was subjected to undergo quantitative analysis of flavonoid, tannin and anthraquinone content using quercetin, tannic acid and emodin as standard respectively.

Estimation of Total Flavonoid Content

The total flavonoid content of highest fraction was determined by UV–visible spectrophotometer using aluminium chloride (Kumar et al., 2008). 5 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the highest antioxidant fraction (10 mg/ml). Absorption readings at 415 nm using UV-VIS spectrophotometer were taken after 10 minutes against a blank sample consisting of a 5 ml of highest antioxidant fraction with 5 ml methanol without $AlCl_3$.

The calibration curve was prepared by using methanol solution of quercetin (standard) with concentrations ranging from 2 to 6mg/ml. The total flavonoid content was expressed in terms mg/g weight of crude extract. The test for concentration, of fraction 3 and 2 were performed three times which obtained from 3 times column chromatographical fractionation.

Estimation of Tannin Content

Content of tannins in highest antioxidant fraction was determined by Folin Denis method (Polshettiwar SA et al., 2007). Spectrophotometric estimation of tannins is based on the measurement of blue colour formed by the reduction of phosphotungstomolybdic acid by tannin like compound in alkaline medium. 1ml of the highest antioxidant fraction and standard solution of tannic acid (50-150 µg/ml) was made up to 7.5ml with distilled water. Then 0.5ml Folindenis reagent and 1ml Na_2CO_3solution were added. The volume was made up to 10ml of distilled water and absorbance was measured at 700nm. The total tannic acid content was expressed as µg of tannic acid equivalent per g of crude extract.

Estimation of Anthraquinone Content

Accurately weighed emodin (5mg) was, transferred to 10ml beaker and dissolved with 3.33ml of methanol to get a standard solution of 1.5mg/ml. Various concentrations (0.5-1.5 mg/ml) of standard solution were prepared using methanol. The absorbance was measured at 437nm against a solvent blank (Suchetaet al., 2011).

RESULTS

Morinda citrifolia under family of Rubiaceae was used for the research. Ten fractions from various concentration of methanol extract of *M.citrifolia* leaves (10-100mg/ml) were tested for their antioxidant activity using in-vitro models. It was observed that free radicals were scavenged by the test compounds in a dose-dependent manner.

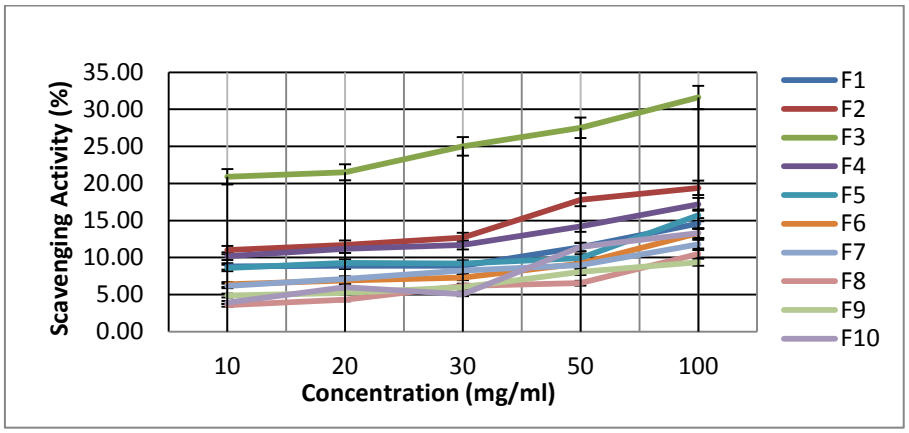


Figure 5: Result of radical scavenging activity of fractions from different concentration of extract against DPPH

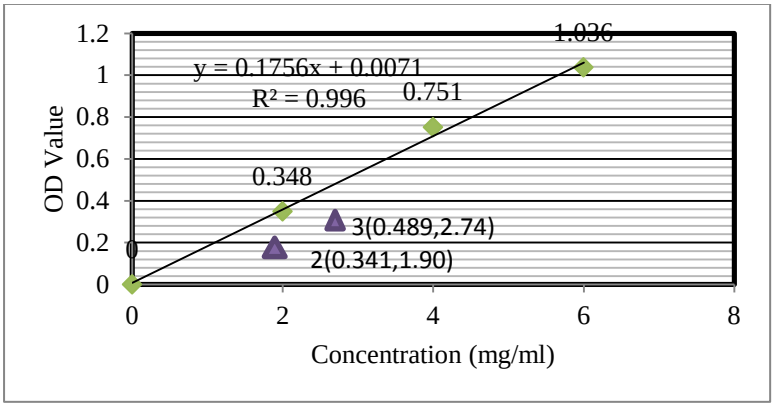
Figure 5 shows that DPPH radical scavenging activity results for ethyl acetate-hexane fractions from methanol extract of *M.citrifolia*and brand product antioxidants Natopherol®. Discolouration of the stable free DPPH radicals by antioxidants in the sample was measuredspectrophotometrically. The IC_{30} value and annihilation of DPPH radical was expressed in % inhibition after 30 minutes. The result showed maximum free radical scavenging activity was observed in 100mg/ml of fraction 3 against the standard dose of marketed α-tocopherol (Natopherol®) of 268mg/ml. The effect of antioxidants on DPPH might to be due to their hydrogen donating ability (Samuel et al., 2012). The DPPH radical scavenging ability of the fractions from the methanolic extracts was significant when exposed comparing to that of tocopherol. It was evident that the extract did show proton donating ability and could serve as a free radical inhibitor or with scavenging activity possibly as a primary antioxidant.

Table 1: Result of preliminary qualitative screening of phytochemicals in fractions 2, 3, 4, 8 and 9 obtained from chromatographical fractionation of *Morinda citrifolia* of 100 mg/ml

Test	Active Fractions				
	2	3	4	8	9
Alkaloid	+	+	-	-	-
Flavonoid	+	+	-	-	-
Glycoside	-	+	+	+	+
Tannin	+	+	-	-	-
Phlobatannin	-	-	-	-	-
Anthraquinone	-	+	+	-	-
Saponin	-	-	-	-	-

Results obtained from preliminary qualitative screening of phytochemicals in leaves of *M.citrifolia*L.are presented in Table 1. Of the seven phytochemicals screened for, five were found present in fraction 3. They are alkaloid, flavonoids, tannins, glycoside and anthraquinone. However, comparing with the least of antioxidant ability, fraction 8 and 9 only demonstrate the present of glycoside by given out brick red precipitate. Remarkably, saponin and phlobatannin were not present in fraction 2, 3, 8 and 9. In addition, anthraquinone is solely present in the fraction 3 by giving light green colour change

Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. Figure 6 shows the contents of total flavonoids were estimated by the standard curves and expressed as quercetin equivalents for flavonoids. The amount of total flavonoid content in the fraction 3 showed 2.74mg/g weight of crude extract and fraction 2 showed 1.90mg/g weight of crude extract, expressed as quercetin equivalents.



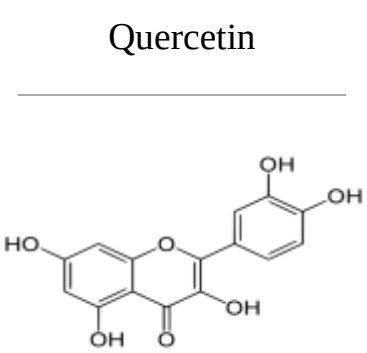


Figure 6: Result of flavonoid contents (Quercetin) in fraction 2 and 3 from standard calibration curve and their respective optical density (OD) at 415nm

Tannic acid is a plant polyphenol which is found, along with other condensed tannins, in several beverages including red wine, beer, coffee, black tea, green tea, and many foodstuffs such as grapes, pears, bananas, sorghum, black-eyed peas, lentils and chocolate (Ilhamiet al., 2009). Similar to many polyphenols, tannic acid has been shown to possess antioxidant (Andrade et al., 2005), antimutagenic (Ferguson, 2001) and anticarcinogenic properties (Sangkilet al., 2001). It was reported that the polyphenolic nature of tannic acid, it's relatively hydrophobic "core" and hydrophilic "shell" are the features responsible for its antioxidant action (Isenburget al., 2006). Also, several authors have demonstrated that tannic acid and other polyphenols have antimutagenic and anticarcinogenic activities. Moreover, the consumption of polyphenol-rich fruits, vegetables, and beverages, such as tea and red wine, has been linked with inhibitory and preventive effects in various human cancers and cardiovascular diseases, which may be related-at least in part-with the antioxidant activity of polyphenols (Andrade et al., 2005). Figure 7shows the contents of tannin were estimated by the standard curves and expressed as tannic acid equivalents for tannin. The amount of tannin content in the fraction 3 showed 47.62µg/g weight of crude extract which lower comparing with fraction 2 showed 113.96µg/g weight of crude extract, expressed as tannic acid equivalents.

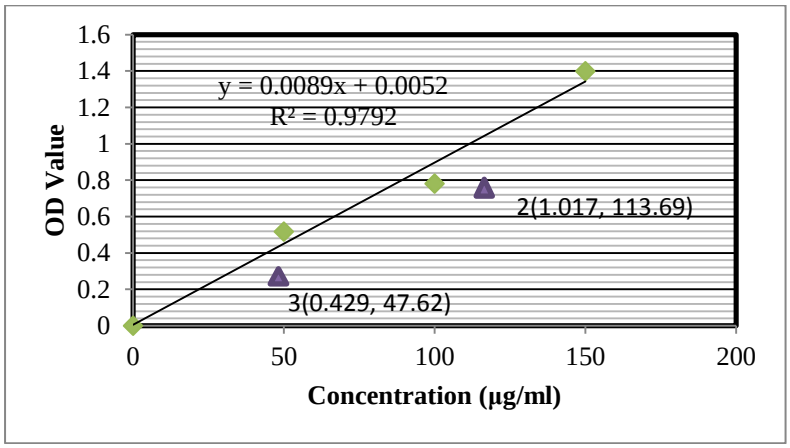


Figure 7: Result of tannin (Tannic acid)contents in fraction 2 and 3 from standard calibration curve and their respective optical density (OD) at 700nm

Emodin is a biologically active, naturally occurring anthraquinone derivative (Subashet al., 2005). These anthraquinone derivatives are well known to exhibit a variety of biological activities, such as antibacterial and antifungal (Chatterjee et al., 2010), antitumor (Koyama et al., 2001), antioxidant (Chatterjee et al., 2013), cytotoxic and hypoglycaemic activities (Choi et al., 2006). Presence of significant amount of tannin, anthraquinones and flavonoids in the methanolic leaf extract of *M.citrifolia* indicates strong antioxidant properties of this plant. Since the phytoconstituent is only available in the fraction 3 through qualitative test, thus quantification is conducted solely to it which the contents of anthraquinone were estimated by the standard curves and expressed as emodin equivalents. Stability of absorbance is of major importance in spectrophotometric measurements. The time which absorbance at 437 nm of emodin in methanol was remain stable after 1 hour. From the UV-VIS spectra it is clarified that the 437 nm was the maximum absorption wavelength for emodin.(Gaikwad A Suchetaet al., 2011). The amount of anthraquinone content in the fraction 3 showed 1.142mg/g weight of crude extract expressed as emodin equivalents presented in Figure 8.

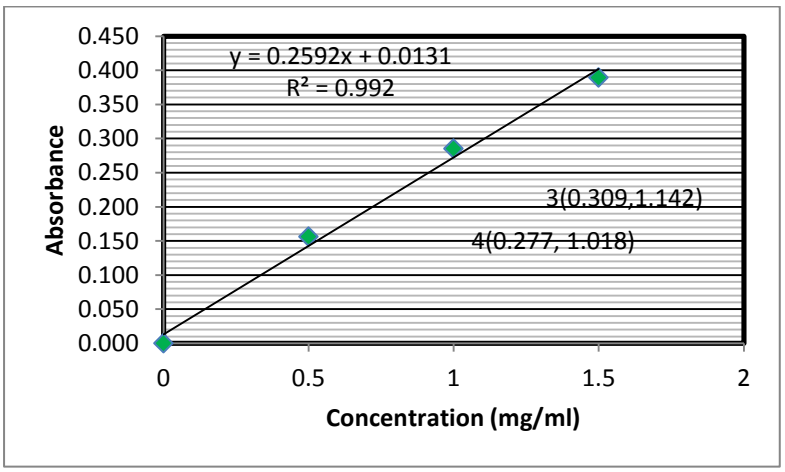


Figure 8: Result of Anthraquinone(Emodin)Content in fraction 3 from standard calibration curve and their respective optical density (OD) at 437nm

DISCUSSION

The model of scavenging, the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Ebrahimzadehet al, 2008). This method is also very convenient for the screening of large numbers of samples with different polarity. The effect of antioxidant on DPPH radical scavenging was thought to be their hydrogen donating ability or radical scavenging. Assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH. DPPH is a stable nitrogen-centred free radical. The colour changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances, which are able to perform this reaction, can be considered as antioxidants and hence they are radical scavengers (Dehpouret al, 2009).

CONCLUSION

It was confirmed that *M.citrifolia* extracts could act as hydrogen and/or electron donors and react with free radicals, converting them into more stable products and subsequently terminating radical chain reactions. *M.citrifolia* could be developed as an effective antioxidant against several kinds of oxidative degenerative diseases such as cancer and liver diseases. Utilization of this plant product will be of advantage to humanity and increased consumption will help in prevention of chronic life style diseases.

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