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Dual COX/ 5-LOX inhibitory potential and antioxidant activities by extracts isolated from Melia Azedarach L. leaves

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Abstract

This study was aimed at evaluating the dual cyclooxygenase (COX) and 5-Lipoxygenase (5-LOX) enzyme inhibition and antioxidant activity of the crude extract (CE) and various solvent fractions from Melia azedarach leaves. Multiple solvents were used to fractionate the 80% ethanolic extract of the leaves, which was then examined for enzyme inhibitory effectiveness against COX and 5-LOX enzymes. All fractions and CE were examined for antioxidant activity, and phenolic acids and flavonoids were identified using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). The ethyl acetate fraction (EaF) had the greatest COX/5-LOX inhibitory impact among tested fractions. In vitro, COX activity studies showed that EaF and butanol fraction (BF) possess a higher COX-2 selectivity index than Indomethacin. Besides, the EaF exhibited the highest reduction potential among the control and the CE when tested for antioxidant activities. These findings support the traditional use of *Melia azedarach* as an anti-inflammatory drug, as the plant inhibited both COX/5-LOX and COX-2 with high selectivity.

1. Introduction

The most frequent chemical species that cause oxidative damage in the body are reactive oxygen species (ROS) like peroxides, superoxide, singlet oxygen, hydroxyl radical, hydrogen peroxides, and so on. These species produced in the body can cause various cardiovascular and other chronic diseases [1-3], neurodegenerative diseases [4,5], and inflammatory diseases [6,7]. Different inflammatory diseases are linked to oxidative stress (an imbalance between ROS and their removal by protective mechanisms such as antioxidants) [7-9]. Inflammatory mediators such as prostaglandins produced by the arachidonic acid pathway need to be reduced and inhibited as they mainly cause inflammation, pain, and fever in the body [10].

Currently, the symptomatic treatment of inflammation is mainly done by non-steroidal anti-inflammatory drugs (NSAIDs). However, the traditional NSAIDs used in COX reduction are non-selective, lead to gastrointestinal side effects, heart strokes and renal failure. On the other hand, the currently used selective COX-2 inhibitors like 'coxibs' are reported for cardiovascular side effects [11]. In addition to COX-2, the increased level of leukotriene from the 5-LOX enzyme can result in bronchospasm [11]. In this regard, medicinal preparations that inhibit both Cyclooxygenase (COX-2) and 5-Lipoxygenase (5-LOX) pathways are superior to commonly used NSAIDs since they could produce a synergetic effect to achieve optimal anti-inflammatory activities [10,11]. This can be done by blocking the production of both leukotrienes and prostaglandins. Hence, indeed, there is a need for new, effective, and safe anti-inflammatory drugs with dual COX-2/5-LOX inhibition capacities, which may obliterate the side effects caused by the inhibition of COX-2 alone [12,13].

Melia azedarach L. (known commonly as "Chinaberry, bead tree, Bakain and Persian lilac") is a deciduous tree belonging to the Meliaceae family that is native to tropical Asia and Australia but has now spread throughout the world. [14-16]. The antioxidant and anti-inflammatory potential of *Melia azedarach* L. from various regions were reported by other groups [14,15, 17-21]. The plant with different species and some species of different cultivars markedly differ in their phenolics, flavonoids and antioxidant activities [18,20]. The antioxidant and anti-inflammatory activities of plant extracts are closely linked to phytoconstituents like total phenolics and flavonoids [22-25]. There is no scientific report on the anti-inflammatory action and antioxidant potential of Taiwanese-origin *Melia azedarach*. Therefore, the purpose of the current study is to investigate the dual COX-2 and 5-LOX inhibitor potential, antioxidant potential, quantification of key flavonoids and phenolic acids, as well as total phenolic and flavonoid content of *Melia azedarach*.

2. Materials and methods

2.1. Plant material

The leaves of *Melia azedarach* L. (Meliaceae) were obtained from National Taiwan University's natural forestry farm in Da'an District, Taipei, Taiwan. The farm location is N 25°00'59.40"; E 121°32'25.1" and the voucher specimen was deposited at the Herbarium of National Taiwan University, voucher no. K.F. Chung 1930. The leaves were powdered for extraction after freeze-drying (LABCONCO, 2.5 Free Zone, USA).

2.2. Extraction

200 g of Melia azedarach leaves powder was extracted thrice, each with 2L of 80% ethanol at 25 on an orbital shaking incubator (Cherng Huei, orbital shaking incubator model 5010, Taiwan) for 24 h. To obtain the crude extract (CE), each extract was filtered using Whatman No.1 filter paper, the three filtrates were mixed, and the solvent was evaporated using a rotary evaporator (EYELA Rotary Evaporator, N-1000, Japan) under reduced pressure at 45°C. The extract yield was calculated, and CE was suspended in water for solvent fractionations. To obtain the n-hexane soluble fraction (HF), hexane was added first, then the suspension was thoroughly agitated and separated in a separating funnel. The remaining water suspension was extracted with ethyl acetate following the same protocol so that the ethyl acetate soluble fraction (EaF) obtained. Lastly, butanol was used to get the butanol soluble fraction (BF). After liquid-liquid extractions, the remaining water suspension was filtered using cotton to obtain the water fraction (WF). A freeze drier was used to dry the water fraction (LABCONCO, 2.5 Free Zone, USA). Finally, the percentage yield was calculated using the weight of dry extracts obtained with each solvent.

2.3. Phytochemical analysis

The leaf extract of *Melia azedarach* was subjected to phytochemical screening, using standard methods discussed in Harborne [26].

2.4. Total phenol content determination

The total phenol content was determined using the Folin-Ciocalteu assay [27] with minor modifications. In a test tube, 200 mL of each solvent fraction (1 mg/mL) was combined with 1.8 mL of deionized distilled water. The solution was thoroughly vortexed after adding 200 µL Folin-Ciocalteu's phenol reagent and allowed to stay for 3 min. Then 400 µL of 7% Na_2CO_3 solution was added with stirring, and it was thoroughly mixed after immediately diluting to a total volume of 4 mL with deionized distilled water. The solution was incubated for 90 min at 25 °C, and the absorbance was measured at 750 nm using a spectrometer (JASCO UV-Vis spectrometer, V-550, Japan). The deionized distilled water was used as blank in this experiment, and a gallic acid standard solution (10-50 µg/mL) was used to prepare the standard calibration curve for total phenolics determination. The extracted samples' total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dried extracts. Finally, Equation 1 was applied to determine the total phenolic compound content of the extracts.

$$\text{Total phenol content} = \text{GAE} \times V/m \quad (1)$$

Where, GAE is the gallic acid equivalence (mg/mL) established from the calibration curve (Y=4.669x-0.013, R2 = 0.998); V is the volume of sample (mL) and m is the weight (g) of a sample.

2.5. Total flavonoid content determination

The total flavonoid content of the extracts was determined by applying the Aluminium chloride colorimetric method [28]. Accordingly. in a test tube, 1 mL of extract (1 mg/mL) was mixed with 4 mL of deionized water, then 0.3 mL of a 5 % NaNO2 solution was added. After allowing the solution to sit for 6 minutes, 0.3 mL of 10% AlCl3 solution was added and left to sit for another 5 minutes. Then, 2 mL of 1.0 M NaOH was added and the mixture was properly vortexed to be thoroughly mixed, and the absorbance was measured immediately at 510 nm using a spectrophotometer (JASCO UV-Vis spectrometer, V-550, Japan). The total content of flavonoids in each fraction and crude extract was calculated using Equation (2).

$$\text{Total flavonoid content} = \text{CE} \times V/m \quad (2)$$

Data were recorded as mean ± SD (standard deviation) of three replications. The mean SD (standard deviation) of three replications was recorded. The results were represented in milligrams of CE per gram of dried fraction. Where; CE is the catechin equivalent (mg/mL) established from the calibration curve (Y=1.0315x+0.0135, R2=0.993); V is the volume of the fraction (mL) and m is the weight (g) of the dried solvent fraction.

2.6. HPLC phenolic acids and flavonoids analysis

The reverse-phase (RP)-HPLC system was used to study the phenolic compounds of different extracts of the plant. The system comprises a JASCO PU-2089Plus Quaternary gradient pump, a JASCO UV-2077Plus 4-λ Intelligent UV/Vis detector, and a Luna 5µ C-18 (2) 100A column (250 x 4.6 mm) following composition of the solvents and the gradient elution conditions used were specified by Zhu and co-workers [29] with slight modifications. The mobile phase consisted of purified water with acetic acid (100:1, v/v) (solvent A), and methanol: acetonitrile: acetic acid (75:25:1, v/v/v) (solvent B) at a flow rate of 1.0 mL/min. The following conditions were used to produce gradient elution: from 0 to 2 min, linear gradient to 5% solvent B; from 2 to 10 min, 5 to 25% solvent B; from 10 to 20 min, linear gradient from 25% to 40% solvent B; from 20 to 30 min, linear gradient from 40% to 50% solvent B; from 30 to 40 min, from 50% to 100% solvent B; from 40 to 45 min, 100% solvent B; from 45 to 55 min, 100 to 5% solvent B. Other working conditions of the systems were established as follows: column temperature (30 oC), injection volume (20 µl), and UV detection at 280 nm for simultaneous detection of hydroxybenzoic and hydroxycinnamic acids. Flavonoids were determined using a 360 nm detection method. The fractionated samples' phenolic components were identified by comparing their relative retention times and UV spectra to those of authentic compounds.

2.7. In vitro COX-1 and COX-2 inhibitory activity

All fractions and the extract were evaluated for COX-1 and COX-2 inhibitory activity using a COX (ovine)-inhibitor screening kit (Catalog No.560101, Cayman Chemical, USA) according to the manufacturer's instructions. The assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a restricted amount of PG antiserum, and it detects PGF2 produced from PGH2 by reduction with stannous chloride. All of the experiments were done twice, and the percent inhibition was calculated by comparing the compounds treated to the control incubations. The quantity of PG tracer bound to the well, which is inversely proportional to the amount of free PG present in the well during incubation, is proportional to the intensity of the spectrophotometrically determined yellow color. The concentration of the samples that inhibited PGF2 synthesis by 50% (IC50, g/mL) was estimated using the

concentration–inhibition response curve. Indomethacin was used as reference standards. Previously, the EIA kit was utilized to assess COX-1 and COX-2 inhibitory activities [30].

2.8. In vitro 5-LOX inhibitory activity

The inhibition of the enzyme 5-LOX was measured with the help of lipoxygenase inhibitor screening assay in which, EIA kit (Cayman Chemicals, MI) (Catalog No. 760700, Cayman Chemical, USA) and 5-lipoxygenase (potato) screening enzyme (Catalog No. 60401, Cayman Chemical, USA) was used according to the manufacturer's instructions. In brief, 90 µL of lipoxygenase enzyme and 10 µL of inhibitor (extract samples and the standard Nordihydroguaiaretic acid, NDGA,) were added to the wells. The reaction was started in each well by introducing 10 µL of the substrate Linoleic Acid and vortexed for 5 minutes. The reaction was stopped by adding 100 µL of chromogen, vortexed for 5 minutes, and measuring the absorbance at 500 nm with an ELIZA reader (BioTek, Power wave XS, USA). By applying Equation (3), the percent inhibition activity, %IA and the IC50 values were calculated.

$$\% \text{ IA} = [\text{Inhibitor}/ \text{ IA}] \times 100 \quad (3)$$

2.9. Antioxidant assay

The antioxidant assay was performed by preparing a series of concentrations by properly diluting each sample and compared to the reference chemicals in all assays.

2.9.1. Reducing power

The reducing powers of leaf extract samples (CE, HF, EaF, BF and WF) and standard α-tocopherol (α-Toc) were studied according to the method specified by Gülçin et al. [31]. Accordingly, samples at various concentrations (50, 100, 250 and 500 µg/mL) were combined with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 1%). The resultant mixture was incubated for 20 minutes at 50°C. After adding 10% TCA (2.5 mL) the mixture was centrifuged (3000 x g) (Hettich zentrifugen, Mikro 20, Germany) for 10 minutes. About 2 mL upper layer was mixed with 2.5 mL distilled water and 0.5 mL FeCl3 (0.1%), and the absorbance was recorded at 700 nm in a spectrometer (JASCO UV-Vis spectrometer, V-550, Japan). The higher the absorbance of the reaction mixture, the greater is its reducing power.

2.9.2. Radical scavenging activity

The radical scavenging activity of the crude extract and fractionated samples of Melia azedarach leaves was studied by using the stable free radical 2-2-diphenyl-1-picrylhydrazyl (DPPH). The technique using 96-well microplates was employed [32]. 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). The sample or standard was mixed with 100 µl of DPPH (0.051 mM in methanol) in each well. The contents in the plate were thoroughly mixed by shaking the plate before being wrapped with aluminium foil. Then the plates are kept in the dark place at 25 for 30 min. Finally, using an ELISA reader (BioTek, Power wave XS, USA) and a methanol solution of DPPH as a control, the absorbance of the solution was measured at 517 nm. Finally, Equation 4 was used to compute percentage inhibition.

$$\text{Scavenging effect } (\%) = [(\text{Ac-As})/ \text{ Ac}] \times 100 \quad (4)$$

Where, As = absorbance of the sample; Ac = absorbance of the control From the above equation, the concentration needed to lower the initial DPPH concentration by 50% (IC_{50}) was calculated.

2.9.3. Hydroxyl radical scavenging activity

The activity of hydroxyl radical scavenging was determined using the method described by Kumar and co-workers [33]. In this context, the competition for the hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) between deoxyribose and samples was investigated. The following reaction mixture was prepared in a test tube to give a final volume of 1.0 mL: 100 µL 2-deoxy-2-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH 7.4), 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), 200 µL mixture of (1.04 mM) EDTA and (200 µM) $FeCl_3$ (1:1 v/v), 100 µL 1.0 mM H_2O_2, and 100 µL 1.0 mM ascorbic acid, and the mixture was incubated at 37 °C for 1 h. The test tube was filled with 1 mL of 1% thiobarbituric acid and 1.0 mL of 2.8% trichloroacetic acid (TCA) and incubated at 100°C for 20 min. This was allowed to cool to room temperature and absorbance was recorded against a control preparation containing deoxyribose and buffer at 532 nm using a spectrophotometer (JASCO UV-Vis spectrometer Model: V-550, Japan). As a positive control, quercetin was used. The percent inhibition was determined by comparing the results of the test and control compounds using Equation (4).

2.9.4. Hydrogen peroxide scavenging activity

The capability of samples to scavenge hydrogen peroxide (H2O2) was studied by following the method described by Alara et al. [34] with slight modification. Accordingly, a 40 mM H_2O_2 solution was produced in phosphate buffer (pH 7.4), and the concentration was determined using absorbances measured at 230 nm with a spectrophotometer (JASCO UV-Vis spectrometer, V-550, Japan). The sample (0.9 mL) prepared in distilled water with a concentration of 50-250 µg/mL was added to 0.6 mL H2O2 (40 mM). Just after 10 min, the absorbance of H_2O_2 was determined at 230 nm against a blank solution comprising phosphate buffer without H_2O_2. Equation (4) was used to compute the hydrogen peroxide scavenging activity of all solvent fractions, the crude extract, and the standard compound, BHT.

2.10. Statistical analysis

The inhibitory activities of 5-LOX and COX, DPPH radical scavenging activity, reducing power activity, and hydroxyl radical and hydrogen peroxide scavenging activities of the extract and solvent fractions of *M. azedarach* leaves are expressed as mean±standard deviation of three replicates per sample. The statistical significance of differences between groups was assessed by student's t-test using Microsoft Excel 2007 (Roselle, IL, USA). Significance was accepted at $P < 0.05$.

3. Results and discussion

3.1. Extraction yield

After repeated extraction (3 times) with 80% ethanol on 200 g dried leaves, 28 g of crude extract was obtained. The percentage yields of different solvent fractions vary in the range from 4.55% to 25.82% with descending order WF > HF > EaF > BF (Table 1).

Table 1. Total phenolics and flavonoids contents and extraction yield of solvent fractions of *Melia azedarach*.

Leaf solvent fraction	Extract yield (%)	TPC (mg of gallic acid equivalent/g)	TFC (mg of catechin equivalent/g)
HF	7.52±3.2^{b}	38.8±0.9^{c}	23.1±1.2^{c}
ETF	7.3±0.5^{b}	389.1±0.7^{a}	367.34±0.6^{a}
BF	4.55±0.3^{c}	161.91±0.4^{b}	146.2±1.2^{b}
WF	25.82±1.1^{a}	18.2±0.1^{d}	11.3±1.1^{d}

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction. TPC: total phenolic content; TFC: total flavonoid content. Each value is stated as mean ± SD of triplicate measurement. Figures on the same column with different letters are significantly different p<0.05.

3.2. Phytochemicals

The preliminary phytochemical screening of the leaves of *Melia azedarach* demonstrated the existence of phenols, saponins, tannins, steroids, cardiac glycosides and terpenoids (Table 2). However, the total phenol content (TPC) varies among the fractions (Table 1). The EaF had the largest TPC (389.1 ± 0.7 mg GAE/g), followed by BF and HF, while WF had the lowest (18.2 ± 0.1 mg GAE/g). A similar concentration trend was obtained for total flavonoid content (TFC) with the highest value of 367.34±0.6 CE/g in EaF (Table 1).

Table 2. Preliminary phytochemical screening of Melia azedarach leaf.

Tested for	Presence/absence	Test performed
Phenols	+++	Ferric chloride; Fast blue B salt
Tannins	+	Braemer's test
Alkaloids	-	Dragendorff's test
Steroids and Terpenoids	++	Liebermann-Burchardt test
Saponins	+++	Froth test
Cardiac glycosides	++	Salkowski test; Acetic acid and ferric chloride test

– Absent; + Traces; +++ Abundant

3.3. Dual COX/5-LOX inhibitory potential

The inhibitory properties of Melia azedarach extracts on 5-LOX, COX-1, and COX-2 were investigated as a mechanism for anti-inflammatory action. In a dose-dependent way, HF, EaF, BF, WF, and CE significantly reduced 5-LOX activity (Fig. 1).

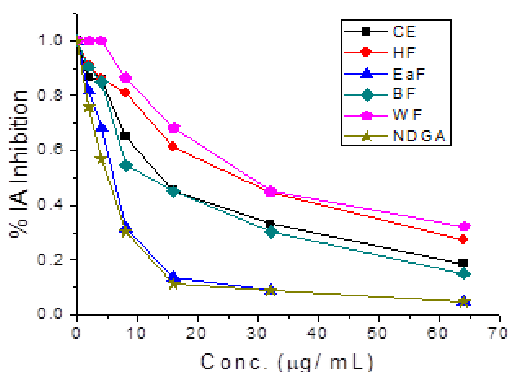


Fig. 1. Fig.1. Inhibitory potential of various solvent fractions of Melia azedarach on 5-LOX.

The EaF of Melia azedarach (IC50 =13.44 ±0.3 g/ml) has the strongest 5-LOX inhibitory action (Table 3). BF and CE also inhibited the enzyme at the low concentration range in a statistically significant manner (p<0.05). Reference drug NDGA had an IC50 value of 10.55±0.8 µg/ml. In the same way, EaF and the other fractions including CE inhibited both COX-1 and COX-2, the effects of the extracts were compared with Indomethacin (INDO).

Table 3. Inhibitory effects (IC50 in µg/ml) of Melia azedarach fractions and reference drugs on 5-LOX, COX-1 and COX-2.

Sample	5-LOX[a]	COX-1[b]	COX-2[b]	COX-1/COX-2[c]
HF	36.4±0.64	55.77±0.38	51.16±0.77	1.09±0.72
EaF	13.4±0.3	27.13±0.5	8.2±0.8	3.31±0.53
BF	26.1±0.27	44.7±0.22	16.9±0.43	2.64±0.28
WF	40.7±0.41	68.38±0.39	52.6±0.48	1.3±0.71
CE	28.2±0.35	47.3±0.74	22.3±0.5	2.12±0.65
INDO	-	4.9±0.28	4.5±0.23	1.08±0.56
NDGA	10.6±0.8	-	-	-

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction; CE: crude extract; INDO: Indomethacin; NDGA: Nordihydroguaiaretic acid. a. Initial activity inhibition by 50% concentration. b. Initial PGF2α production inhibition by 50%. c. ratio index of the two IC50 results.

In the like way as 5-LOX, the EaF exhibited the highest inhibition on COX activity of prostaglandin synthesis (Fig. 2). IC_{50} values for COX-2 were found to be 8.2±0.8 µg/ml and 16.9±0.43 µg/ml for EaF and BF, respectively. It's observed that the BF and CE exhibited an immediate inhibitory effect on COX-1. Moreover, the COX studies showed that EaF and BF possess a higher COX-2 selectivity index (COX-1/COX-2 ratio) of 3.31±0.53 and 2.64±0.28, respectively, compared to INDO (1.08±0.56).

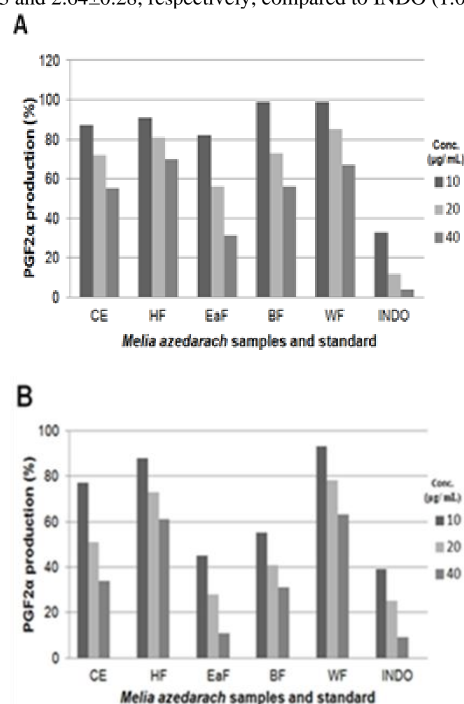


Figure 2: Inhibitory potential of various solvent fractions of Melia azedarach on COX-1(A) and COX-2 (B).

3.4. Antioxidant Activities

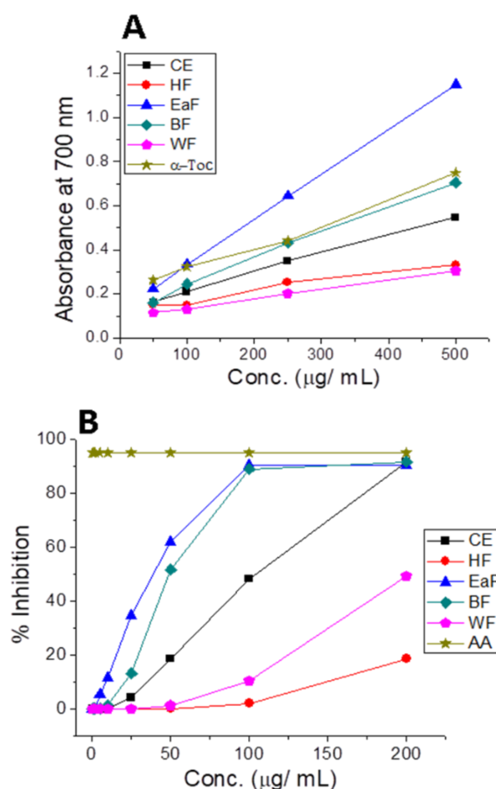
The ferric reductive capabilities of the extract, fractions and the control, α-tocopherol, were measured (Fig. 3A). The graph shows that EaF exhibited the highest reduction potential, followed by α-tocopherol (α-Toc), BF, CE,

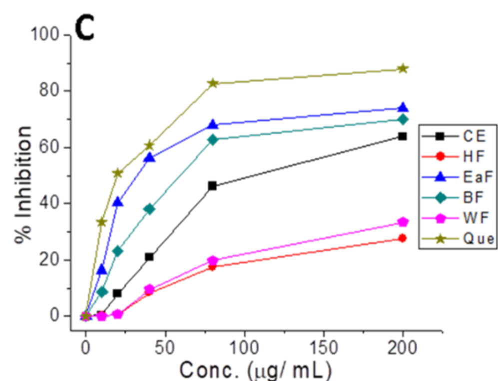
HF, and WF. Similarly, using 96-well microplates, the ability to act as a hydrogen atom donor in the transformation of the DPPH radical to its reduced form was studied (Fig. 3B). At a lower concentration (< 100 µg/mL), a highly significant difference ($p<0.01$) in inhibition capacity between the active fractions (EaF, BF) and CE was observed.

Though the standard ascorbic acid (AA) had ≥95% inhibition in the tested concentration ranges, the active fractions EaF and BF had comparable inhibition at a concentration ≥100 µg/mL, and CE at a concentration ≥200 µg/mL. The amounts of sample required to scavenge 50% of DPPH, (IC50 Values) followed the order: AA < EaF < BF < CE < WF < HF (Table 4).

Table 4. Scavenging effect (IC_{50}) on DPPH radicals, hydrogen peroxide, and hydroxyl radicals of Melia azedarach leaf samples.

Sample	IC_{50} DPPH assay (µg/mL)	IC_{50} Hydroxyl radical assay (µg/mL)	IC_{50} H_2O_2 assay(µg/mL)
HF	>>200^{f}	>200^{e}	>250^{e}
EaF	78.86±0.7^{b}	74.59±0.3^{b}	89.03±0.4^{b}
BF	86.35±0.6^{c}	106.72±1.3^{c}	136.79±0.6^{c}
WF	>200^{e}	>200^{f}	>250^{f}
CE	111.23±1.8^{d}	120.1±1.2^{d}	145.41±0.71^{d}
AA	<2^{a}	-	-
BHT	-	-	71.87±0.24^{a}
Quercetin	-	50.68±1.1^{a}	-





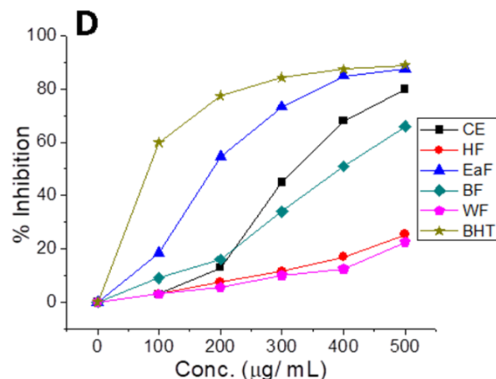


Figure 3: Various solvent fractions and crude extract of Melia azedarach and the standards antioxidant activity measured with Reduction potential (A), DPPH radical scavenging (B), Hydroxyl radical scavenging (C), and Hydrogen peroxide inhibition (D).

The hydroxyl radical assay result (Figure 3C) illustrates the increase of the hydroxyl radical inhibition by the samples in a concentration-dependent manner. The calculated IC50 values increase in the order: Que < EaF < BF < CE < HF < WF (Table 4). In general, the EaF and BF of Melia azedarach leaf extract exhibit far better inhibition of DNA damage than the crude extract. The inhibition potential increases with concentration. In the same way, the ability to scavenge H2O2 was determined and the corresponding IC50 values of samples follow the order: BHT < EaF < BF < CE < HF < WF (Table 4). Hence, similar to the other assays, EaF exhibited the highest inhibition among all samples in all concentrations on H_2O_2 assay (Figure 3D). BF showed higher inhibition than CE at the lower concentrations tested.

Antioxidant activity has been reported to be directly associated with the development of reducing power [35]. We explored the Fe^{3+} to Fe^{2+} transformation in the presence of samples for reductive ability assessments, and the experiment functioned as a major indicator of possible antioxidant activity. From the results of this study, it was found that the EaF exhibited stronger reducing power than the control (α-tocopherol), implying strong antioxidant potential and in all cases studied, the reduction potential of samples increased with the concentration used.

Even though hydrogen peroxide is not very reactive, it may give rise to hydroxyl radicals in the cell and may pose toxic effects. [36, 37]. The structural properties of a sample's active ingredients, particularly phenols,

which determine their electron-donating and scavenging capacities due to their hydroxyl group, can be ascribed to its H2O2 scavenging ability [38, 39]. Hydroxyl radicals produced via the Fenton`s reaction are reactive biological molecules [37] in living systems. They can result in lipid oxidation and lead to various biological damage [40]. Thus, their scavenging potential may offer a significant therapeutic approach against oxidative stress-induced diseases. When the extract fraction of Melia azedarach leaf or the reference chemical quercetin (Que) was added to the reaction mixture, the sample or Que eliminated hydroxyl radicals from the sugar and stopped it from degrading. In our investigation, TPC levels were higher than those found in a study on Melia of Indian origin [17].

3.5. Phenolic acids and flavonoid profiles

By comparing their retention times in the HPLC chromatogram and regression statistics with the respective standards, the four solvent fractions (HF, EaF, BF, and WF) from the crude extract of Melia azedarach leaves were identified and quantified for their phenolic acids and flavonoids contents. The retention times (in a minute) of standard phenolic acids and flavonoids in the HPLC chromatogram are gallic acid (6.74), catechin (13.1), 3,4-dihydroxy cinnamic acid (15.75), syringic acid (16.4), p-coumaric acid (19.5), ferulic acid (20.6), rutin (22.5), myricetin (26.3), quercetin (32.1), luteolin (34.0), apigenin (37.2) and kaempferol (38.4). From the chromatograms (Figure 4), it was possible to observe three major flavonoids among them two (myricetin and rutin) were identified and quantified.

Rutin was the major one in all analyses with the highest concentration in EaF (203±2.1 mg/g), followed by BF (113.1±1.8 mg/g), HF (36.05±0.55 mg/g), and WF (7.51±0.67 mg/g). Myricetin was the second major component in all four fractions. Catechin was also detected in EaF and WF at lower concentrations. Moreover, phenolic acids such as cinnamic acid, syringic acid, ferulic acid, and p-coumaric acid were identified, albeit in small concentrations (Tables 5 and 6). The trace amount of Luteolin, quercetin, apigenin and kaempferol were identified, hence couldn't be quantified. By comparing concentrations of the identified flavonoids among all fractions, it can be seen that EaF contains the highest concentration of total flavonoids (331.2±0.43 mg/g). Cinnamic acid was in the highest concentration among the phenolic acids in all fractions, while gallic acid was not detected.

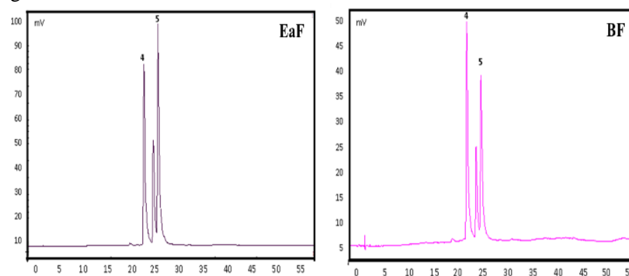


Figure 4: HPLC chromatograms of the most active solvent fractions (Ethyl acetate, and Butanol) of Melia azedarach. Major Peaks: 4, Rutin; 5, Myricetin.

Phenols are widely distributed and essential plant constituents because of the potential health benefits of their anti-oxidative activity. As reported by several research works, the total phenols content and the antioxidant activity of plant extracts were highly correlated [22-24]. Our study highly supports these scientific facts; the solvent fractions of the Melia azedarach with high TPC and TFC exhibited strong antioxidant activity. Though the extraction yields of EaF and BF were less than those of the HF and WF (Table 2), both EaF and BF showed higher antioxidant activity in all assays, which implies that their main constituents were antioxidant compounds. In this study, the high concentrations of rutin and myricetin flavonoids in EaF are believed to contribute to the observed strong antioxidant activity. Their COX and 5-LOX inhibitory actions are likely due to their antioxidant activities [41]. Besides the flavonoids and other phenolics present in these species, saponins and terpenes in the extract may also play a role in the overall antioxidant and anti-inflammatory effects observed [42,43].

The most widely recommended medications for the treatment of inflammation and pain are non-steroidal anti-inflammatory drugs or NSAIDs. The toxic effects of these medications, on the other hand, are a source of concern. As a result, various novel techniques for designing and developing superior anti-inflammatory drugs with fewer adverse effects are being examined. The major catalysts for the biological synthesis of prostaglandin H2 from the arachidonic acid substrate are COX-1 and COX-2. COX-1 inhibition has certain negative side effects, whilst COX-2 inhibition has therapeutic effects in pain, inflammation, cancer, glaucoma, Alzheimer's disease, and Parkinson's disease [44]. The COX-2 selectivity index of ETF and BF of CE of Melia azedarach is higher, which explains the favourable effects and dual inhibition of COX/5-LOX, as well as the good selectivity toward COX-2 inhibition, and also demonstrates the lack of COX-2 specificity of INDO, one of the most often used NSAIDs.

Table 5. Flavonoid content of Melia azedarach leaf (mg/g dried solvent fraction).

Sample	Catechin	Rutin	Myricetin	Quercetin	Luteolin	Apigenin	Kaempferol	Total
HF	ND	36.05±0.55^{c}	0.00443±0.0000^{c}	Nq.	Nq.	Nq.	Nq.	36.054±0.55^{c}
EaF	1.35	203±2.1^{a}	128.2±0.73^{a}	Nq.	Nq.	Nq.	Nq.	331.2±2.83^{a}
BF	ND	113±1.8^{b}	33.5±0.94^{b}	Nq.	Nq.	Nq.	Nq.	146.5±2.74^{b}
WF	0.439	7.51±0.67^{d}	0.000885±0.000001^{d}	Nq.	Nq.	Nq.	Nq.	7.511±0.67^{d}

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction; CE: Crude Extract. Values are expressed as mean ± SD of triplicate measurement. Numbers on the same column with different letters are significantly different p<0.05. Nq. = Not quantified because too trace.

Table 6. Phenolic acid content of Melia azedarach leaf (mg/g dried solvent fraction).

Sample	Gallic acid	3,4-Dihydroxy cinnamic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Total
HF	ND	ND^{d}	ND^{d}	ND^{c}	ND^{d}	ND^{d}
EaF	ND	1.58±0.0032^{a}	0.8±0.001^{a}	0.233±0.0001^{a}	0.625±0.002^{a}	3.238±0.0062^{a}
BF	ND	0.251±0.004^{c}	0.267±0.00011^{b}	0.002669±0.000002^{b}	0.0215±0.0005^{b}	0.5422±0.0046^{c}
WF	ND	0.797±0.0021^{b}	0.01386±0.00003^{c}	0.002119±0.0000019^{b}	0.000717±0.00000^{c}	0.814±0.00213^{b}

HF: hexane fraction; EaF: ethyl acetate fraction; BF: butanol fraction; WF: water fraction; CE: Crude Extract. Values are expressed as mean ± SD of triplicate measurement. Numbers on the same column with different letter are significantly different p<0.05. ND = Not Detected.

4. Conclusions

In addition to oxidative stress, the cyclooxygenase (COX) and lipoxygenase (LOX) pathways are the principal inflammatory mediators that can induce a variety of degenerative disorders in humans. Non-steroidal anti-inflammatory medicines (NSAIDs) are routinely used to treat inflammations; however, they inhibit the cyclooxygenase pathway non-selectively, causing gastric mucosal damage due to COX-1 inhibition and allergic reactions. The dual COX/5-LOX inhibition, antioxidant activities and HPLC chromatogram-based identification and quantification of phenolic metabolites were conducted for various fractions and crude extracts of Melia azedarach leaves. These findings justify the traditional use of the leaves against inflammatory diseases; moreover, it depicts higher total phenolic content than earlier reports of similar species from other localities. These activities in this plant are mainly attributed to several phenol components especially, flavonoids (rutin and myricetin). Other phenolics compounds such as 3, 4-dihydrocinnamic acid, ferulic acid, syringic acid, p-coumaric acid might also have contributed to these activities.

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