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Isolation and Characterization of Quercetin Glycosides and Antioxidant Phytochemicals from *Azadirachta indica* Leaf Extract

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Abstract

Neem (Azadirachta indica) is consumed as vegetable in some parts of Southeast Asia, and as medicine and insecticides. This study reports the quantitative analysis of the major and minor antioxidant phytochemicals from its leaves, isolation and characterization process of them. Antioxidant activities were characterized by the ability of reducing power, DPPH scavenging, hydroxyl radicals scavenging and hydrogen peroxide scavenging. All antioxidant assay results showed that ethyl acetate fraction exhibited the strongest antioxidant activities and the highest total phenol content. Two major compounds (quercetin-3-O-galactoside and quercetin-3-rhamnoside) and four minor compounds (ferulic acid, myricetin, kaempferol and quercetin) were isolated and identified from the ethyl acetate extract using silica gel and Sephadex LH-20 column chromatography. The structures of the major compounds were identified from UV-Vis and LC-MS-MS chromatograms.

1. Introduction

Various parts of the neem tree have been used for food, medicine, and insecticides since ancient times [1]. Sithisarn, et al. reported that in Thailand the tree tender shoots and flowers are eaten as a vegetable after the fresh young leaves and flowers are soaked in boiled water for a few minutes, they are eaten with sweet sauce, cooked fish and rice; and in each meal a total amount of 300 - 500 g of the material is consumed [2]. There are studies on incorporating neem products in diet [3,4]. In India neem is very popular for its use in Ugadi Pachhadi (soup like pickle). It also used in parts of mainland Southeast Asia, particularly in Cambodia; in Myanmar the young leaves and flower buds are boiled with tamarind fruit to soften its bitterness and eaten as a vegetable. Moreover, the plant has a wide spectrum of biological activities such as larvicidal, anti-bacterial, anti-diabetic, anti-ulcer, anti-malarial, anti-tumour, anti-inflammatory, anti-oxidant and more [5,6].

Active oxygen and, in particular, free radical is considered to induce oxidative damage in biomolecules and plays an important role in aging, cardiovascular diseases like atherosclerosis hypertension, asthma, diabetes mellitus, cancer, and inflammatory diseases [7]. In recent years, there has been increasing interest in the use of natural food additives and incorporation of health-promoting substances into the diet. Natural phenolic compounds, such as flavonoids and phenolic acids, are known to have high antioxidant activity. Hence there is an increasing interest in antioxidants from natural rather than from synthetic sources [8] and a diet, which includes fruit and vegetable in every meal seems to be important in order to control oxidative stress.

A large number of compounds have been isolated from various parts of neem, but only a few of them have been studied for their biological activity. The most studied compounds are the limonoids, azadirachtin and nimbolide, and several triterpenes (Pankaj, Lokeshwar, Mukesh & Vishnu, 2011). Sithisarn et al. [2] reported an antioxidant potential of neem from various localities of Thailand, hence, the aim of this work was to investigate the antioxidant potential of various solvent extracts of Indonesian neem leaves and to isolate and characterize antioxidant compounds from solvent extract with the highest antioxidant activity.

2. Materials and methods

2.1. Sample and standards

Powder of dried neem leaf was purchased from PT. Intaran (Bali, Indonesia) with a moisture content of 6%. The obtained neem powder was dried at 30°C. Thin layer chromatography (TLC) silica gel (60 F254) aluminum sheet (20 x 20 cm) was purchased from Merck (Darmstadt, Germany). Advantec filter paper was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). Silica gel (70-230 mesh) was obtained from Silicycle (Quebec, Canada). Characteristics of silica gel according to the manufacturer were: particle size = 60-200 µm; pore size = 60 Å; pH = 7; water content = 6%; and specific surface area = 500 m2/g. Phenolic acid standards (ferulic acid, quercetin, myricetin, and kaempferol) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Aldrich Co. (St. Louis, MO). All solvents were HPLC grade and obtained from

commercial sources.

2.2. Extraction and solvent partitioning

For crude extract (CE) preparation, dried sample (200 g) was extracted three times with 2 L of 80% ethanol at 25 ºC on an orbital shaking incubator for 24 h. Each extract was filtered using filter paper (Whatman No.1). All three filtrates were combined and solvent was removed by using a rotary evaporator (EYELA Rotary Evaporator, N-1000, Japan) under reduced pressure at 45 ºC. The filtrate obtained was suspended in water and n-hexane was added. The mixture was well mixed and then allowed to separate in a separating funnel to obtain the n-hexane fraction (HF). The remaining water fraction was extracted with ethyl acetate using the same protocol to obtain ethyl acetate fraction (EaF). Butanol was then used to extract the resulted water fraction to get the butanol fraction (BF). The final water suspension was filtered using cotton to obtain the water fraction (WF). The water fraction was dried using a freeze dryer (LABCONCO, 2.5 Free Zone, USA). These solvents with varying polarities will theoretically extract different plant constituents. Four partition fractions of extract were obtained, viz. hexane fraction (0.87 g), ethyl acetate fraction (2.84 g), butanol fraction (9.89 g), and aqueous fraction (25.01 g) with an operation loss of 9.49 g.

2.3. Antioxidant activity

2.3.1. Reducing power

The reducing powers of the extract fractions and standard α-tocopherol were determined according to the method previously described [10]. Samples (CE, HF, EaF, BF or WF) at different doses (50, 100, 250 and 500 µg/ mL) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 ºC for 20 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture, which was centrifuged for 10 min at 3000 x g (Hettich zentrifugen, Mikro 20, Germany). The upper layer of the solution (2 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrometer. Higher absorbance of the reaction mixture indicated greater reducing power.

2.3.2. Radical scavenging activity

The technique using 96-well microplate [11] was employed, in a slightly modified way, to measure the ability of sample in quenching the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The stock solution (300 µg/ mL) was diluted to varying concentrations (0.2-200 µg/ mL) in a 96-well microplate (a final volume of 100 µL for each well). Sample or standard was mixed with 100 µL of DPPH (0.051 mM in methanol) in each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminum foil and placed in dark for 30 min at 25 ºC. Then the absorbance was measured at 517 nm using an ELIZA reader. A methanol solution of DPPH served as a control. Percentage inhibition was calculated using equation 1. From the equation, the concentration necessary to decrease by 50% the initial DPPH concentration (IC50) was calculated.

$$Scavenging\ effect\ (\%) = \frac{(Control\ absorbance - Sample\ absorbance)}{(Control\ absorbance)}\ X\ 100 \quad \text{……..(1)}$$

2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and samples for hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system, Fenton reaction [12]. The reaction mixture in a test tube containing, in a final volume of 1.0 mL, 100 µL 2-deoxy-2-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH7.4), 500 µL of various concentrations of samples and standard (10, 20, 40, 80, and 200 µg) in KH_2PO_4-KOH buffer (20 mM, pH7.4), a mixture of 100 µl EDTA (1.04 mM) and 100 µl $FeCl_3$ (200 µM), 100 µl H_2O_2 (1.0 mM), and 100 µl ascorbic acid (1.0 mM), was incubated at 37 ºC for 1 h. 1 mL of 1% thiobarbituric acid and 1.0 mL of 2.8% trichloroacetic acid were added to the test tube and incubated at 100 ºC for 20 min. After cooling, absorbance was measured at 532 nm using a spectrophotometer, against a control preparation containing deoxyribose and buffer. Quercetin was used as a positive control. Reactions were carried out in triplicate. Percent inhibition was determined by comparing the results of the test and control compounds in equation 1.

2.3.4. Hydrogen peroxide-scavenging activity

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined from absorption at 230 nm in a spectrophotometer. Sample (0.9 mL) with a concentration of 100-500 µg/ml in deionised distilled water, was added to 0.6 ml hydrogen peroxide solution (40 mM) [13]. Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution of phosphate buffer without hydrogen peroxide. The hydrogen peroxide scavenging activity of the all solvent fractions, the crude extract, the oil and the standard compound BHT was calculated using equation 1.

2.4. Total phenol content

The total phenol content was measured using the Folin-Ciocalteu following the method described by Kim et al. [14] in a slightly modified way. In brief, a sample of 200 µL of each sample (1 mg/ mL) was added to a test tube, and deionized distilled water (1.8 mL) was added. Folin-Ciocalteu's phenol reagent (200 µL) was added and the solution thoroughly mixed and allowed to stand for 3 min. To further continue the reaction, 400 µL of 7% Na2CO3 solution was added with mixing. The mixed solution was immediately diluted to a total volume of 4 mL with deionised distilled water and mixed thoroughly. After 90 min at 25 ºC the absorbance was read at 750 nm using a spectrophotometer. Deionized distilled water was used as blank. The standard curve for total phenolics was made using Gallic acid standard solution (10-50 µg/ mL) under the same procedure as above. The total phenolic was expressed as milligrams of Gallic acid equivalents (GAE) per gram of dried extracts. Total content of phenolic compounds in the plant extract was calculated using equation 2:

Total phenol content = GAE x V/m ……(2)

Where GAE is the Gallic acid equivalence (mg/mL) or concentration of Gallic acid established from the calibration curve (Y=4.669x-0.013, R^2=0.998); V is the volume of sample (mL) and m is the weight (g) of sample.

2.5. Total flavonoid content

The total flavoinoid content was determined by using aluminum chloride colorimetric method [15]. Briefly, 1 mL of extract (1 mg/ mL) was mixed with 4 mL of deionised distilled water in a test tube, followed by adding 0.3 mL 5% $NaNO_2$ solution. After 6 min, 0.3 mL of 10% $AlCl_3$ solution was added and allowed to stand for 5 min before 2 mL of 1.0 M NaOH was added. The mixture was thoroughly mixed well using vortex. The absorbance was measured immediately at 510 nm using a spectrophotometer. Total content of flavonoids was calculated using equation 3.

Total flavonoid content = CE x V/m ……(3)

Where CE is the Catechin equivalent (mg/mL) or concentration of Catechin

solution established from calibration curve (Y=1.0315x+0.0135, R^2=0.993); V is the volume of the fraction (mL) and m is the weight (g) of the dried solvent fraction. Data was recorded as mean ± SD for three replications. Results were expressed as mg Catechin equivalents per g of dried fraction.

2.6. TLC screening

TLC was used for qualitative screening of the constituents in crude extract. TLC analysis for ethyl acetate extract screening was performed on Silica gel F254 TLC plates using ethyl acetate: dichloromethane (9:1, v/v) as the mobile phase. Reactive compounds were visualized after spraying the plates with a methanol solution of 0.02% w/v DPPH.

2.7. Column chromatography

Column chromatography using silica gel and Sephadex LH 20 stationary phases were used to separate active compounds present in the extracts by activity assay-guided fractionation. The ethyl acetate extract was subjected to silica gel column chromatography after optimizing proper solvent system on TLC, and eluted with ethyl acetate: dichloromethane (4:1, v/v). A total of 21 fractions were collected. Fractions 1-3 were less reactive against DPPH free radical spray. Fraction 4-6, 7-9, 10-12, and 13-14 were combined and re-chromatographed separately on Sephadex LH-20 column with ethyl acetate: dichloromethane (4.5:0.5, v/v). From combined fractions 4-6 ferulic acid, from fractions 7-9 myricetin, from fractions 10-12 kaempferol and from fractions 13-14 quercetin were isolated. All of the compounds were identified by comparing with standards HPLC fingerprints. Similarly Fractions 14-20 were combined re-chromatographed on Sephadex LH-20 column with methanol: chloroform (8:3.5, v/v) eluent. The same chromatography system was applied for isolating from Fraction 21. Hence from combined fraction 14-20 yielded Compound B and from Fraction 21 yielded Compound A.

2.8. HPLC analysis

Phenolic compounds were identified using HPLC following the modified method of [16]. A 20 µL aliquot of sample was separated using a Jasco HPLC System (Model USA). Peaks were detected with a multi-wavelength UV-vis detector (Jasco, USA) operated at 280 and 360 nm. Two wavelengths were used because, at 360 nm, UV absorbance of ferulic acid is optimum and at 280 nm simultaneous detection of hydroxybenzoic and hydroxycinnamic acids is possible. Separations were achieved on a 5-µm Phenomenex Luna C-18 column (240 mm x 4.6 mm; Phenomenex, USA). Gradient elution was performed using solvent mixture A (water: acetic acid, 100:1, v/v) and solvent mixture B (methanol: acetonitrile: acetic acid, 75:25:1, v/v/v) as follows: 0-2 min, 5% B; 2-10 min, 5-25% B; 10-20 min, 25-40% B; 20-30 min, 40-50% B; 30-40 min, 50-100% B; 40-45 min, 100% B; 45-55 min, 100-5% B. Solvent flow rate was 1.0 mL/ min. Peak identities were confirmed from retention data and by spiking of extracts with authentic standard.

2.9. UV-Vis analysis

The absorption spectra of Compound A and Compound B in methanol were recorded in 200-800 nm and their absorption maxima were determined.

2.10. LC-MS/MS analysis

The analysis was performed with a API 4000Q-TRAP LC-MS/MS System, equipped with an electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany), including an 1100 Series diode array detector (DAD). Enhanced mass spectra (EMS) and enhanced product ion (EPI) scans were used to gain structural information about the molecules. Electrospray mass spectra data were recorded on a negative ionization mode for a mass range of 0-500 m/z. CE was set at -35 eV and -55 eV. Column, mobile phase, and gradient elution used were the same as HPLC analysis.

3. Results and discussion

3.1. Extraction and separation

In this study, aqueous methanol (methanol: water, 80:20 v/v) was used to extract antioxidant compounds because phenolics are often extracted in higher amounts in more polar solvents such as aqueous methanol. After the powdered leaf of Azadirachta indica (200 g, mesh 60) was repeatedly extracted three times with 80% methanol, the amount of crude extract (CE) was 48.1 g. Separation of compounds present in crude extract can eliminate undesirable chemicals and help identifying the most important phenolic compounds present. In this study, solvent partitioning method was used. Four solvent fractions (hexane, ethyl acetate, butanol, and aqueous fraction) were obtained from crude methanol extract using solvent-solvent fractionation.

3.2. Total phenolics and flavonoids content

The total phenolics contents (TPC) of the solvent fractions (HF, EaF, BF and WF) were evaluated using the Folin–Ciocalteu assay, which is a fast and reliable method to quantify phenolics and the results are presented in Table 1. The highest TPC value was obtained from the EaF (233.09±2.7 mg GAE/g) followed by BF and WF, whereas HF had the lowest TPC (32.51±0.9 mg GAE/g). Total flavonoid contents (TFC) in various solvent fractions follow the BF > EaF > HF > WF.

Table 1. Total phenolics and flavonoids contents and extraction yield of solvent fractions of Azadracta indica

Neem leaf solvent fraction	Extract yield	TPC (mg of gallic acid equivalent/g)	TFC (mg of catechin equivalent/g)
HF	21.9±3.2%^{b}	32.51±0.9^{d}	25.6±2.2^{b}
EaF	3.6±0.5%^{d}	233.09±2.7^{a}	51.3±7.6^{a}
BF	7.02±0.3%^{c}	135.88±1.2^{b}	53.6±5.2^{a}
WF	39±2.1%^{a}	47.01±1.1^{c}	13.4±3.1^{c}

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction. TPC: total phenolic content; TFC: total flavonoid content. Values are expressed as mean ± SD of triplicate measurement. Numbers on the same column with different letter are significantly different $p<0.05$.

3.3. Antioxidant activity

Figure 1A shows the reductive capabilities of various extract fractions against α-tocopherol. For quantifying reductive ability, the transformation of Fe3+ to Fe2+ in the presence of sample was measured. The reducing capacity of a compound may serve as a indicator of its potential antioxidant activity [10]. It was found that, the reducing power of the extracts increased with its concentration dependently. At all concentrations studied, EaF and BF showed higher reductive potential than the CE, the HF, the WF and the control (α-tocopherol, Toc) in a high statistically significant ($p<0.01$) manner.

The DPPH radical is stable and is widely used to evaluate the radical scavenging activity of antioxidant compounds. The ability to act as donor of hydrogen atoms in the transformation of the DPPH radical to its reduced form was investigated for CE, HF, EaF, BF, WF and the standard (ascorbic acid, AA). Figure 1B shows that the DPPH radical scavenging ability of all extract fractions. It can be seen that EaF has the highest activity among all extract fractions. At lower concentration (<100 µg/ mL) high significant

difference was observed ($p<0.01$). Similar trend was also observed for BF but lower with $p<0.05$. For instance at 25 µg/ mL concentration the CE didn't show free radical activity but 52.82±0.7% and 38.03±0.67% inhibition were exhibited by EaF and BF, respectively. Though the standard (AA) had ≥95% inhibition in the tested concentration ranges, the active fractions (EaF, BF and CE) had comparable inhibition at a concentration ≥200 µg/ mL. The IC50 Values follow the order: AA < EaF < BF < CE < WF < HF (Table 2). The IC50 values varied from 64.33±0.7 µg/ml (EaF) to 220.92±2.7 µg/ mL (HF). The corresponding values for CE and HF are 112.29±1.8 µg/ mL and 125.61±1.7 µg/ mL, respectively. The observed differences in scavenging activities of the samples against the DPPH system is as a result of differences in concentrations of phenolic compounds contained in the samples.

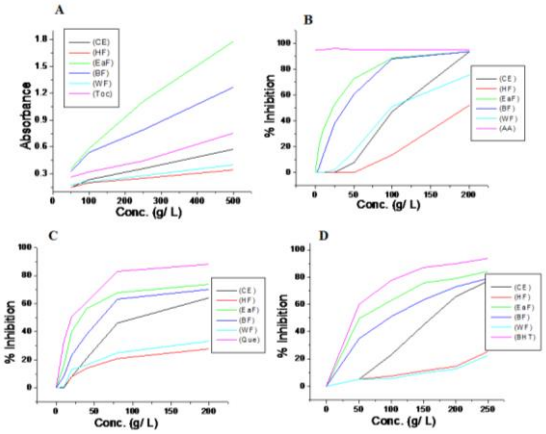

Figure 1. Neem leaf 80% methanol extracts solvent partition antioxidant assays: Reduction potential (A), DPPH free radicals inhibition (B), Hydroxyl radical inhibition (C), Hydrogen peroxide inhibition (D).

Table 2. Scavenging effect (IC50) on DPPH radicals, hydrogen peroxide, and hydroxyl radicals of Azadracta indica leaf.

Neem leaf solvent fraction	IC_{50} DPPH assay (µg/ml)	IC_{50} Hydroxyl radical assay (µg/ml)	IC_{50} H_2O_2 assay(µg/ml)
HF	220.92±2.3^{f}	>200^{f}	>500^{e}
EaF	64.33±0.7^{b}	81.77±0.3^{b}	191.6±1.1^{b}
BF	77.39±0.6^{c}	107.36±1.3^{c}	247.8±2.2^{c}
WF	125.61±1.7^{e}	>200^{e}	>500^{f}
CE	112.29±1.8^{d}	139.88±1.2^{d}	258.12±2.8^{d}
Ascorbic acid	<2^{a}	-	-
BHT	-	-	143.88±1.9^{a}
Quercetin	-	50.68±1.1^{a}	-

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction; CE: Crude Extract; BHT: Butylated hydroxyl toluene. Values are expressed as mean ± SD of triplicate measurement. Numbers on the same column with different letter are significantly different $p<0.05$.

The H_2O_2 scavenging capacity of a sample may be attributed to the structural features of the active components in the sample, which determine their electron donating abilities. Hydrogen peroxide is not very reactive, but it may be toxic to cell since it may give rise to hydroxyl radicals in cells [17]. Hydroxyl radicals, which are reactive biological molecules produced via the Fenton`s reaction in living system, can cause lipid oxidation and enormous biological damage, thus their scavenging property may provide an important therapeutic approach against oxidative stress induced ailments. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [18]. In this study, hydroxyl radicals scavenging activity was quantified by measuring the inhibition of the degradation of the deoxyribose by free radicals. Deoxyribose levels were determined by reaction with thiobarbituric acid. Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH. When extract fraction of neem leaf and the reference compound quercetin were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented their degradation. The results are shown in Figure 1C. All extract fractions exhibited significant reducing ($p<0.05$) on DNA damage at all concentrations. The control Quercetin (Que) was highly effective in inhibiting the oxidative DNA damage with an IC50 value of 50.68±1.1 µg/ mL. The difference between CE and the control was statistically highly significant ($p<0.01$). Similarly, the IC50 value for the EaF in the hydroxyl radical scavenging assay was 81.77±0.3 µg/ mL and the difference from that of CE was statistically highly significant ($p<0.01$). BF has an IC50 value 107.36±1.3 µg/ mL and is statistically significantly deferent ($p<0.05$) from that of CE. The inhibition ability increases with concentration. The hydroxyl radicals scavenging effect of various extract fractions of neem leaves and standards decreased in the order of: Que > EaF > BF > CE > WF > HF. Moreover the ability to scavenge H_2O_2 was determined and the results are shown in Figure 1D. Extract fraction was capable of scavenging H_2O_2 in a dose-dependent manner, especially the EaF and the BF. Fifty micrograms per milliliter of the CE exhibited only 5.6±0.3% scavenging activity on H_2O_2. On the other hand, at the same concentration; EaF, BF and the standard BHT showed 49.43±2.01, 35±1.1 and 59.95±0.8% activity, respectively. Significant differences ($p<0.01$) were exhibited between all extract fractions and the CE at lower concentration ranges. These results indicate that neem leaf possesses H_2O_2 scavenging activity but its activity is lower than that of BHT and there is statistically a significant correlation between H_2O_2 scavenging activity of extract fractions and control ($p<0.01$). The IC50 values, the sample concentration required to decrease H_2O_2 by 50%, are summarized in Table 2. They follow the order: BHT < EaF < BF < CE < HF < WF. HF and WF have lower activities, which may be due to the lower concentration of active metabolites in these fractions.

Table 3. Phenolic acid content of neem leaf (mg/g).

Neem leaf solvent fraction	Gallic acid	Dihydroxy Cinammic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Unidentified phenolics	Total
HF	ND^{d}	ND^{d}	0.0134±0.002^{c}	ND^{d}	0.365±0.002^{b}	3.4±0.3^{d}	3.7784±0.304
EaF	1.074±0.001^{a}	80.89±0.37^{a}	6.61±0.05^{a}	0.029±0.004^{a}	0.013±0.001^{d}	140.01±1.23^{a}	228.616±1.66
BF	0.3299±0.0005^{c}	0.003±0.0009^{c}	0.00134±0.0005^{d}	0.023±0.002^{b}	3.12±0.07^{a}	6.56±0.1^{c}	10.037±0.174
WF	0.786±0.004^{b}	1.52±0.06^{b}	0.516±0.004^{b}	0.01153±0.0001^{c}	0.0157±0.0003^{c}	9.516 ±0.8^{b}	12.365±0.87

Table 4. Flavonoid content of neem leaf (mg/g)

Neem leaf solvent fraction	Catechin	Rutin	Myricetin	Quercetin	Luteolin	Unidentified phenolics	Total
HF	ND[d]	36.06±1.05[b]	0.0226±0.0002[c]	ND	ND	3.4±0.3[d]	3.7784±0.304
EaF	3.9±0.33[a]	4.01±0.74[d]	23.8±1.12[a]	ND	ND	140.01±1.23[a]	228.616±1.66
BF	0.0088±0.0004[c]	80.13±1.96[a]	0.08132±0.0002[b]	ND	ND	6.56±0.1[c]	10.037±0.174
WF	0.135±0.002[b]	20.03±1.6[c]	0.000887±0.000001[d]	ND	ND	9.516 ±0.8[b]	12.365±0.87

The four extract fractions contained several antioxidant compounds (Table 3 and Table 4). These antioxidant compounds were present in higher concentrations in the EaF which gave the highest antioxidant activity and total phenolic contents. Hence the EaF was selected for further purification and isolation of the antioxidant compounds.

3.4. Isolation of Compounds

The HPLC profile of ethyl acetate fraction exhibits 8 well resolved signals indicating the 8 compounds of the fraction (Figure 2). Compounds corresponding to no. 4 and no. 6 exist at higher concentrations while the remaining compounds correspond to no. 1, 2, 3, 5, 7 and 8 were at lower quantities. Silica gel column chromatography was used to isolate and purify chemical constituents. Gradient elution with ethyl acetate: dichloromethane (4:1, v/v) was applied and 21 fractions were collected. Each column fraction was tested for its activity against 0.02% DPPH on TLC plate. The fractions with similar spots were combined and subjected to further purification on Sephadex LH-20 column, and finally yielded antioxidant compounds. All 8 compounds were efficiently isolated in this work. The two major components, compound no. 4 (C4) and no. 6 (C6) were identified based on UV and LC/MS/MS method (Section 3.5 and 3.6). While the rest of minor compounds were identified by RP-HPLC, comparing their retention times with those of authentic standards. Compound no. 1 at a retention time of 20.5 min was identified as ferulic acid (Figure 2). Ferulic acid exhibits a wide range of therapeutic properties because of its antioxidant potential [19]. Compound no. 3 at a retention time of 21 min was identified as myricetin (Figure 2). Myricetin is a phenolic compound with anticarcinogenic effect, and is an effective cancer chemopreventive agent [20]. Compound no. 8 at a retention time of 37 min was identified as kaempferol (Figure 2). Kaempferol showed an antioxidative activity against metal-induced lipid peroxidation [21]. Compound no. 7 at a retention time of 32 min was identified as quercetin (Figure 2). Quercetin, a flavonoid present in neem leaf, has been demonstrated to inhibit buccal pouch carcinogenesis by its radical scavenging property [22]. However, the trace components (Compound no 2 and no. 5) couldn't be identified since HPLC standards were unavailable and the isolates were too dilute for NMR analysis.

3.5. UV-visible analysis

C4 and C6 were partially characterized from their UV-Visible spectra and compared with standard compounds in references. UV spectra of flavonoids are particularly informative, providing considerable structural information that can distinguish the type of phenol and the oxidation pattern. Typical UV-Vis spectra of flavonoids include two absorbance bands. For flavonols, band A lies in 350-385 nm and band B in 250-290 nm [23]. The results for C4 (Figure 3A) showed that there are 2 main peaks (λmax) at 262 and 358 nm, which suggests that C4 could be flavonol. However, there is no reference report of UV spectra close to the absorbance of C4. C6 has maximum absorbance (λmax) at 267 and 349 nm (Figure 3B), which suggests that it could be flavone. From previous study, C6 was considered as luteolin derivative [24]. Zhu, Zhang and Lo [25] isolated a compound with similar UV spectra to that of C6 and identified it as luteolin-7-O-β-D-glucopyranoside. C6 is yellow powder. However, UV-vis analysis can't provide complete information about the structures but it can be used as an indicative tool for characterization of C-ring, whereas the MS spectra could provide additional, significant information.

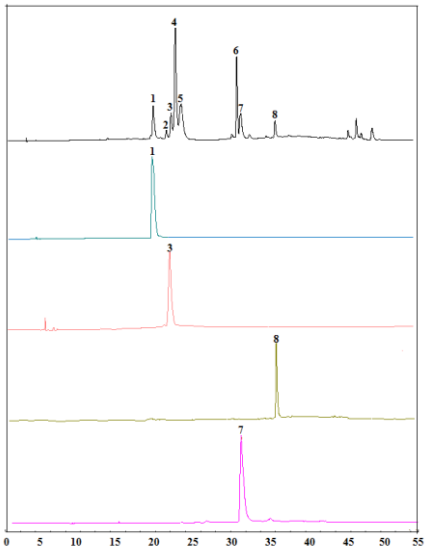

Figure 2. HPLC chromatogram of the ethyl acetate fraction and isolated compounds from neem leaves. The peaks identified (1) Ferulic acid, (3) Myricetin, (7) Quercetin, and (8) Kaempferol

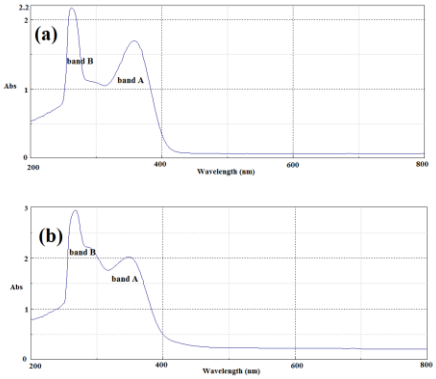

Figure 3. UV-Visible spectra of the two major isolated compounds in ethyl acetate fraction of 80% methanol extract of neem leaf. Spectra (a) is for Compound A and spectra (b) is for Compound B recorded in the range of 200-800 nm.

3.6. LC-MS-MS Analysis

LC-MS/MS can screen more than one compound in one injection. When standards were unavailable, different MS/MS experiments of product ion scan were carried out in order to confirm the structure of the deprotonated molecules [26]. Since polyphenols contain one or more hydroxyl and/or carboxylic acid groups, MS data were acquired in negative ionization mode [27]. Prasain, Wang and Barnes [28] also recommended negative ion mode for flavonoid analysis in biological samples because of its better sensitivity and limited fragmentation. ESI in negative ion mode combined with tandem mass spectrometry (MS/MS) has been proven useful for compound identification of flavonoid glycosides. ESI-MS/MS is powerful enough to distinguish several methylated flavonoid isomers based on ion spectra.

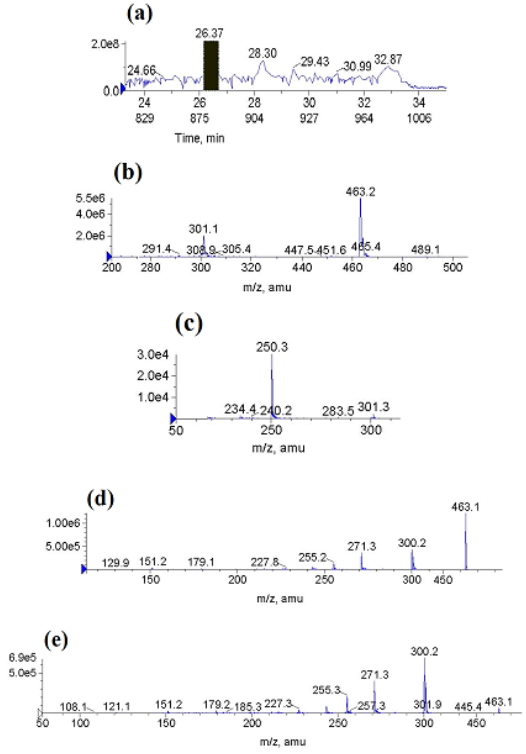

Figure 4. (a) LC-MS total ion chromatograms (TIC), (b) EMS spectra, (c) full-scan mass (MS) spectra, and product ion mass (MS/MS) spectra with collision energy (d) -35 eV and (e) -55 eV of Compound A obtained during LC/MS/MS analysis under optimized instrumental conditions.

Identification of C4, and C6 was carried out by comparing m/z values of their molecular ions and their MS/MS spectra with MS/MS spectra library. Data of the LC-MS total ion chromatogram (TIC) obtained in the negative enhanced full scan mode (EMS) confirmed the formation of C4 at 26.37 min (Figure 4a). After subtraction of the culture control TIC chromatograms, the resulting full mass spectra of C4 was evaluated in detail. EMS (Figure 4b) and MS/MS (Figure 4d) spectra of this compound show a major ion fragment at m/z 463. Scanning between 50 and 600 amu shows the molecular ion of C4 at m/z 250 (Figure 4c).

The resulting full scan mass spectra, Figure 4d & 4e, obtained at collision energiess of -35 eV and -55 eV, respectively shows a major fragment ion at m/z 300, probably corresponding to the loss of a rhamnose, glucose or galactose moiety from glycoside.

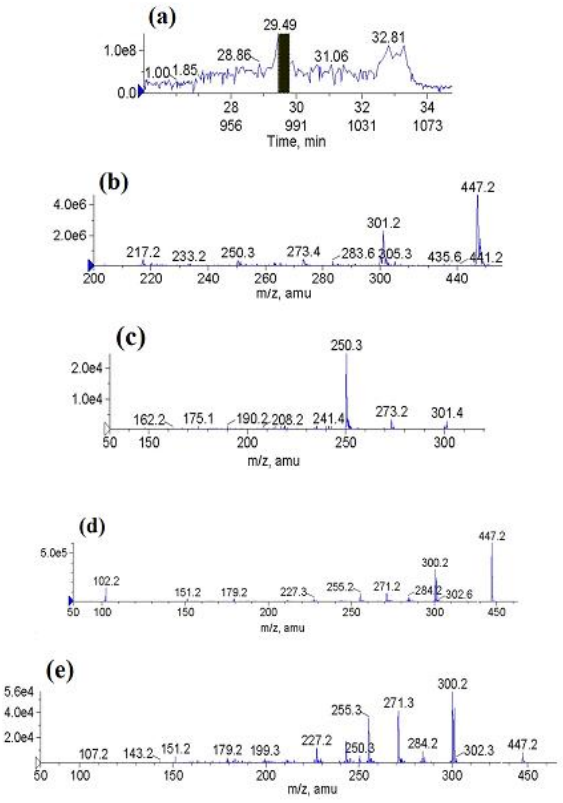

Figure 5. (a) LC-MS total ion chromatograms (TIC), (b) EMS spectra, (c) full-scan mass (MS) spectra, and product ion mass (MS/MS) spectra with collision energy (d) 35 eV and (e) 55 eV of Compound B obtained during LC/MS/MS analysis under optimized instrumental condition.

Fragmentation pattern of MS/MS spectra from EPI scan shows ions at m/z 463, m/z 301, m/z 300, m/z 271, m/z 255, and m/z 228 (Figure 4d). The MS/MS spectra show similarities to the MS/MS spectrum of quercetin-3-O-galactoside (glucoside/hexoside) [27]. Hence C4 was identified as quercetin-3-O-galactoside or hyperoside (m/z 463) by neutral loss scan of 162 mass units from m/z 463 to m/z 301, respectively. The loss of 162 mass units may be due to the loss of galactose, glucose or mannose [29].

Neutral loss scan of 162 and 132 mass units revealed the presence of compounds with a hexose (galactose or glucose) or a pentose (arabinose or xylose) unit [30]. The mass measured for the [M – H]- ion of C4 was m/z 463.1, whereas the calculated value of quercetin-3-O-galactoside (C21H20O12) is m/z 464.38.

Total ion chromatogram shows C6 at 29.49 min (Figure 5a). Scanning between 50 and 600 amu shows the molecular ion of C6 at m/z 250 (Figure 5c). EMS (Figure 5b) and MS/MS (Figure 5d) spectra of C6 show a major ion fragment at m/z 447. The EPI scan produced fragment ions such as m/z 447, 300, 271, and 255. Some characteristic fragments of quercetin, such as ions at m/z 271 and 255 could be also found in the MS/MS spectrum of C6. The corresponding MS/MS spectrum, Figures 5d and 5e, obtained at collision energies of -35 eV and -55 eV, respectively shows a major fragment ion at m/z 300. The loss of 147-unit mass from m/z 447 to 300 is most likely due to the breakdown of pentose and deoxyhexose, respectively [29]. From this evidence, C6 was identified as quercitrin or quercetin-3-

rhamnoside. The mass measured for the [M – H]- ion of C6 was m/z 447.2, whereas the calculated value of quercetin-3-rhamnoside (C21H20O11) is m/z 448.09. Hvattum and Ekeberg [31] reported this compound with the same fragment ions.

Conclusions

Azadirachta indica leaf exhibited strong antioxidant effect and 80% methanol extract partitioned with ethyl acetate is a good source for phenolic compounds. Searching for antioxidant compounds resulted in successful isolation of four minor compounds, namely, ferulic acid, myricetin, kaempferol, quercetin and two major compounds characterized as quercetin-3-O-galactoside and quercetin-3-rhamnoside. Effective separation method was needed to obtain high purity compounds for specific NMR analysis. The potency of these compounds could provide a chemical basis for some of the health benefits claimed for *Azadirachta indica* in folk medicine and warrant further studies to assess their potential as an effective natural medicine.

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