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In Vitro Antioxidant Activity of *Feronia Limonia* Bark Relevant to the Treatment of Oxidative Stress Mediated Neurodegenerative Disorders

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Abstract

The present study was designed to explore the potent antioxidant of crude methanol extract (CME) of *Feronia limonia* bark and its four fractions such as petroleum ether (PEF), chloroform (CLF), ethyl acetate (EAF) and aqueous fractions (AQF) in the treatment of neurodegenerative disorders, caused by oxidative stress. The highest phenolic and flavonoid content were detected in CLF (18.16 µg of gallic acid equivalent/gm of dried sample &160.20 µg of GAE/gm of extract) suggestive for good source of antioxidant. To the best of the knowledge, the compound C-1, Umbelliferone (7-hydroxycoumarin), reported for the first time from this plant source, was isolated & characterized from the chloroform fraction by column chromatography, PTLC and by ^{1}H-NMR, ^{13}C-NMR and HMBC respectively. All the fractions and the compound isolated were investigated for in vitro antioxidant activity by total antioxidant capacity assay, ferric reducing power assay, 1,1-diphenyl-2-picrylhydrazyl(DPPH) free radical & hydroxyl radical scavenging assay and lipid peroxidation assay. The total antioxidant activity and reducing capacity of CLF & EAF was higher than CME, AQF & PEF. In case of DPPH assay, all the extracts tested exhibited strong and similar free radical scavenging activity (IC_{50} values 15.8-17.4 µg/ml) compared with the reference standard ascorbic acid(IC_{50} value 12.6 µg/ml). The AQF had the highest hydroxyl radical scavenging activity with IC_{50} value 12.5 µg/ml. The isolated compound and seven column fractions (F2 to F8) of CLF also possessed good antioxidant activity. The highest lipid peroxidation inhibition capacity was found from F7 (IC_{50} value 14 µg/ml) appearing more potent than the standard catechin. Irrefutably, the study revealed that the plant bark inhibits multiple components of the oxidative stress pathway, which is suggestive for using in the effective and safe treatment of neurodegenerative diseases after in vivo effectiveness test is done.

Keywords: Neurodegenerative disorders, Umbelliferone, DPPH, Lipid peroxidation inhibition, *Feronia limonia*

Contents

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1. Introduction

Neurodegenerative disorders are a heterogeneous group of chronic and progressive diseases that are characterized by abnormalities of relatively specific regions of the brain and the specific population of neurons [1-3]. The mechanism of neuronal damage in neurodegenerative disorders that have been elucidated include neuronal apoptosis, abnormal protein deposition, mitochondrial mechanisms of cell death, generation of reactive oxygen species(ROS) and oxidative stress related signaling [4]. One of the major changes found in essentially all neurodegenerative diseases is the over expression of ROS like superoxide anion radical(O_2^-), hydrogen peroxide(H_2O_2) & hydroxyl radical(OH^o), singlet oxygen, nitric oxide radical, hypochlorite radical, various lipid peroxides and other free radicals that lead to oxidative stress and cell death [5]. All the markers of oxidative stress have been documented in neurodegenerative disorders including Alzheimer's diseases [6-8] & Parkinson's disease [9-11]. Over production of ROS induces cellular and molecular abnormalities in these diseases including peroxidation of lipid, oxidation of protein, enzyme as well as DNA along with decrease in superoxide dismutase (SOD), catalase, glutathione peroxidation and reduced glutathione level [12].

The human body's antioxidants are capable to mop up free radical by neutralizing them, as a result free radical or ROS induced cell damage is prevented. But the process is effective in case of vast production of free radicals that may not happen always [13, 14]. Antioxidants in food have received a great amount of attention as they possess primary preventive ingredients against various diseases [15, 16]. Therefore, antioxidant therapies foreseeing the reduction of oxidative damage and the increase of endogenous antioxidant defenses have been suggested to prevent, delay or ameliorate the diseases symptoms [4].

Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene(BHT), tertiary butylated hydroquinone and gallic acid esters are efficient against ROS, but concurrently cause prompt negative health effects like liver damage and mutagenesis. As a result, strong restriction has been placed on their application [17-20]. At present there is special interest on naturally occurring antioxidants derived from the plant sources which are candidates in the treatment of oxidative damage. Compounds isolated from plant possess ideal structural chemistry for free radical scavenging activity, and shown to be more effective antioxidants in vitro are used for the treatment of neurodegenerative disorders [4]. Recently, a number of plant products including phenolic and flavonoid contents as well as various crude extracts of plants were reported for antioxidant actions [21-25].

Feronia limonia bark ,locally called Kath bel bark, belonging to the Family, Rutaceae, widely distributed in indomalya ecogone, Bangladesh, India, Pakistan, Sri Lanka, Indochinese ecoregion, Java, Malaysia ecoregion, has been used for centuries in folk medicine to treat anorexia, bronchitis, calculus, cardiac debility, cough, diarrhea, gastropathy, fever, tuberculosis , toothaches and as antiemetic, aromatic, astringent, carminative, cardiotonic, expectorant, purgative, alexipharmic and are useful in pruritus and pharyngodynia. The plant is also useful for asthma, consumption, tumors, opthalmia, leucorrhoea, scurvy and sore throat. Fruit pulp is sour, sweet, edible stomachic, stimulant and astringent. The bark is aromatic, having cooling sensation and is useful in vitiated conditions of pitta. The bark is occasionally prescribed for biliousness and useful in liver disease [26-28]. The different parts of the plant have been investigated by several workers and found to contain coumarins, furanocoumarins, lignans, alkaloids, steroids and flavonoids. The unripe fruits contain stigmasterol. Root bark yieldedosthol, geranyl umbelliferone, marmin, marmesin, aurapten,bergapten, isopimpinellin and fernoil. The heartwood contains ursolic acid and a flavanone glycoside 7-methylporiol-b-Dxylopyranosyl-D-glucopyranoside. The stem bark of *F. limonia* yielded flavanone, alkaloids, coumarins, lignan, sterols and triterpene. Psoralen, bergapten, orientin, vitexin and saponarin have been isolated from leaves [29-35].

Previous studies have shown that the methanolic extract of the bark exhibit strong antioxidant activity [35]. In continuation of the study, the present work was designed to investigate the methanolic extract of the bark using conventional chromatographic technique to explore the potent antioxidants which may have the sufficient capacity to fight against the neurodegenerative disorders.

2. Materials and Methods

2.1. Plant material and Extraction Preparation

Fresh Bark of *Feronia limonia* were collected in the vicinity of Rajshahi University, Bangladesh in the month of February, 2012. Authentication was achieved by the comparison with the herbarium specimen deposited in the herbarium of the Dept. of Botany of the same university. The plant materials were washed with water, cut into pieces, shade dried for several days , then dried in an oven for 24 hours below 60°C and pulverized in coarse powder using a grinder. The powdered plant material (700 gm) was extracted with cold methanol(2 L), then filtered, squeezed off and evaporated off under reduced pressure in a rotary evaporator to obtain crude methanol extract (CME, 20gm). An aliquot of the concentrated methanolic extract was further partitioned with petroleum ether, chloroform, ethyl acetate and water. The resultant fractions i.e., pet ether (PEF, 4.54 gm), chloroform (CLF, 9.28 gm), ethyl acetate (EAF, 5.68 gm) and water (AQF, 8.5 gm) soluble fractions were used for next experimental work.

2.2. Phytochemical Screening

The presence of various classes of active chemical constituents such as alkaloids, steroids, glycosides, saponins, tanins etc. in all extractives were determined by color test, Libermann-Buchard's test, general test, lead acetate test, frothing test respectively using standard procedures [36].

2.3. Determination of Total Phenolic Content

Total phenolic content of different fractions of *F. limonia* were measured according to the Folin-Ciocalteu method [37]. Briefly, the samples solution (0.5 mL) at different concentrations (ranging from 100 to 1100 μg/mL) was mixed with 2.58 mL of Folin-Ciocalteu's phenol reagent. After 3 min, 0.3 mL of saturated sodium carbonate

solution was added to the mixture. The reaction mixtures were incubated at room temperature (25°C) for 20 min. The absorbance was measured at 760 nm with a spectrophotometer. Gallic acid solutions with concentrations ranging from 25 to 400 µg/mL were used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance and the levels in the samples were expressed as gallic acid equivalents (mg of GAEs/g of extract). The estimation was performed in triplicate, and the results were expressed as mean ± SD.

2.4. Determination of Total Flavonoid Content

The total flavonoid content of different fractions so prepared was estimated by following the procedure by Garrett and Grisham [38]. Briefly, one ml of aqueous extract containing 0.1 gm/m of dry matter was placed in a 10 ml volumetric flask, then 5 ml of distilled water added followed by 0.3 ml of 5% $NaNO_2$. After another 5 minutes, 6 ml of 10% $AlCl_3$ was added and volume made up with distilled water. The solution was mixed well and absorbance was measured at 510 nm. The total flavonoid content was calculated using standard catechin calibration curve. The results were expressed as milligrams of catechin equivalents (CE) per gram of dried extract.

2.5. Isolation and Identification of Compound

The chloroform fraction (9gm) was subjected to column chromatography for further fractionation. A well – stirred suspension of silica gel (120 gm in n-hexane) was poured into column (height 36 cm and diameter 5.5 cm). When the adsorbent was well settled, the excess of n-hexane was allowed to pass through the column followed by applying the CLF as free flowing mass made from silica gel to top of column. The column was eluted with a mobile phase of increasing polarity: n-hexane/chloroform/EtOAc/MeOH. Depending on the TLC behavior eight different column fractions (F-1 to F-8) were finally obtained for further analysis from 339 fractions. The pure Compound-I (C-I) dark brown gummy mass was isolated from F-3(47.4 mg) & F-6(43.7 mg) fractions in solvent system, n-hexane: chloroform (1:3) with R_f ¯value 0.552 using TLC followed by PTLC technique. The other fractions were not undergone any further chemical investigation due to small quantity.

2.6. In Vitro Antioxidant Assay

2.6.1. Determination of Total Antioxidant Capacity

Total antioxidant capacity of different extractives and isolated compound, column fractions(F-2 to F-8) of *L. feronia* were measured spcetrophotometrically thorough phosphomolybdenum method by Prieto, et al. [39] with some modifications. The assay was based on the reduction of Mo(VI) – Mo(V) by all extractives and subsequent formation of a green reagent phosphate/ Mo(V) complex at acidic pH [40]. Briefly, an aliquot of 0.5 ml of sample solution was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate). The tubes were incubated at 95^0 c for 90 minutes. The mixture was cooled to room temperature and the absorbance of aqueous solution of each was measured at 695 mm against a blank (3 ml of reagent solution). The assay were carried out in triplicate and expressed as mean ± SD. The antioxidant activity was expressed as the absorbance of the sample.

2.6.2. DPPH Free Radical Scavenging Assay

The free radical scavenging activity of the extract, isolated compound as well as their various fractions was evaluated according to Braca *et al*. [41, 42]. Briefly, sample solution with different concentrations (ranging from 0 to 200 µg/mL) was mixed with 0.3% of DPPH methanol solution. The reaction mixtures were incubated at room temperature and allowed to react for 30 minutes in the dark. After 30 min, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity. Ascorbic acid (AA) was used as a positive control. The percentage of inhibition of DPPH (%) was calculated as follows:

% inhibition of DPPH = Diff x 100/Absorbance of control

Where, Diff = Absorbance of control – Absorbance of test sample

The concentration of sample required to scavenge 50% of the DPPH free radical (IC_{50}) was determined from the curve of % inhibitions plotted against the respective concentration.

2.6.3. Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging activity of different extractives of this plant bark was determined by the method described by Elizabeth and Rao [43]. 1 ml of reaction mixture was made by adding 2-deoxy-D-ribose (2.8 mM); KH_2PO_4-KOH (20 mM, pH 7.4); $FeCl_3$ (100 µM) and various conc. (6.25 -100 µgm/ml) of the test sample or reference compound. After incubation for 1 hr. at 37^0 c, 0.5 ml of the reaction mixture was added to 1 ml of 2.8% trichloroacetic acid (TCA). Then 1 ml of 1% aqueous thiobarbituric acid (TBA) was added and the mixture was incubated at 90^0 c for 15 min to develop the color. After cooling the absorbance was measured at 532 nm against an appropriate blank solution (the same solution mixture without plant extract or std. undergone same incubation). The percentage inhibition activity was calculated from the following equation:

% I = $\{(A_0 – A_1)/A0\}$ x 100, where, A_0 and A_1 are the absorbance of the control and extract/standard respectively. IC 50 was measure from the graph of % inhibition against various concentrations.

2.6.4. Lipid Peroxidation Inhibition Assay

The inhibition of lipid peroxidation assay of the isolated compound and all extractives of said plant bark was determined against according to the method as described by Liu [44] with slight modification. In assay, the brain of adult long Evan rats (av. weight 150 gm) were homogenized with a homogenizer in ice-cold phosphate buffer (50 mM,pH 7.4) to produce 1/10 homogenate followed by centrifugation at 10000 rpm for 15 min at 4^0 c. The supernatant so produced was used as liposome. The ability of plant extract to inhibit lipid peroxidation was studied by incubating homogenates treated with hydrogen peroxide (10 µM) and different conc. of plant extracts. Hydrogen

peroxide induces lipid peroxidation in rat brain homogenates. Lipid peroxides react TBA to form a pink product, thiobarbituric acid reacting substances measurable to calorimetrically at 532 nm. The difference between the control and plant extract treated sample is the measurement of decrease in TBARS formation, reflecting the reduction of hydroxyl radical induced lipid peroxidation.

2.6.5. Reducing Power Capacity Assessment

The reducing power of bark extract of the plant was determined according to the method as described by Oyaizu [45]. Aliquot (1 ml) of samples solution at different concentrations (ranging from 12.5 to 100 µg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) solution of potassium ferricyanide. After mixing well, all the mixtures were warmed in a water bath at 50°C for 20 min. Then, 2.5 ml of 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 2000 rpm for 10 min. The supernatant (2.5 ml) was combined with 2.5 ml of distilled water, and 0.5 ml 0.1% (w/v) ferric chloride solution was added. The absorbance was measured at 700 nm with a spectrophotometer against blank (the same solution mixture without the plant extract or positive control). Ascorbic acid was used as positive control. All the tests were run in triplicate and results were reported as mean ± SD. A higher absorbance of reaction mixture indicates a higher reducing power.

3. Result

The phytochemical screening of all the extracts of *Feronia limonia* showed that saponin, tannin, alkaloids, glycosides & steroids were present in CME & EAF in varying extent but, AQF, PEF & CLF contained glycosides & steroid only in moderate amount with absence of other bioactive compounds. All the extractives contained reportable amount of phenol and flavonoid (Table 1). Total phenolic and flavonoid content of CME were 13.26 mg of GAE/gm & 78.37 mg of CE/gm. Among the different fractions of CME, highest phenolic & flavonoid content was found from CLF (18.16 µg of GAE/gm of dried extract & 160.20 µg of GAE/gm of extract, respectively) (Table 1).

Table-1. Total phenolic and flavonoid content of all extractives of *Feronia limonia* bark.

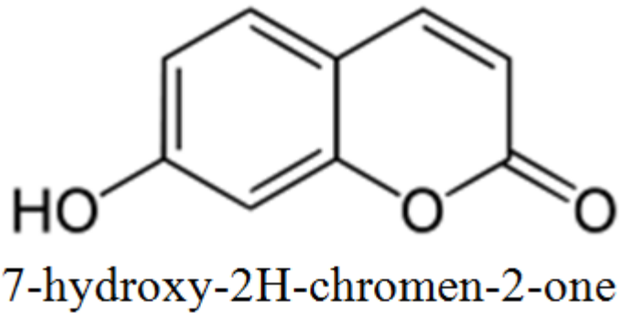
Extracts	Total Phenolic content	Total Flavonoid content
CME	*13.26±0.71(mg)*	78.37*±1.614(mg)*
CLF	*18.16±0.90*(µg)	*160.20±0.90*(µg)
EAF	*12.15±1.5*(µg)	*120.56±3.86*(µg)
PEF	*12.09±0.93*(µg)	*98.33±3.34(*µg)
AQF	*10.81±0.77*(µg)	*109.44±1.93*(µg)

CME= Crude methanol extract, CLF, EAF, PEF, AQF= Chloroform, Ethyl acetate, Pet-ether, Aqueous fraction respectively.

Characterization of the Compound, C-1

Repeated chromatographic separation and purification of chloroform fraction of crude methanol extract of the same plant provided a pure compound, C-1, the structure of which was characterized by ^{1}H-NMR, ^{13}C-NMR and HMBC spectral data. The compound was isolated as gummy mass, dark brown in color, soluble in acetone, chloroform, ethyl acetate, and methanol (slightly); but insoluble in pet-ether with R_f value 0.552 in n-Hexane/chloroform (1:3) solvent system and showed positive test with vanillin/sulfuric acid spray reagent indicating the character of higher alcohol or phenol or essential oil.

Umbelliferone: Dark brown, gummy mass; ^{1}H (500 MHz, $CDCl_3$) δ ppm: 7.62 (1H, d, J= 7.6 Hz), 7.34 (1H,d, J= 6.8 Hz), 6.83 (1H,d, J 6.8 Hz), 6.80 (1H, s), 6.23 (1H, d, J= 7.6 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_c, ppm: 162.2(C-2), 161.3(C-7), 155.1(C-9), 144.5(C-4), 129.2(C-5), 118.4(C-10), 113.2(C-6), 112.9(C-3) & 101.5(C-8)



7-hydroxy-2H-chromen-2-one

Fig-1. Structure of umbelliferone

The ^{1}H spectrum showed the presence of impurities with major peaks that were identified based on coupling constants as three protons on substituted benzene ring at δ (in ppm) 7.34 (1H,d, J= 6.8 Hz), 6.83 (1H,d, J 6.8 Hz) and 6.80 (1H, s). These three protons signals are probably due to the presence of 1, 2, 4-trisubstituted benzene ring. The ^{1}H spectrum also showed the presence of 1, 2,-disubstituted cis-olefin protons at δ 7.62 ppm (1H, d, J= 7.6 Hz) and 6.23 (1H, d, J= 7.6 Hz). Considering these spectral data with ^{13}C-NMR spectral data extracted (δ 162.2, 161.3, 155.1, 144.5, 129.2, 118.4, 113.2, 112.9 & 101.5) from impurities suggested the presence of 7-hydroxy coumarin moiety in the skeleton. Also the HMBC spectrum showed no correlation between aromatic parts of the spectrum with the up fielded carbons. Hence, based on the above short discussion, the most probable structure consistent with all these date seemed to be 7-hydroxycoumaring or umbelliferone (Fig. 1). The NMR data of FL-8-3 was in good concurrence with those reported in literature of umbelliferone [258-259]. However, to confirm the complete structure

of the impure part, more purification is needed to take 2D NMR data (gHMBC, gHSQC and NOE) that will be reported elsewhere.

The results of DPPH free radical scavenging assay used as one of the test method of antioxidant activity demonstrated that CME, its CLF, EAF, PEF & AQF fractions and C-1 exhibited better free radical scavenging activity(IC_{50} values 17.4µg/ml, 16.8 µg/ml, 17 µg/ml, 16.2 µg/ml, 15.8 µg/ml, 17.4 µg/ml respectively) than that of, Column fractions, F-2 – F8(IC_{50} values, 22.4 µg/ml, 27 µg/ml, 31 µg/ml, 23.6 µg/ml, 18.6 µg/ml, 33.2 µg/ml, 26 µg/ml respectively) (Fig.-2). The scavenging activity was increased with increment of concentration (Fig.-2). The total antioxidant capacity was measured by phosphomolybdate radical scavenging test. For crude extract, all fractions and for compound, phosphomolybdate radical scavenging activity was found increased with higher concentrations. Highest phosphomolybdate radical scavenging activity was found for F-8 fraction (Fig. 3). The CLF also showed significant phosphomolybdate radical scavenging activity. In case of hydroxyl radical scavenging activity test, among all samples, F-2 fraction showed highest activity (IC_{50} value 6 µg/ml) which appeared to more potent than reference standard catechin (IC_{50} value 8.8 µg/ml). Other samples also showed considerable activity, shown in Fig. 4. The result of lipid peroxidation inhibitory activity was presented in Fig. 5. In comparison, CME possesses the highest inhibitory effect (IC_{50} value 29 µg/ml) against brain lipid peroxidation than its fractions CLF, EAF, AQF, PEF (IC_{50} value 49µg/ml, 61µg/ml, 63µg/ml, 80 µg/ml). Among all samples, F-7 showed the highest inhibition with IC50 value of 14 µg/ml which appeared to be more potent than standard catechin (IC_{50} value 16µg/ml). Reductive capabilities of CME, all fractions, & the compound were shown in Fig.6. The reducing power of CME, all fractions, & the compound was increased gradually with the increase in concentrations. Among all samples, at highest test conc. (100 µg/ml), the highest reducing power capacity was shown by C-1 & F-2 fraction with absorbance of 3.04 & 3.03 respectively proving approximately as potent as standard catechin (absorbance 3.5). Higher absorbance is an indicator of high reducing power.

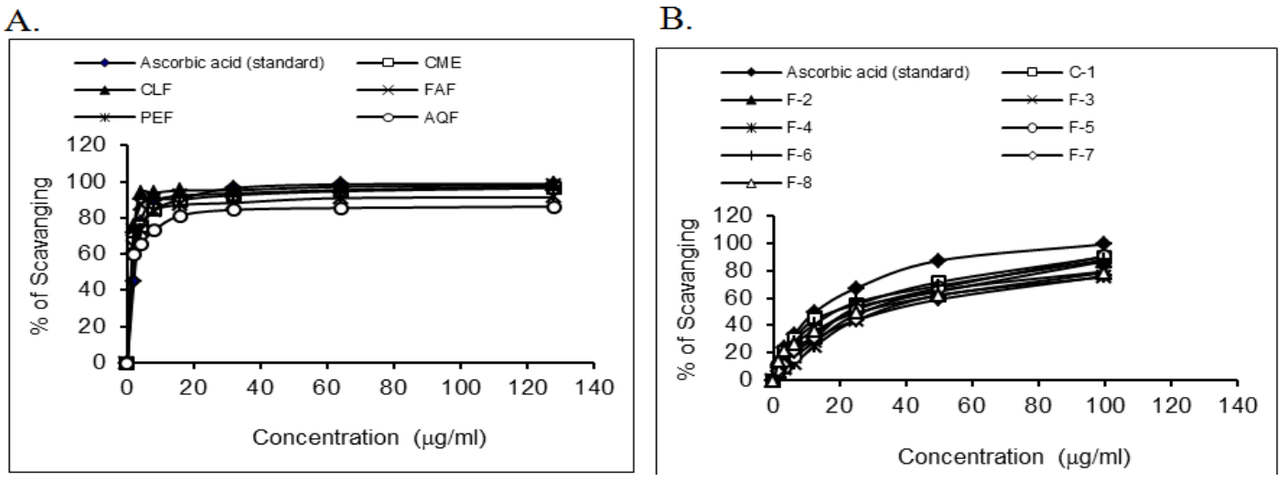


Fig-2. A. DPPH radical scavenging activity of crude methanolic extract of Feronia limonia, its four fractions and std. ascorbic acid. B. DPPH radical scavenging activity of compound (C-1) & column fractions (F 2 to F8)

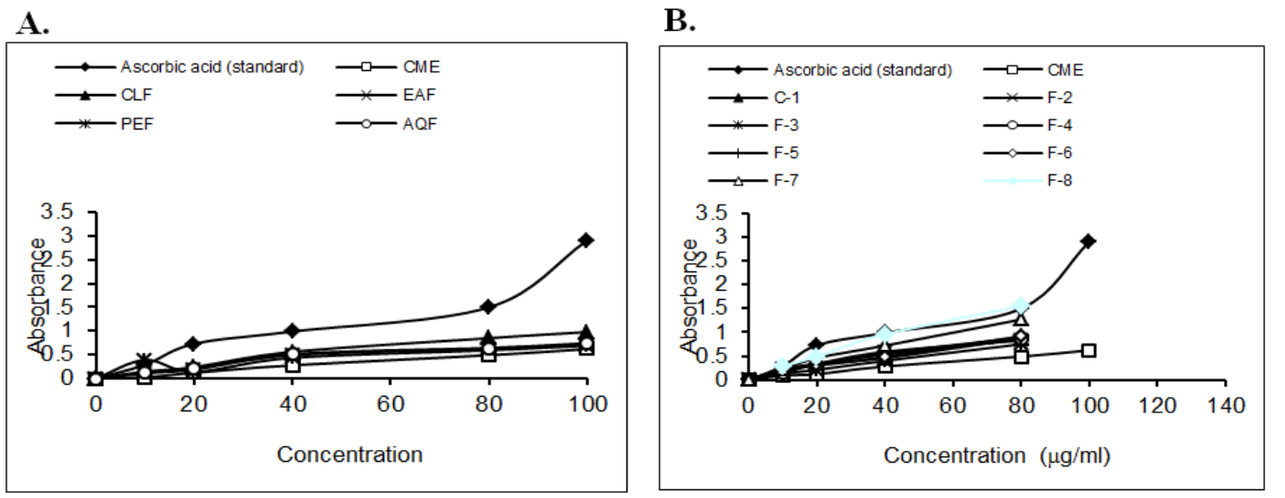


Fig-3. A. Total antioxidant activity of crude methanolic extract of Feronia limonia, its four fractions and std. catechin. B. Total antioxidant activity of compound (C-1) & column fractions (F 2 to F8).

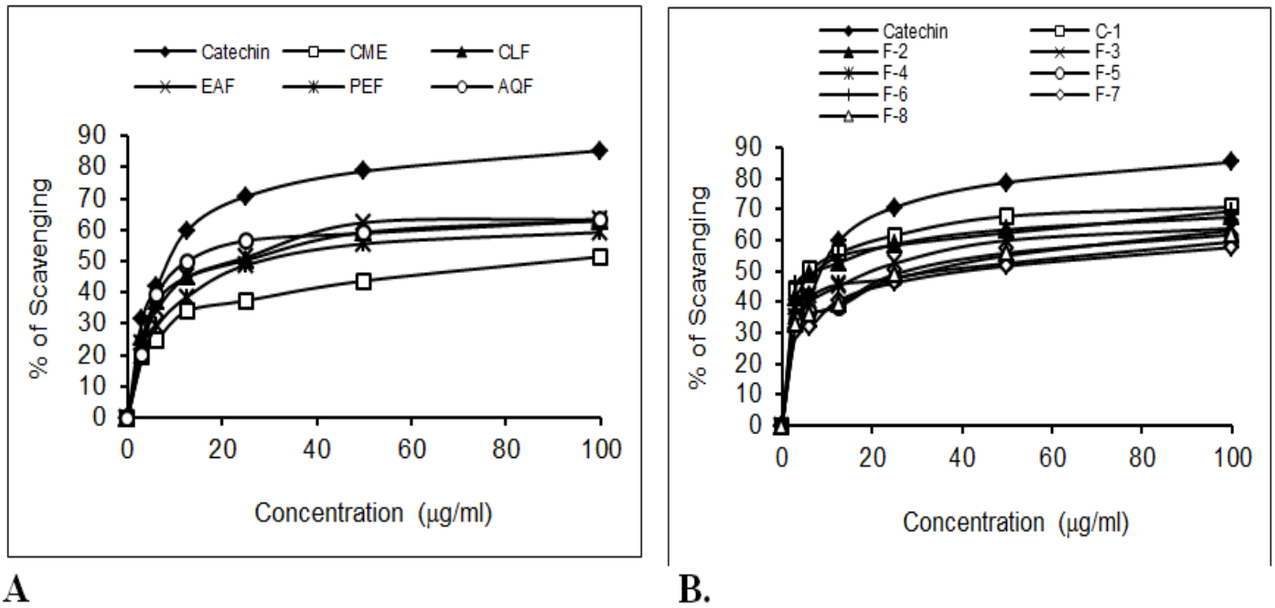


Fig-4. A. Hydroxyl radical scavenging activity of crude methanolic extract of Feronia limonia, its four fractions and std. catechin.. B. Hydroxyl radical scavenging activity of compound (C-1) & column fractions (F 2 to F8).

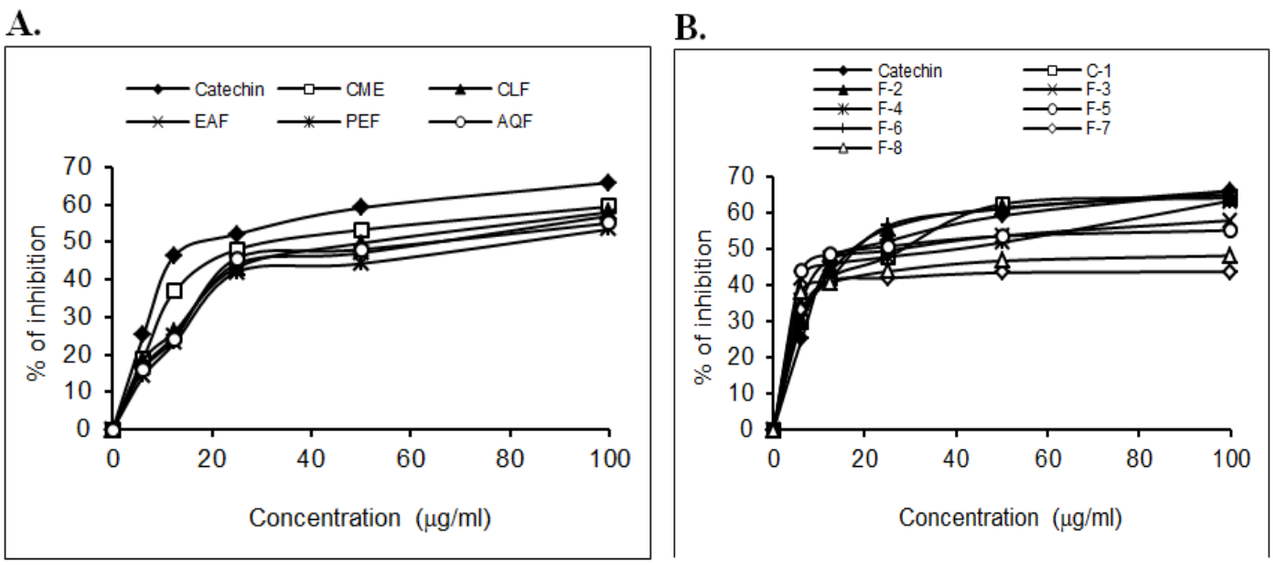


Fig-5. A. Lipid peroxidation inhibitory activity of crude methanolic extract of Feronia limonia, its four fractions and std. catechin.. B. Lipid peroxidation inhibitory activity of compound (C-1) & column fractions (F 2 to F8).

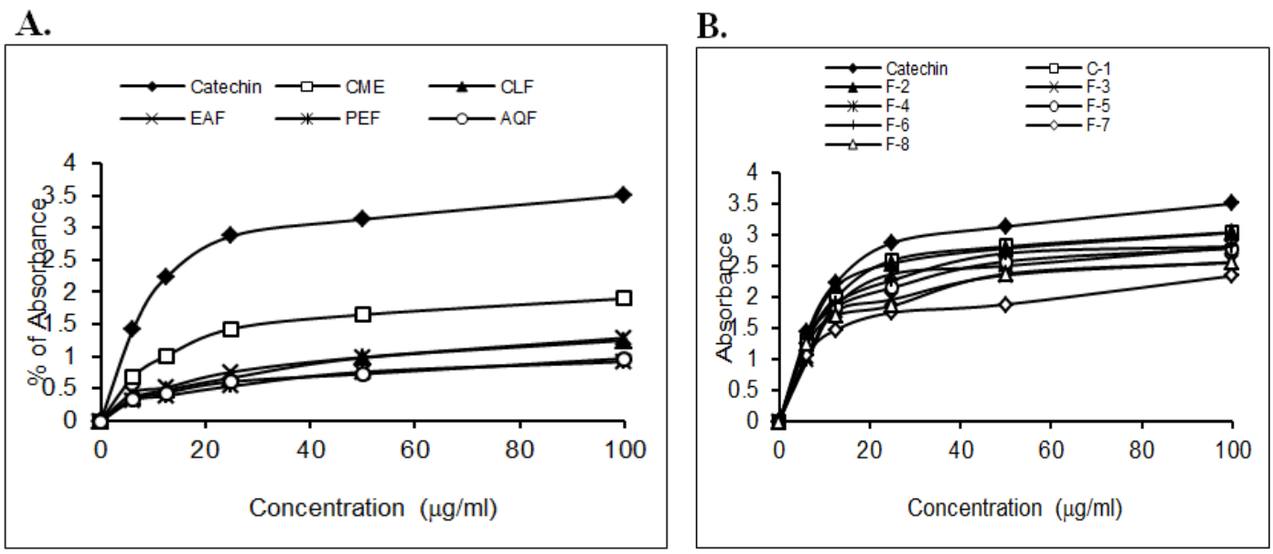


Fig-6. A. Reducing power capacity of crude methanolic extract of Feronia limonia, its four fractions and std. catechin.. B Reducing power capacity of compound (C-1) & column fractions (F 2 to F8).

4. Discussion

Although several mechanism of neurodegeration have been proposed, the generation of reactive oxygen species and oxidative damage is implicated in the pathogenesis of neurodegenerative disorders [46-48]. Antioxidants therapy plays an colossal role in the reduction of oxidative damage and increase of endogenous antioxidant defenses suggestive for prevention, delay or upgrade the disease symptoms. Considering this phenomena the present study

was done with a view to isolate completely new plant source's antioxidant which may have capacity in the management of oxidative stress mediated any kind of disorders. Crude methanolic extract of bark of this plant, its four fractions were undergone the phytochemical analysis which showed the presence of various bioactive compounds like phenolic, flavonoid, alkaloid, saponin, glycoside, tannin and steroids. Due to high content of phenolics & flavonoid, the chloroform fraction was further investigated by column chromatography followed by PTLC that yielded Compound, C-1, which was identified as umbelliferone by ^{1}H-NMR, ^{13}C-NMR & HMBC and seven column fractions, F-2 to F-8. One of the most noticeable matters of this study is that umbelliferone is isolated for the first time from this particular plant source.

DPPH free radical scavenging activity assay, the most popular spectrophotometric methods for determination of the antioxidant capacity of food, beverages and vegetable extracts was used for evaluating the antioxidant proper of all extractives, compound and the column fractions [49]. The isolated compound showed the most significant DPPH free radical scavenging activity. In total antioxidant activity, chloroform fraction exhibited the highest activity. As free hydroxyl radical is considered a factor in neurodegenerative diseases due to its toxicity and role in various chemical reaction, all the plant samples were undergone hydroxyl scavenging activity test, the result of which demonstrated that the column fraction, F-2, C-1 and F-6 had the strong activity. Although all the fractions exhibited appreciable lipid peroxidation activity, the highest activity was shown by F 7 foreshadowing more potent than the standard catechin. These results point that *Feronia limonia* is a good source of lipid peroxidation scavenging.

To end with, the in vitro studies portends that *Feronia limonia* inhibits multiple components of the oxidative stress pathways that can be the cause of neurodegenerative diseases. The chloroform fraction of the plant appears to be good source of strong antioxidant possibly due to high content of phenolic compounds and flavonoids. Although the in vivo effectiveness remains to be investigated, the strong antioxidant activity of the compound C-1 which is appeared for the first time in this plant and potent column fractions isolated from the chloroform fractions may prove useful for an effective and safe treatment of neurodegenerative diseases.

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