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# Comparative Phytochemical Investigation, Antioxidant and Anticancer Properties of Leave Extracts of Four Medicinal Plants from Chhattisgarh, India

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## ABSTRACT

*Background: The present study investigates comparative antioxidant, anticancer activity and qualitative, quantitative and spectral characterization of phytochemicals present among aqueous and ethanolic/hydro-ethanolic leave extracts of four medicinal plants viz. Anthocephalus cadamba, Aegle marmelos, Emblica officinalis and Moringa oleifera.*

*Methods and Findings: In vitro antioxidant capacities were evaluated via free radical scavenging assays: DPPH, FRAP, ABTS, phosphomolybdate assay and total reducing capability. In vitro anticancer activity was evaluated against three human cancer cell lines SK-OV-3, A498 and T-24 by SRB assay. Quantitative estimation of phenolic, flavonoids, flavonols and Vitamin C content was done calorimetrically. Spectral characterization of phytochemicals was done via UV-Vis, FTIR and GC-HRMS analysis. Among all the leave extracts, aqueous and hydro-ethanolic leave extracts of E. officinalis has highest reducing power and lowest $IC_{50}$ value for DPPH free radical with higher phenolic content. Evaluation of anticancer activity of leave extracts showed no significant activity against above cell lines. Spectral characterization of phytochemicals showed the presence of carbohydrates, carboxylic acid, phenols, flavonoid and Vitamins.*

*Conclusion: The phytochemicals present may responsible for antioxidant and other pharmacological effects of above plants.*

**Keywords:** *Anthocephalus cadamba*; *Aegle marmelos*; *Emblica officinalis*; *Moringa oleifera*; Antioxidant; Anticancer.

**Abbreviations:**

ABTS:2, 2' azino bis (3-ethylbenz-thiazoline-6-sulphonic acid) diammonium salt; ADR:Adriamycin; A498:Human Kidney Carcinoma cell line; BEL-7404:Human Hepatocellular Carcinoma Cells; DPPH:2,2-diphenyl-1-picrylhydrazyl; DTC:2,4-Dinitrophenylhydrazine-Thiourea-Copper sulphate reagent; FRAP:Ferric Reducing Antioxidant Power; $FeCl_3$:Ferric Chloride; FTIR:Fourier Transforms Infrared Spectroscopy; GC-HRMS: Gas Chromatograph- High Resolution Mass Spectrometer; GAE:Gallic Acid Equivalent; HCL:Hydrochloric Acid; $H_2SO_4$:Sulphuric Acid; HT-29:Human Colon Cell Line; KBr:Potassium Bromide; Mo:Molybdenum; NIST:National Institute of Standards and Technology; RT:Room Temperature; RPMI 1640:Roswell Park Memorial Institute 1640 medium; SK-OV-3:Human Ovarian Cancer Cell Line; SRB:Sulforhodamine B; T-24:Human bladder cancer cell line; TCA:Trichloroacetic acid solution; TFC:Total Flavonoid Content; TLC:Thin Layer Chromatography; TPC:Total Phenolic Content; TPTZ:2,4,6-tripyridyl-s-triazine; UV-Vis:Ultra Violet-Visible; WHO:World Health Organization.

## INTRODUCTION

In human history, especially in developing countries plants are used for numerous healing purposes and numerous present drugs are prepared from plant sources [1]. According to WHO among 122 pure compounds identified from plant 80% were used in some medicinal purposes [2,3]. Now a day's medicinal plants are widely evaluated to find natural antioxidants for treating oxidative stress related diseases [4,5]. The phytochemicals with antioxidative properties

such as Vitamin C, E and β-carotene, phenolics and flavonoids exhibit a wide range of pharmacological actions and medicinal properties including anti-carcinogenic, anti-inflammatory and protecting degenerative diseases [6].

*Anthocephalus cadamba* belongs to Rubiaceae family,commonly known as "Kadamba" in Sanskrit and Hindi. Majorly the bark and leaves of plant are reported to have medical significance. The pharmacological effects of plant and phytochemicals of Kadamba include antidiabetic, antioxidant, antitumor, analgesic, anti-inflammatory, antibacterial, antidiarrheal, antihepatotoxic, diuretics and laxative activities [7,8]. Chandel et al. reported the antioxidant activity and phytochemicals of 95% ethanolic and other fractions of leaves extracted under refluxed conditions [9]. Ganjewala et al. reported the antioxidant activity and phyto-constituents of methanolic extracts of leaves of *Neolamarckia cadamba* [10]. Chandel et al. showed antioxidant, antigenotoxic and anticarcinogenic activity against COLO-205 cancer cell line of flavonols isolated from *A. cadamba* leaves [11]. Also Singh et al. reported cytotoxic potential of leaves against human cancer cell lines [12].

*Aegle marmelos* belongs to Rutaceae family, commonly known as Bael. All parts of the plant (leaves, fruit pulp, flower, stem bark, root bark etc.) are medicinally useful. The leaves are used as mild laxative, as an anti-inflammatory agent of conjunctiva and other body parts, its juice is given in constipation and jaundice, and used in treatment of wound, lecorrhoea and deafness [13,14]. Ariharan and Prasad reported the phytochemicals in chloroform leave extract of *Aegle marmelos* [15]. *Emblica officinalis* belongs to family Euphorbiaceae, commonly known as Indian gooseberry or Amla. It is the most important medicinal plant in Indian traditional system of medicine. Several parts of plant have variety of medicinal properties among which the fruit part is more important and explored more for its medicinal value [16]. The plant parts show antioxidant, antibacterial, antidiabetic, hypolipidimic, hepatoprotective, antiulcerogenic, gastroprotective, and chemopreventive properties [17]. Nain et al. reported the antioxidant and antibacterial activity of hydro-methanolic leave extract of *E. officinalis*. [18]. Other studies have reported anti-inflammatory [19,20] antidiabetic [21] activity and nephroprotective activity against cisplatin nephrotoxicity in Amla leaves [22].

*Moringa oleifera* belongs to Moringaceae family. Different parts of the plant have medicinal values. The pharmacological effects of plant include antitumor, antioxidant, cholesterol lowering, anti-inflammatory, antipyretic, antidiabetic, nephroprotective and hepatoprotective properties. The leaves juice is believed to control glucose levels, applied to reduce glandular swelling, eye and ear infections, scurvy and catarrh, rubbed on the temples for headaches [23,24]. Goswami and Singhai investigated antioxidant activity *via* DPPH assay of successive solvent extracts (Petroleum ether, chloroform, ethyl acetate and 70% ethanolic extracts) of *M. oleifera* leaves [25]. Shahrirar et al. investigated Phytochemical, antioxidant activity *via* DPPH assay, and cytotoxic activity of five different extracts (n-hexane, ethanol, chloroform and methanol) of *M. oleifera* leaves [26]. Patel et al. investigated phytochemical and antifungal activities of 90% ethanolic and aqueous extract prepared *via* maceration method of *M. oleifera* leave extracts [27]. Sanganna et al. investigated antiproliferative activity against human HT-29 colon cell line and antioxidant activity *via* DPPH assay of ethanolic and aqueous extract prepared *via* soxhlet extracts of *M. oleifera* leaves [28]. Imohiosen et al. investigated Phytochemical and antimicrobial studies of 100% ethanolic extracts of *M. oleifera* leaves [29]. Torres-Castillo et al. investigated phytochemical, antioxidant enzymes and antifungal properties of different parts of *M. oleifera* plant extracted *via* maceration method [30]. Jayawardana et al. evaluated antioxidant and antimicrobial activity in of *M.oleifera* leaves in chicken sausages [31].

The present study investigates comparative *in vitro* antioxidant activities *via* different assays, anticancer activity *via* SRB assay, comparative phytochemical investigation *via* qualitative, quantitative and spectral identification (UV-Vis, FTIR and GC-HRMS) of phytochemicals in aqueous and different ethanolic/hydro-ethanolic leave extracts of above medicinal plants.

## MATERIALS AND METHODS

### Chemicals and Reagents

Sodium carbonate ($Na_2CO_3.10H_2O$), Aluminum chloride ($AlCl_3$) Potassium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl) ABTS (2, 2' azino bis (3-ethylbenz-thiazoline-6-sulphonic acid) diammonium salt), Potassium persulphate, TPTZ (2,4,6-tripyridyl-s-triazine), Ammonium molybdate, Potassium ferricyanide, Trichloro Acetic Acid (TCA) were purchased from Hi-Media Chemical Pvt. Ltd. Mumbai, India. Folin–Ciocalteu Reagent, Ferric chloride ($FeCl_3$), Potassium bromide (KBr), Quercetin, Gallic acid, ascorbic acid were purchased from Sigma-Aldrich (USA). All other reagents were of analytical grade and were purchased commercially from local vendors supplying scientific grade chemicals. Ultra-pure water (Elix, Merck Millipore, India) was used throughout the experiments.

**Collection of leave samples**

The leaves of four plants *i.e. Anthocephalus cadamba* (Kadam), *Aegle marmelos* (Bael), *Emblica officinalis* (Amla), *Moringa oleifera* (Munga) were collected from areas near Bilaspur District and Guru Ghasidas University campus, Bilaspur (Chhattisgarh) India. The collected leaves were identified and authenticated by qualified Botanist from Guru Ghasidas Vishwavidyalaya, Bilaspur (Chhattisgarh) India.

**Extraction of bioactive components**

The collected leaves were washed with tap water to remove dirt and air dried at room temperature for 4-7 days. The dried leaves were grounded in to small parts. 30 g of grounded leaves were extracted by aqueous and ethanolic/ hydro ethanolic solvents using soxhlet extractor for 8 h at temperature below the boiling point of the solvent. After extraction the extracts were concentrated by evaporating the solvent until it got reduced to solid or semi-solid mass. The concentrated extracts were weighed and percentage yield was recorded and stored in air tight container at 4ºC for further use.

$$percentage\ yeild = \frac{Weight\ of\ the\ extract}{Weight\ of\ the\ powdered\ leaf\ sample} \times 100$$

**Preliminary phytochemical screening of leave extracts**

Each of aqueous and ethanolic/hydro-ethanolic extract of all the four plants were subjected for qualitative phytochemical analysis for the detection of various active phytochemicals such as alkaloids, saponins, phytosterols, flavonoids, phenols, terpenoids, phlobatannis, anthraquinones, carbohydrates, glycosides, amino acids, proteins and vitamins by standard methods [32,33].

**Determination of total phenolic content**

Total phenolic content present in each of aqueous and ethanolic/hydro-ethanolic extract of leaves was evaluated by Folin- ciocalteu assay (FC), gallic acid was taken as standard [34]. 400 µL of each of the extractives (1 mg/mL) and standard (250 µg/mL to 0.48 µg/mL) was separately mixed with 2 mL of Folin–ciocalteu reagent (10%) and 1.6 mL of sodium carbonate ($Na_2CO_3.10H_2O$) 20.25%. After shaking, the tubes were incubated for 2 h. The absorbance was measured at 738 nm against the blank. From the standard curve total phenolic content of the extractives which is equivalent to Gallic acid was calculated and expressed in terms of Gallic acid equivalent (GAE) in µg/mg of the extractives or µg GAE/mg of extractive.

**Determination of total flavonoid content**

Aluminum chloride ($AlCl_3$) colorimetric method was used for the total flavonoid content using quercetin as standard [35]. Each of leave extracts (0.5 mL of 1 mg/mL) and standard (100 µg/mL to 10 µg/mL) were mixed with 1.5 mL of methanol, 0.1 mL of 1M potassium acetate, 0.1 mL of 10% $AlCl_3$ and 2.8 mL of distilled water and allowed to incubate for 30 min at RT. The absorbance of reaction mixture was taken at 415 nm against blank. From the linear equation of standard curve total flavonoid content of each of extractives was calculated.

**Determination of total flavonol content**

Flavonol content were determined by using rutin as a standard compound. This method is based on the formation of complex with maximum absorption at 440 nm [36]. Each of leave extracts (1 mg/mL) was mixed with 1 mL aluminum chloride (20 mg/mL) and 3 mL sodium acetate (30 mg/mL). After 2.5 h the absorbance was read at 440 nm. The absorbance of standard rutin (1 mg/mL) solution in methanol was measured under the same conditions. The amount of flavonols in plant extracts was calculated by following formula:

$$X = \frac{A\,M_0}{A_0\,M}$$

Where, X is the flavonoid content (µg/mg) as rutin equivalents. $A$ and $A_o$ is the absorption of plant extract and rutin solution, respectively; $M_o$ and M is molecular weight of rutin and weight of leave extract (µg), respectively.

**Determination of Vitamin C content**

Each of leave extracts (1 mg) was treated with 4 mL of 10% Trichloroacetic Acid Solution (TCA) and centrifuged for 20 min at 3500 rpm, then 0.5 mL of supernatant was mixed with 0.1 mL of DTC reagent (2, 4- Dinitrophenylhydrazine-thiourea-copper sulphate reagent) and allowed to incubate for 3 h at 37ºC. 0.75 mL of ice cold 65% $H_2SO_4$ was added and allowed to stand for 30 min at RT. A set of standards (100-20 µg of ascorbic acid) was processed similarly

along with a blank containing 0.5 mL of 10% TCA. The absorbance of colour developed was read at 520 nm. The concentration of Vitamin C was calculated from standard curve and expressed as µg of ascorbic acid equivalents per mg of extract [37].

**UV-Visible spectral analysis of leave extracts**

300 µg/mL solution of each leave extracts were prepared in their extractive solvents and absorption spectra of all the leave extracts were recorded by UV-Vis spectroscopy (UV-1800, Shimadzu, Japan) from 200 to 1100 nm for preliminary characterization of leave extracts.

**Fourier transforms infrared spectroscopy (FTIR) analysis of leave extracts**

FTIR spectra of all the leave extracts were obtained between 4000 and 600 $cm^{-1}$ with a resolution at 4 $cm^{-1}$ using FTIR spectrophotometer (Thermo Scientific, Nicole iS5). Leave extracts (1 mg) were mixed properly with 100 mg KBr and properly grounded before analysis.

**GC-HRMS analysis of Leave Extracts**

The Gas Chromatography with High Resolution Mass Spectrometer (GC-HRMS) analysis of all leave extracts was performed using Aligent technology GC System (model 7890) equipped with high resolution mass spectrometer (Jeol, model: Accu TOF GCV). The experimental conditions involved HP-5MS Capillary Standard non-polar column (column length-60 m, column diameter-0.32 m and phase thickness-0.25 µm). Spectroscopic detection involved an electron ionization system which utilized high energy electrons (70 eV). Pure Helium (He) gas was used as a carrier gas with flow rate of 1.0 mL/min. The initial oven temperature was set at 20ºC with a hold of 5 min then increase of 100ºC at 10ºC/min then 200ºC at 4ºC/min and finally to 280ºC at 10ºC/min. The relative percentage amount of each component was calculated by comparing the peak area of component with the total areas. The results were compared with NIST Library search programme.

***In vitro* antioxidant activity of leave extracts:**

**DPPH radical scavenging assay**

The antioxidant activity of aqueous and ethanolic /hydro ethanolic extractives of leaves of above plants was assayed on the basis of free radical scavenging effect of the stable DPPH free radical [38]. 1 mg/mL (1000 µg/mL) stock solutions of each of the aqueous and hydro ethanolic/ethanolic leave extracts of above plants were prepared in their respective extractive solvents. From the above stock solution further 7 dilutions were made from 500 µg/mL to7.8 µg/mL by reducing the concentration to just half of previous concentration. Ascorbic acid was used as standard. The stock solution of DPPH was prepared by dissolving 24 mg DPPH in 100 mL ethanol and the working solution was prepared by mixing 12 mL stock solution with 45 mL ethanol so as to obtain an absorbance of 1.1 ± 0.05 at 517 nm. 200 µL of above sample dilutions were mixed with 2800 µL of working DPPH solution and incubated in dark for 20 min at room temperature and reading was taken by using UV/Visible Spectrophotometer at 517 nm against blank. Percentage inhibition was calculated by using the formula as follows:

$$\%\,\text{inhibition} = \left[\frac{A_0 - A_i}{A_0}\right] \times 100$$

Where $A_o$=absorbance of the control, and $A_i$=absorbance of the sample. $IC_{50}$ Value (Concentration of compound required to quench 50% of the DPPH free radical) was calculated by plotting the graph, taking % inhibition on Y axis and concentration on X axis.

**Antioxidant activity by DPPH staining**

TLC plate was prepared by silica gel and 3 µl of each standard (ascorbic acid) and sample (1 mg/mL) were carefully loaded into the plate and allowed to dry for 3 min. The TLC plate was sprayed with 0.2% DPPH solution in methanol. Discoloration of DPPH indicates scavenging potential of the leave extracts [39].

**ABTS radical cation decolourisation Assay**

The method involves scavenging of ABTS [2, 2' azino bis (3- ethylbenz-thiazoline-6-sulphonic acid) diammonium salt] radical cation, a blue green chromogen which is produced by a reaction between ABTS and potassium persulphate. The coloured radical is converted to colourless ABTS in the presence of antioxidant reductant [40]. ABTS radical cation ($ABTS^{\bullet+}$) stock solution was produced by reacting 7.4 mM ABTS and 2.6 mM potassium persulphate in equal ratio (1:1 v/v) and incubated overnight (12-16 h) at dark in RT. The working solution of ABTS radical cataion was prepared by diluting 1 mL $ABTS^{\bullet+}$ stock solution with 50 mL methanol so as to obtain absorbance of ≥ 0.7 at 734

nm. 1 mg/mL (1000 µg/mL) stock solutions of each of the aqueous and hydro ethanolic/ethanolic leave extracts were prepared in their respective extractive solvents. From the above stock solution further dilutions were made such as 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, and 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and 0.625 µg/mL. 200 µL of different dilutions of sample solution was mixed with 2800 µL of working $ABTS^{\bullet+}$ solution and incubated for 1 to 2 h in dark at RT. Absorbance was taken at 734 nm against blank. Tannic acid was taken as standard. Percentage scavengity was calculated by using the formula:

$$\%scavengity = \left[\frac{A_0 - A_i}{A_0}\right] \times 100$$

Where $A_o$=absorbance of the control, and $A_i$=absorbance of the sample. $IC_{50}$ Value was calculated by plotting the graph, taking % Scavengity on Y axis and concentration on X axis.

**Ferric reducing antioxidant power assay**

The FRAP assay was carried out by method of Benzie and Szeto with some modifications [41]. The Stock solution included 300 mM acetate buffer, pH 3.6 (3.1 g Sodium acetate tri-hydrate in 16 mL glacial acetic acid), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCL and 20 mM anhydrous $FeCl_3$. The working FRAP reagent was prepared by mixing the above solution in the ratio of 10:1:1 respectively at the time of use. 200 µL of each sample (1 mg/mL) were allowed to react with 2800 µL of working FRAP reagent for 30 min. in dark. Absorbance of the colored product (Ferrous tripyridyltrazine complex) was taken at 593 nm against blank. Gallic acid was taken as standard and results were expressed in terms of µg/mg of GAE.

**Phosphomolybdate assay for evaluation of antioxidant capacity**

Phosphomolybdate assay, a quantitative method to evaluate the antioxidant capacity, has invariably been used for determination of antioxidant activity of many plant extracts [42]. To the 200 µL of samples (1 mg/mL) 1 mL of test reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The tubes were then incubated in water bath at 70ºC temperature for 90 min. Samples were cooled at ambient temperature and the absorbance was measured against blank at 695 nm. Antioxidant capacity of each sample was calculated form graph and expressed as equivalent of ascorbic acid (µg/mg).

**Determination of reducing power**

Transformation of $Fe^{3+}$ to $Fe^{2+}$ in the presence of extractives under study was taken as the parameter to study the measurement of the reductive ability. The reducing power was determined according to the method of Oyaizu [43]. 1 mL of leave extracts (200 µg/mL)/standard (different dilutions), 2.5 mL of potassium ferricyanide and 2.5 mL of phosphate buffer were allowed to incubate at 50ºC for 20 min to reduce ferricyanide to ferrocynaide. The reaction was stopped by adding 2.5 mL of 10% Trichloro Acetic Acid (TCA) followed by centrifugation at 1000 rpm for 10 min. The upper layer was collected and mixed with distilled water and ferric chloride (0.1% w/v in water) in the ratio 1:1:0.2. After 20 min. absorbance was measured at 700 nm and concentration of ferric–ferrocynaide was determined using ascorbic acid as standard. Increase in absorbance of the reaction mixture indicated a high reducing power of the samples.

***In vitro* anticancer activity of leaves extract**

*In vitro* anticancer activities of hydro-ethanolic leaf extracts of all the above plants was evaluated against three cancer cell lines (Human Kidney Carcinoma cell line A498; Human ovarian cancer cell line SK-OV-3 and Human bladder cancer cell line T-24) by Sulforhodamine B (SRB) assay [44,45].

**Cell culture**

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates, at densities depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5 % $CO_2$, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.

**Sulforhodamine B (SRB) assay**

All the leave extracts were initially solubilized in dimethyl sulfoxide at 100 mg/mL and diluted to 1 mg/mL using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1 mg/mL) was thawed and diluted to 100 µg/mL, 200 µg/mL, 400 µg/mL and 800 µg/mL with complete medium containing test article. Aliquots

of 10 µL of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µL of medium, resulting in the required final drug concentrations i.e. 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL. After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µL of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µL) (0.4% (w/v) in 1% acetic acid) was added to each of the wells, and incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells*100. GI 50 value is concentration of drug causing 50% inhibition of cell growth.

### Statistical analysis

All antioxidant assays, anticancer and quantitative test were performed in triplicates and values are presented as mean ± SD. The results were validated statistically using one way ANOVA. Significant difference between mean values of each test were compared by Tukey's test at the significant level $p<0.05$ using Graph pad prism software version 5.

## RESULTS AND DISCUSSION

### Percentage yield of leaf extracts

For the recovery of bioactive phytochemicals from plant sources extraction is the main step. The yield of extracts depends on many factors such as nature of solvent with varying polarity, nature of phytochemicals, the extraction method used, sample particle size, presence of interfering substances, sample to solvent volume ratio, pH, temperature and extraction time. Commonly used solvents for extraction of polar compounds include alcohols (methanol, ethanol), acetone, ethyl acetate and mixture of alcohol and water [46]. In the present study among the aqueous and ethanolic/ hydro ethanolic extraction greater yield were obtained with hydro ethanolic extraction as compared as to aqueous extraction. These results are in agreement with other studies which showed higher extraction values in alcoholic/ hydro-alcoholic extracts than aqueous extracts [47]. The percentage yield of each of aqueous and ethanolic/ hydro ethanolic extract of leaves of all the four plants (i.e. *Anthocephalus cadamba, Aegle marmelos, Emblica officinalis, Moringa oleifera*) are presented in Table 1.

**Table 1:** Percentage yield of leave extracts.

| S.NO. | Leave Sample | % yield in aqueous solvent | % yield in ethanolic/hydro-ethanolic solvent (% of solvent used) |
|---|---|---|---|
| 1. | *Anthocephalus cadamba* (Kadam) | 20.4% | 15.85% (100%) |
| 2. | *Aegle marmelos* (Bael) | 6.0% | 30.5% (30%) |
| 3. | *Emblica officinalis* (Amla) | 22.4% | 26.4% (70%) |
| 4. | *Moringa oleifera* (Munga) | 28.8% | 29.0% (50%) |

### Qualitative phytochemical screening

It is reported that most active phytochemicals in plants include tannins, saponins, phenols, alkaloids, flavonoids, glycosides, steroids and terpenoids in varying concentrations which are responsible for its pharmacological actions. Specifically phenolic compounds act as antioxidants and are responsible for varying range of medicinal values such as anticancer, anti-inflammatory, and diabetes etc [48,49]. Comparative qualitative phytochemical screening of all leave extracts were shown in Table 2. All the leave extracts showed the presence of alkaloids, saponins, phytosterols, flavonoids, phenolic compounds, terpenoids. Although, carbohydrates are present in all leave extracts except in *A. cadamba*, glycosides are present in *Moringa* and *Emblica* leaves, amino acids are absent in all leaves, presence of proteins are detected in Amla leaves only, phlobatannis are absent in all leaves, presences of anthraquinones was in

**Table 2:** Comparative preliminary phytochemical screening.

| S.No. | Phytochemicals | Kadam Leaves | | Bael Leaves | | Munga Leaves | | Amla Leaves | |
|---|---|---|---|---|---|---|---|---|---|
| | | Aqueous extract | Ethanolic extract | Aqueous extract | Ethanolic extract | Aqueous extract | Ethanolic extract | Aqueous extract | Ethanolic extract |
| 1. | Alkaloids | + | + | + | + | + | + | + | + |
| 2. | Saponins | + | + | + | + | + | + | + | + |
| 3. | Phytosterols | + | + | + | + | + | + | + | + |
| 4. | Flavonoids | + | + | + | + | + | + | + | + |
| 5 | Phenolic compounds | + | + | + | + | + | + | + | + |
| 6 | Terpenoids | + | + | + | + | + | + | + | + |
| 7 | Carbohydrate | - | - | + | + | + | + | + | + |
| 8 | Starch | - | - | - | - | - | - | + | + |
| 9 | Glycosides | | | - | - | - | + | + | - |
| 10 | Amino acids | - | - | - | - | - | - | - | - |
| 11 | Proteins | - | - | - | - | - | - | + | - |
| 12 | Phlobatannins | - | - | - | - | - | - | - | - |
| 13 | Anthraquinones | - | - | - | + | - | - | - | - |
| 14 | Vitamin C | - | + | - | - | - | - | + | + |

Bael leaves only, although vitamin C was detected in Amla and Kadam leaves only. The results are in similarity with other studies [15,50].

**Quantitative estimation of Phenolic, Flavonoid, Flavonols and Vitamin C content**

The total phenolic content was estimated by Folin-Ciocalteu (FC) method in which electrons from phenolic component are transferred to FC reagent in alkaline medium [51,52]. The phenolic content in all the extractives varied between 396.00 ± 0.0 and 46.07 ± 1.18 µg of GAE per mg of dried leaf extract. Among all the leaf extractive the highest phenolic content was observed in AET (Amla Ethanolic Extract) (396.00 ± 0.0 µg/mg), followed by AAQ (Amla Aqueous Extract)>KET (Kadam Ethanolic Extract))>KAQ (Kadam aqueous ectract)>BET (Bael Ethanolic Extract)>BAQ (Bael Aqueous Extract)>MET (Munga Ethanolic Extract)>MAQ (Munga Aqueous Extract) (Table 3). The phenolic content in extracts of above leaves are higher when compared to leaves of some other medicinal plants [52,53,54]. The higher value of phenolic content in alcoholic extracts in compare to their respective aqueous extract was may be due to more phenolic group in phenols extracted in ethanolic extract than phenolic compound extracted in aqueous extract also, it may be due to specific complex formation of some phenolic compounds in ethanolic extract which are soluble in alcoholic solvents.

The flavonoid content of all the extracts is shown in Table 3 and is expressed as µg quercetin equivalent per mg of plant extracts. The value of flavonoid varies from 62.20 ± 2.42 to 13.27 ± 0.12, with highest value in KET followed by AET>KAQ>AAQ>BET>MET>MAQ>BAQ. Again the flavonoid content was higher in the present leaves extracts when compared to leaves of some other medicinal plants [53]. The effect of solvents on flavonoid content was similar to phenolic content. The value of Flavonols was expressed as µg/mg as rutin equivalent, ranging from 0.207 ± 0.004 to 0.059 ± 0.004 and it was found to be highest in MET followed by AAQ>AET>BET> >BAQ>KAQ>MAQ>KET. The Value of Vitamin C was expressed as µg/mg and ranged from 348 to 18.5 with the highest value in AET and AAQ. It is reported that 80% of antioxidant activity of plant was due to its antioxidative vitamins (Vitamin A, E and C).

**Table 3:** Total Phenolic, Flavonoid, Flavonol and Vitamin C content of leave extracts.

| Plant Sample | TPC[1] | TFC[2] | Total Flavonol[3] | Vitamin C[4] |
|---|---|---|---|---|
| KAQ | $189.07 \pm 1.86^{d}$ | $44.60 \pm 0.40^{b}$ | $0.099 \pm 0.005^{c}$ | $54.5 \pm 4.50^{c}$ |
| BAQ | $53.37 \pm 0.99^{f}$ | $13.27 \pm 0.12^{d}$ | $0.100 \pm 0.000^{c}$ | $28.0 \pm 8.17^{c}$ |
| AAQ | $306.47 \pm 1.33^{b}$ | $43.00 \pm 2.31^{abc}$ | $0.149 \pm 0.005^{b}$ | $140.0 \pm 8.17^{b}$ |
| MAQ | $46.07 \pm 1.18^{g}$ | $19.33 \pm 0.50^{c}$ | $0.081 \pm 0.002^{c}$ | $18.5 \pm 6.13^{c}$ |
| KET | $208.23 \pm 1.90^{c}$ | $62.20 \pm 2.42^{a}$ | $0.059 \pm 0.004^{d}$ | $66.0 \pm 1.63^{c}$ |
| BET | $75.13 \pm 1.96^{e}$ | $25.27 \pm 1.36^{c}$ | $0.145 \pm 0.007^{b}$ | $30.5 \pm 11.04^{c}$ |
| AET | $396.00 \pm 0.0^{a}$ | $51.53 \pm 0.61^{a}$ | $0.145 \pm 0.004^{b}$ | $348.0 \pm 9.81^{a}$ |
| MET | $47.1 \pm 1.61^{g}$ | $23.60 \pm 0.72^{c}$ | $0.207 \pm 0.004^{a}$ | $23.5 \pm 6.13^{c}$ |

Values represent mean ± standard de*via*tion (SD) of three independent experiments (n=3).Values within in a column followed by different alphabets (in superscript) are significantly different among each other according to ANOVA (Turkey test). (i.e. the highest value was superscripted with alphabet a, next highest value was superscripted with b and vice versa. The values within the column superscripted with different alphabets are significantly different like value superscripted with a is significantly different from b,c,d,e,f,g and vice versa, the values superscripted with same alphabet are non-significantly different, like for e.g. two values with same superscript 'a' are non-significantly different. Values less than $P<0.05$ are considered statistically significant).

1. Total phenol content expressed as concentration of polyphenols (µg) in term of Gallic acid equivalent (GAE) per mg of leave extracts.
2. Total flavonoid content expressed as concentration of flavonoids (µg) in term of Quercetin per mg of leave extracts.
3. Total Flavonol content expressed as concentration of flavonol (µg) in term of Rutin per mg of leave extracts.
4. Vitamin C content expressed as µg/mg of leave extracts.

AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

**UV-Vis Spectral analysis**

Figure 1(a and b) shows the UV-Vis Spectra of aqueous and ethanolic/hydro-ethanolic leave extracts of all four plants. The characteristics peak in all extracts varies from range 200 nm to 350 nm between which lies majorly the phenolics and flavonoids. The maximum absorbance with varying wavelength and its corresponding predicted compound was shown in Table 4. The maximum absorbance for *E. officinalis* aqueous extract obtained at wavelength 349, 261 and 211 nm, similar absorption maximum obtained for hydro-ethanolic extract at 354, 265 and 228 nm, absorption at this range was may be due to the presence of yellow flavonols (chalcones & aurones), Phenolic compounds, P- hydroxyl benzoic acid (phenolic acids) and O-coumaric (Phenolic acid) [55] thus, conforming the qualitative test of phenols and flavonoids. The maximum absorbance for *A. marmelos* and *A. cadamba* aqueous and hydro-ethanolic extracts was obtained between 321 and 281 nm depicting the presence of Flavanones & Dihydroflavonols, dihydrochacones, flavan-3-ols, Proanthocynidins or phenolic compounds [56,57,58]. While the absorption maxima of *M. oleifera* aqueous and hydro-ethanolic extracts were 305, 261 and 266, 259 nm respectively, depicting the presence of flavonoids and yellow flavonols conforming the qualitative and quantitative test for flavonoids. The UV-Vis spectral analysis supports the above biochemical observation for the presence of phenols and flavonoids which may be responsible for the antioxidant properties of the above leaves.

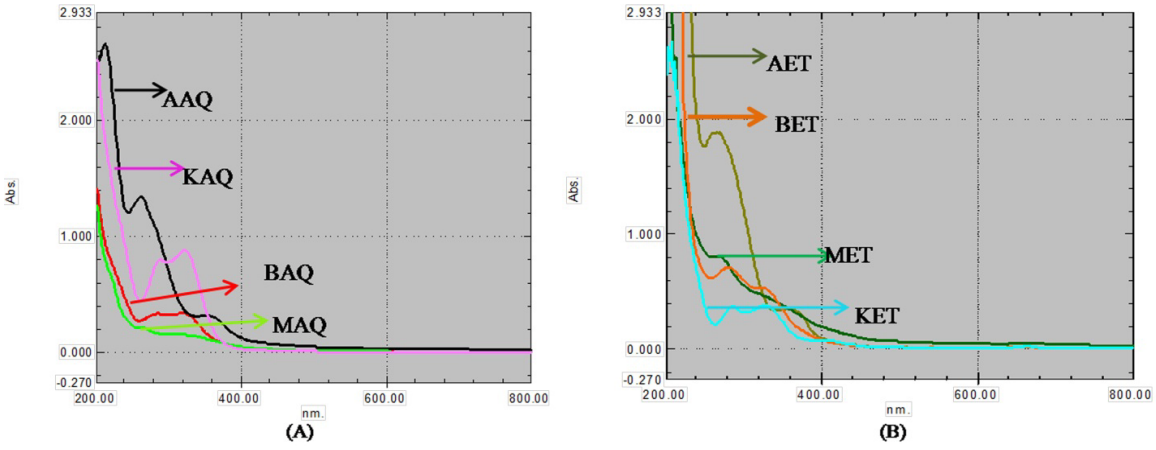


**Figure 1:** UV-Vis Spectral Analysis of leave extracts: (A) Aqueous leave extract of all four Plants (B) Ethanolic/Hydro-ethanolic leave extract of all four Plants. AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

**Table 4:** UV-Vis Spectral Analysis of Leave Extracts.

| Sample | Peaks λ (nm) | Absorbance | Predicted Compounds |
|---|---|---|---|
| **AAQ** | 349,261, 211 | 0.310,1.340, 2.666 | Yellow flavonols (chalcones & aurones),Yellow Flavonols, Phenolic compounds |
| **BAQ** | 318, 284 | 0.333, 0.329 | Flavanones & Dihydroflavonols, dihydrochacones,, Flavan-3-0ls, Proanthocynidins. Or phenolic compounds. |
| **KAQ** | 321, 288 | 0.875, 0.791 | Flavanones & Dihydroflavonols, dihydrochacones,, Flavan-3-0ls, Proanthocynidins or phenolic compounds. |
| **MAQ** | 305, 261 | 0.148, 0.216 | Flavonoids, Yellow flavonols |
| **AET** | 354, 265, 228 | 0.349, 1.885, 3.918 | Yellow flavonols (chalcones & aurones)<br>P- hydroxyl benzoic acid (phenolic acids)<br>O- coumaric (Phenolic acid) |
| **BET** | 318, 281 | 0.387, 0.506 | Flavanones & Dihydroflavonols, dihydrochacones,, Flavan-3-0ls, Proanthocynidins. Or phenolic compounds. |
| **KET** | 327, 285 | 0.376 0.367 | Flavanones & Dihydroflavonols, dihydrochacones,, Flavan-3-0ls, Proanthocynidins. Or phenolic compounds. |
| **MET** | 266, 259 | 0.804 0.800 | Flavonols, p- hyroxyl bezoic acid.<br>Flavonols |

AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

### Fourier transforms infrared spectroscopy (FTIR) analysis

FTIR Spectra identifies functional groups present in active components based on the peak value at infrared region. FTIR Spectra of aqueous and ethanolic/hydro-ethanolic leave extracts of all four plants was shown in Figure 2. The major functional groups present in *E. officinalis* aqueous (AAQ) and hydro ethanolic extract (AET) include alcohol, alkane, carbonyl, nitro, amides, ether and aromatic rings. FTIR spectra of *A. marmelos* aqueous and hydro-ethanolic extract showed the presence of alkane, amines, amides, ether, alcohol, alkenes, aromatic rings, carbonyl and ether groups. Whereas, in *A. cadamba* aqueous and ethanolic group functional groups present were alkane, alkene, alcohol, carbonyl, nitro, ether, and aromatic rings. Also the similar functional groups present in *M. oleifera* aqueous and hydro-ethanolic extract. The characteristics peak value and functional group present in all extracts was summarized in Table 5. The functional groups observed in all leave extracts indicated the presence of carbohydrates, carboxylic acid, amides, phenolics, flavonoids, etc. as confirmed by GC-HRMS analysis of all extracts (see section 3.6) which are acting as antioxidant and showed other pharmacological effects. Among the functional groups observed in aqueous and hydro-ethanolic extracts the O-H group is present only in ethanolic extracts. Presence of OH group in ethanolic extracts may be responsible for its higher antioxidant activity as OH group has the ability to form hydrogen bonding.

**Table 5:** FTIR Analysis of Leave Extracts

| Leave Extracts | Characteristic Wavelength ($cm^{-1}$) | Functional Group |
|---|---|---|
| **AAQ** | 1979.96 | Unknown |
| | 1716.79 | C=O (aldehydes, ketones, carboxylic acids, esters) |
| | 1616.21 | C=O group |
| | 1511.16 | $NO_2$ (Nitro compounds) |
| | 1335.55 | C-N (amines , amides) |
| | 1112.30 | C-N (amines , amides) |
| | 1057.79 | C-O (ether, alcohol) |
| | 860.73 | aromatic rings |
| **AET** | 3319.36 | O-H Stretching (Alcohol) |
| | 1720.74 | C=O (aldehydes, ketones, carboxylic acids, esters) |
| | 1612.81 | C=O group |
| | 1511.90 | $NO_2$ (Nitro compounds) |
| | 1447.10 | C-H (Alkane) |
| | 1212.40 | C-N (amines , amides) |
| | 1040.20 | C-O (ether, alcohol) |

| | | |
|---|---|---|
| **BAQ** | 1597.87 | Unknown |
| | 1420.16 | C-H (Alkane) |
| | 1288.87 | C-N (amines , amides) |
| | 1123.82 | C-O (ether, alcohol) |
| | 932.34 | =C-H (alkenes) |
| | 860.97 | aromatic rings |
| **BET** | 3360.48 | O-H Stretching (Alcohol) |
| | 2362.18 | C-H Stretching |
| | 2338.68 | C-H Stretching |
| | 1843.80 | C=O (aldehydes, ketones, carboxylic acids, esters) |
| | 1034.01 | C-O (ether, alcohol) |
| **KAQ** | 1606.34 | C=C group |
| | 1522.94 | $NO_2$ (Nitro compounds) |
| | 1390.90 | C-H (Alkane) |
| | 1047.76 | C-O (ether, alcohol) |
| **KET** | 3387.41 | O-H Stretching (Alcohol) |
| | 2920.08 | C-H (Alkane) |
| | 2849.56 | C-H (Alkane) |
| | 1690.07 | C=O Stretching |
| | 1608.69 | C=O group |
| | 1521.09 | $NO_2$ (Nitro compounds) |
| | 1444.96 | C-H (Alkane) |
| | 1375.06 | C-H (Alkane) |
| | 1066.82 | C-O (ether, alcohol) |
| | 924.39 | =C-H (alkenes) |
| | 875.20 | =C-H (alkenes), aromatic rings |
| | 816.31 | =C-H (alkenes), aromatic rings |
| | 717.52 | =C-H (alkenes), aromatic rings |
| **MAQ** | 1606.11 | C=O group |
| | 1406.24 | C-H (Alkane) |
| | 1031.92 | C-O (ether, alcohol) |
| | 887.32 | =C-H (alkenes), aromatic rings |
| | 809.79 | =C-H (alkenes), aromatic rings |
| **MET** | 3349.90 | O-H Stretching (Alcohol) |
| | 2919.50 | C-H (Alkane) |
| | 2850.31 | C-H (Alkane) |
| | 2361.57 | C-H Stretching |
| | 1733.40 | C=O (aldehydes, ketones, carboxylic acids, esters) |
| | 1609.24 | C=O group |
| | 1507.99 | $NO_2$ (Nitro compounds) |
| | 1405.05 | C-H (Alkane) |
| | 1123.48 | C-O (ether, alcohol) |
| | 913.58 | =C-H (alkenes) |

AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

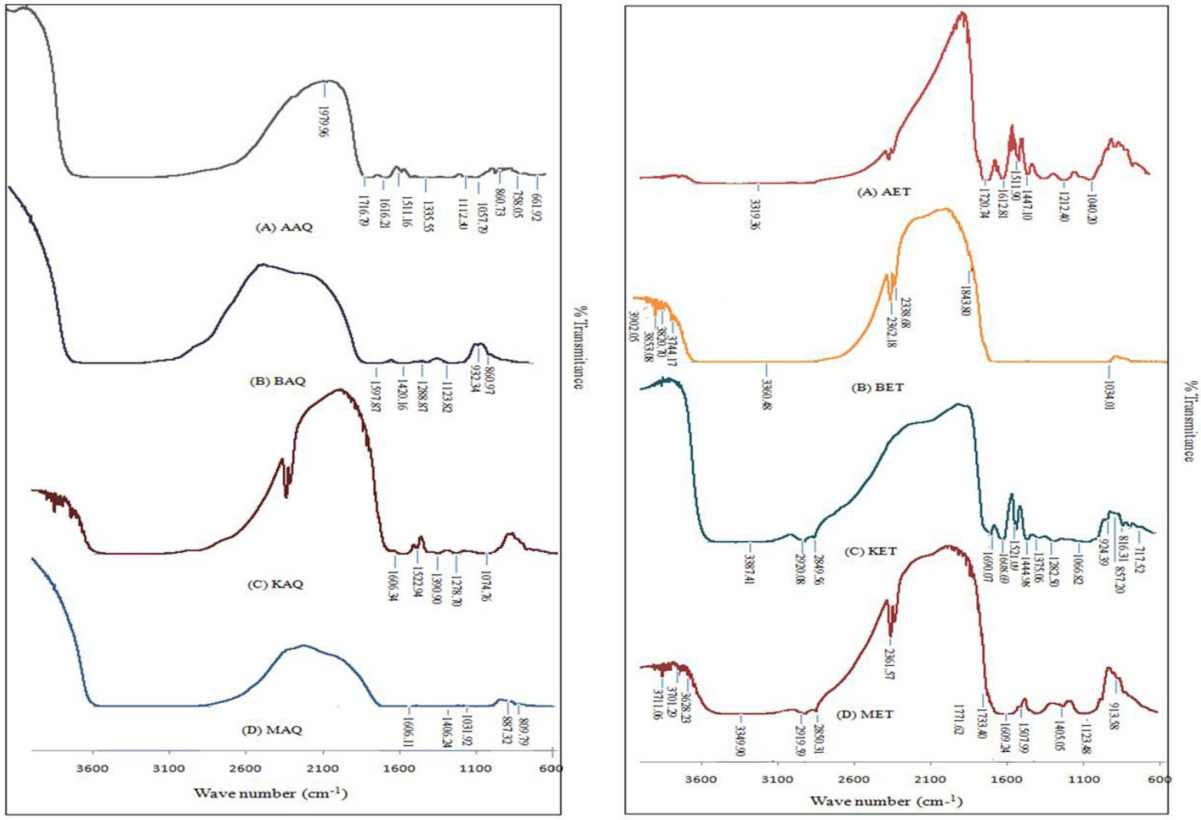


**Figure 2:** Fourier Transform Infrared Spectroscopy (FTIR): (A) Aqueous leave extract of all four Plants (B) Ethanolic/Hydro-ethanolic leave extract of all four Plants. AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

**GC-HRMS analysis of leaf extracts**

Plants are the source of numerous compounds and majority of these compounds have medicinal values [58]. GC-MS is most commonly used technique for separation and identification of compounds, in present study aqueous and ethanolic/hydro ethanolic leave extracts of above four plant species are subjected to GC with high resolution mass spectroscopy study for identification of compounds present. Compounds are identified by comparing MS spectra with standard spectra from NIST library. The compounds identified in aqueous and hydro ethanolic leave extracts of four plants are shown in Table 6, Figures 3 and 4. The major bioactive-compound obtained in *E. officinalis* aqueous extract (AAQ) was 1,2,3-Benzenetriol (Pyrogallol/Phenol) (29.05%), Melezitose (5.8%), whereas major compound in hydro-ethanolic extract (AET) include 1,2,3-Benzenetriol (Pyrogallol/Phenol) (16.6%), Vitamin E; (α-Tocopherol) (6.64%). Pyrogallol is a powerful reducing agent and Vitamin E is a potent antioxidant and it is believed to be protecting cells from oxidative stress [59]. The major bioactive compound detected in *A. marmelos* aqueous extract (BAQ) were Tert-Hexadecanethiol (61.75%), whereas major compound in hydro-ethanolic extract (BET) include Phytol (10.5%), Oxalic acid (10.5%), Oleic acid (19.67%), Vitamin E (11.4%). Phytol is a diterpene alcohol which is used in synthesis of Vitamin E and K which is also a degradation product of chlorophyll [60]. Oxalic acid also act as reducing agent, oleic acid has cholesterol lowering capability [61]. The major phyto-compounds present in *A. cadamba* aqueous extract (KAQ) include 2-piperidnone,N-[4-bromo-n-butyl] (37.7%), 9-Octodecenoicacid [Z], Phenylmethyl ester (35.33%). However, the compound present in hydro-ethanolic extract (KET) include Dodecanoic acid (32.41), Phytol (4.29), Squalene (12.1%), Polyalthic acid (11.34). The major antioxidant compound present in *Moringa oleifera* aqueous extract (MAQ) include Vitamin E (53.21), whereas in ethanolic extract (MET) include n-Hexadecanoic acid (38.35%), Phytol, 9,12,15-octadecatrienoicacid (39.7%).

**Table 6:** GC-HRMS analysis of Leave Extracts.

| Leave Extract | RT (min) | Compound, molecular formula, Common name | % Peak |
|---|---|---|---|
| Emblica officinalis Aqueous extract (AAQ) | 3.96 | 2-Furancaboxaldehyde,5-(hydroxymethyl); $C_6H_6O_3$; Hydroxymethyl-5-furfural | 6.97 |
| | 8.14 | 1,2,3-Benzenetriol; $C_6H_6O_3$; Pyrogallol,Phenol,Piral | 29.05 |
| | 7.82 | α-D-Glucopyranoside,O-α-D-glucopyranosyl-(1-fwdraw.3)-β-D-fructofuranosyl; $C_{18}H_{32}O^{16}$; Melezitose | 5.8 |
| | 24.9 | 1-Hexadecanol,2-methyl; $C_{17}H_{36}O$; 1,2-Methylhexadecan-1-ol | 2.44 |
| Emblica officinalis hydro ethanolic extract (AET) | 3.72 | 4H-Pyran-4-one,2,3-dihydro-3,5dihydroxy-6-methyl; $C_6H_8O_4$; 1,3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-Pyran-4-one. | 2.5 |
| | 8.94 | 1,2,3-Benzenetriol; $C_6H_6O_3$; Pyrogallol,Phenol,Piral | 9.96 |
| | 18.97 | Silane,trichlorodocosyl; $C_{22}H_{45}Cl_3Si$; Docosyltrichlorosilane | 1.53 |
| | 25.04 | Z-8-Methyl-9-tetradecenoic acid; $C_{15}H_{28}O_2$ | 7.74 |
| | 28.83 | 9-Octadecenoic acid (Z),hexylester; $C_{24}H_{46}O_2$; Oleic acid, hexylester. | 4.34 |
| | 31.63 | Vitamin E; $C_{29}H_{50}O_2$; α-Tocopherol | 6.64 |
| | 36.99 | Trans-13-Octadecenoic acid; $C_{18}H_{34}O_2$ | |
| Aegle marmelos aqueous extract (BAQ) | 19 | Oxalic acid, allyl hexadecyl ester; $C_{21}H_{38}O_4$ | 2.0 |
| | 26.5 | Oleic acid; $C_{18}H_{34}O_2$; Red Oil, Emersol 211 | 2.8 |
| | 31.75 | Tert-Hexadecanethiol; $C_{16}H_{34}S$ | 40.08 |
| Aegle marmelos hydro-ethanolic extract (BET) | 17.64 | Phytol; $C_{20}H_{40}O$; trans phytol | 10.5 |
| | 18.98 | Oxalic acid, allyl hexadecyl ester; $C_{21}H_{38}O_4$ | 10.97 |
| | 27.02 | 3-Buten-2-one,4-(3-hydroxy-6,6-dimethyl-2-methylonecyclohexyl | 9.1 |
| | 28.12 | Oleic acid; $C_{18}H_{34}O_2$; Red Oil, Emersol 211 | 19.67 |
| | 30.49 | n-propyl 11-octadecenoate; C21H40O2 | 11.43 |
| | 30.81 | 9,12,15-Octadecatrienoic acid 2- [(trimethylsily)oxy]-1-[[(trimethylsilyl)oxy] methyl] ethylester(ZZZ); $C_{27}H_{52}O_4Si_2$ | 22.41 |
| | 31.61 | Vitamin E; $C_{29}H_{50}O_2$; α-Tocopherol | 11.4 |
| Anthocephalus cadamba aqueous extract (KAQ) | 18.98 | Oxalic acid, allyl Pentadecyl ester; $C_{20}H_{38}O_4$ | |
| | 22.42 | 2,6-Dimethylbenzaldehyde, thiosemicarbazone; $C_{10}H_{13}N_3S$ | |
| | 24.77 | 2-piperidnone,N-[4-bromo-n-butyl]; $C_9H_{16}BrNO$ | 21.86 |
| | 26.11 | 9-Octodecenoicacid [Z], Phenylmethyl ester; $C_{25}H_{40}O_2$; Benzyl oleate | 35.33 |
| Anthocephalus cadamba ethanolic extract (KET) | 4.38 | 4H-Pyran-4-one,2,3-dihydro-3,5dihydroxy-6-methyl; $C_6H_8O_4$ | |
| | 12.35 | Dodecanoic acid,3-hydroxy; $C_{12}H_{24}O_3$ | 32.41 |
| | 13.91 | 10-Methyl-E-11-tridecen-1-ol propionate; $C_{17}H_{32}O_2$ | 2.7 |
| | 14.47 | D-Glucose,4-O-α-D-glucopyranosyl; $C_{12}H_{22}O_{11}$; Maltose. | |
| | 15.65 | n-Hexadecanoic acid;$C_{16}H_{32}O_2$; Palmitic acid | 11.34 |
| | 17.52 | Phytol; $C_{20}H_{40}O$; trans phytol | 4.29 |
| | 18.4 | [1,1'-Bicyclopropyl]-2-octonoic acid,2'hexyl, methyl ester; $C_{21}H_{38}O_2$ | 8.02 |
| | 28.59 | Squalene $C_{30}H_{50}$ | 12.1 |
| | 29.7 | Sulfurous acid, butylactadecyl ester; $C_{22}H_{46}O_3S$ | 8.02 |
| | 29.85 | 1-Napthalenecarboxylic acid,5-[2-(3-Furanyl)ethyl]decahydro-1,4a,dimethyl-6-methylene-,[1R-(1α,4aβ,5β,8aα)]; $C_{20}H_{28}O_3$; Polyalthic acid. | 11.34 |
| Moringa oleifera aqueous extract (MAQ) | 10.44 | 1-Gala-1-ido-octose; $C_8H_{16}O_8$ | |
| | 15.18 | d-Mannose; $C_6H_{12}O_6$; Mannose | 3.22 |
| | 31.65 | Vitamin E; $C_{29}H_{50}O_2$; α-Tocopherol | 53.21 |
| | 30.81 | 9,12,15-Octadecatrienoic acid 2- [(trimethylsily)oxy]-1-[[(trimethylsilyl)oxy] methyl] ethylester(ZZZ); $C_{27}H_{52}O_4Si_2$ | 5.3 |
| | 30.89 | B-Sitosterol; $C_{29}H_{50}O$; Sitosterol | 19.51 |
| Moringa oleifera ethanolic extract (MET) | 13.95 | 3,7,11,15- Tetramethyl-2-hexadecen-1-ol; $C_{20}H_{40}O$ | 6.50 |
| | 15.69 | n-Hexadecanoic acid; $C_{16}H_{32}O_2$ | 38.35 |
| | 17.55 | Phytol; $C_{20}H_{40}O$; trans phytol | 2.5 |
| | 18.20 | 9,12,15-octadecatrienoicacid,[Z,Z,Z];$C_{18}H_{30}O_2$ | 39.7 |

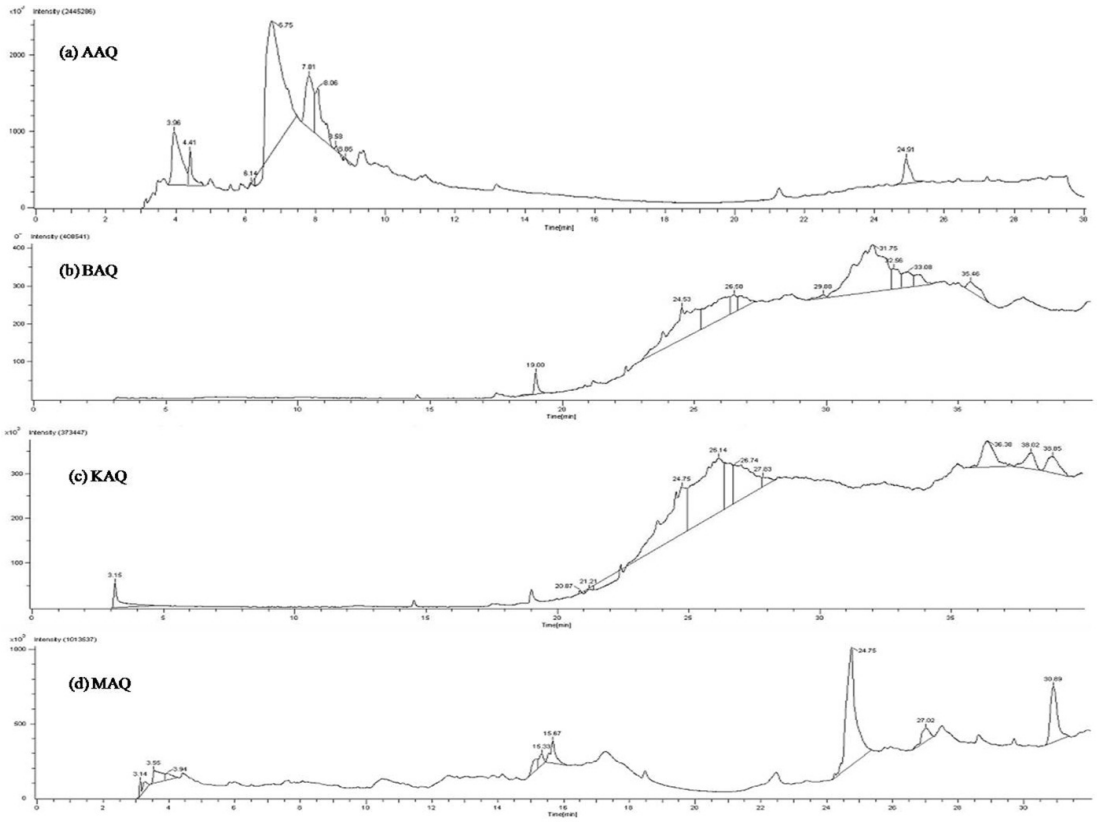


**Figure 3:** Gas Chromatography with High Resolution Mass Spectrometry (GC-HRMS) plot of Aqueous Leave extracts of all four plants. (a) AAQ=Amla Aqueous Extract, (b) BAQ=Bael Aqueous Extract, (c) KAQ=Kadam Aqueous Extract, and (d) MAQ=Munga Aqueous Extract.

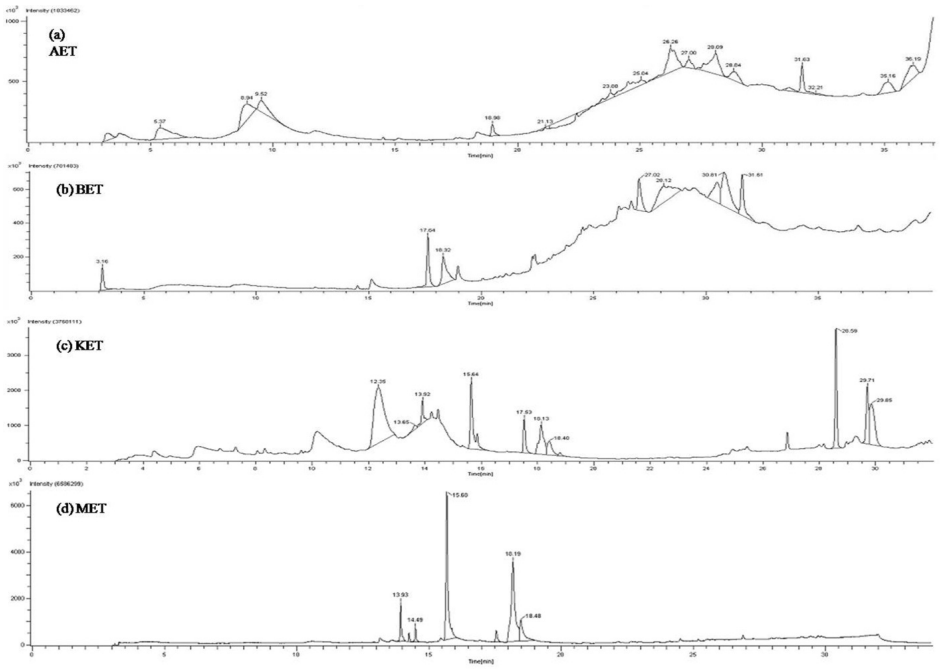


**Figure 4:** Gas Chromatography with High Resolution Mass Spectrometry (GC-HRMS) plot of ethanolic/hydro-ethanolic Leaves extracts of all four plants. AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

### *In-vitro* antioxidant activity

### DPPH free radical scavenging activity

DPPH free radical is commonly used *in vitro* assay for assessment of antioxidant potency. Freshly prepared solution of DPPH in methanol/ethanol is a stable violet colored which upon reduction with hydrogen or electron donors of antioxidant compounds present in extracts changes to yellow colored complex (2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine) resulting in decrease in absorbance at 517 nm [62]. Decolorization of violet colored DPPH to yellow color by leave extracts was clearly depicted in Figure 5. DPPH radical Scavenging activity of aqueous and hydro-ethanolic extracts of all leaves was shown in Table 7. All of the leave extract able to reduce DPPH free radical to yellow colored diphenyl picryl hydrazine. Among all the leaves extracts the minimum concentration require to scavenge 50% of DPPH free radical ($IC_{50}$ value) was observed in AET (46.88 ± 4.1) and AAQ (86.903 ± 2.5) which is lower than positive control ascorbic acid (117.857 ± 147.1). After AET and AAQ the lowest $IC_{50}$ values was observed in KET and KAQ followed by BET and BAQ. The DPPH radical scavengity activity of MET and MAQ was much lower (highest $IC_{50}$ value) than above leave extracts. The results of DPPH radical scavenging activities correlates with TPC (P=-0.8) and TFC (P=-0.78) (Figure 6) of leave extracts. The extracts with higher TPC and TFC have higher DPPH radical scavenging activity. Similar $IC_{50}$ values are reported in other leaves [63,64].

**Table 7:** Antioxidant activity ($IC_{50}$ value) on DPPH radicals, ABTS radical, FRAP value and reducing power of plant extracts.

| Plant Sample | DPPH[1] | ABTS[2] | FRAP[3] | PMA[4] | RP[5] |
|---|---|---|---|---|---|
| **KAQ** | 237.49 ± 1.4$^{d}$ | 39.71 ± 4.6$^{b}$ | 115.95 ± 0.08$^{c}$ | 167.42 ± 12.8$^{b}$ | 57.69 ± 2.9$^{b}$ |
| **BAQ** | 742.83 ± 15.1$^{f}$ | 18.24 ± 3.9$^{ab}$ | 19.96 ± 0.58$^{f}$ | 31.46 ± 0.8$^{e}$ | 3.83 ± 0.4$^{d}$ |
| **AAQ** | 86.903 ± 2.5$^{b}$ | 29.25 ± 1.6$^{b}$ | 117.83 ± 0.29$^{ab}$ | 151.96 ± 2.3$^{b}$ | 117.49 ± 6.9$^{a}$ |
| **MAQ** | 1426.33 ± 120.2$^{g}$ | 101.44 ± 18.0$^{abc}$ | 28.27 ± 1.22$^{e}$ | 64.71 ± 11.8$^{de}$ | 8.40 ± 1.2$^{d}$ |
| **K ET** | 104.913 ± 1.2$^{b}$ | 20.27 ± 7.0$^{ab}$ | 117.31 ± 0.03$^{b}$ | 302.13 ± 36.8$^{ab}$ | 65.11 ± 2.2$^{b}$ |
| **BET** | 503.643 ± 9.0$^{e}$ | 21.35 ± 7.4$^{ab}$ | 54.51 ± 2.84$^{d}$ | 131.04 ± 3.1$^{ab}$ | 20.74 ± 0.7$^{c}$ |
| **AET** | 46.88 ± 4.1$^{a}$ | 21.05 ± 0.8$^{b}$ | 119.61 ± 0.16$^{a}$ | 195.83 ± 26.1$^{abcd}$ | 95.04 ± 2.5$^{a}$ |
| **MET** | 1477.63 ± 147.1$^{fg}$ | 155.88 ± 9.0$^{c}$ | 21.25 ± 0.25$^{f}$ | 92.42 ± 12.9$^{bce}$ | 21.87 ±1.5$^{c}$ |
| **Standard** | 117.857 ± 147.1$^{c}$ | 13.04 ± 0.6$^{a}$ | | | |

Values represent mean ± standard de*via*tion (SD) of three independent experiments (n=3).Values within in a column followed by different alphabets (in superscript) are significantly different among each other according to ANOVA (Turkey test) (i.e. the highest scavenging activity was superscripted with alphabet a, next highest scavenging activity was superscripted with b and vice versa. The values within the column superscripted with different alphabets are significantly different like value superscripted with a is significantly different from b,c,d,e,f,g and vice versa, the values superscripted with same alphabet are non-significantly different, like for e.g. two values with same superscript 'a' are non-significantly different. Values less than P<0.05 are considered statistically significant.

AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

1 concentration of leave extracts/standard (ascorbic acid) required scavenging of 50% of DPPH radical.

2 concentration of leave extracts/standard (tannic acid) required scavenging of 50% of ABTS radical.

3 FRAP value was expressed as equivalent of gallic acid (µg/mg).

4 Phosphomolybdate assay of each leave extracts were expressed as equivalent as ascorbic acid (µg/mg).

5 Reducing power of all leave extracts expressed as equivalent as ascorbic acid (µg/200µg of sample)

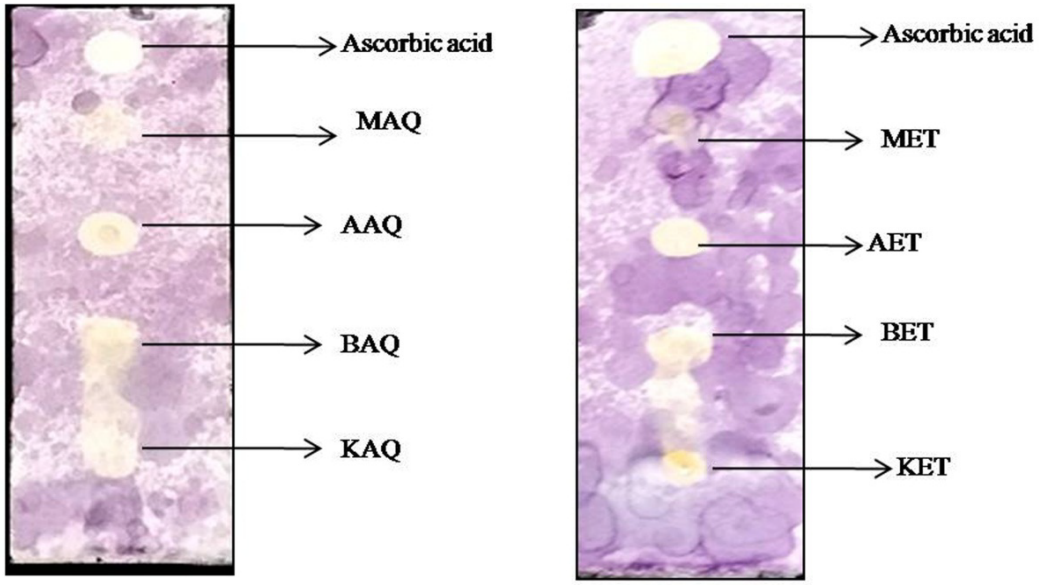


**Figure 5:** DPPH Spot Assay of leave extracts. (A) Aqueous leave extract of all four Plants (B) Ethanolic/Hydro-ethanolic leave extract of all four Plants. AAQ=Amla Aqueous Extract, BAQ=Bael Aqueous Extract, KAQ=Kadam Aqueous Extract, MAQ=Munga Aqueous Extract, AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.

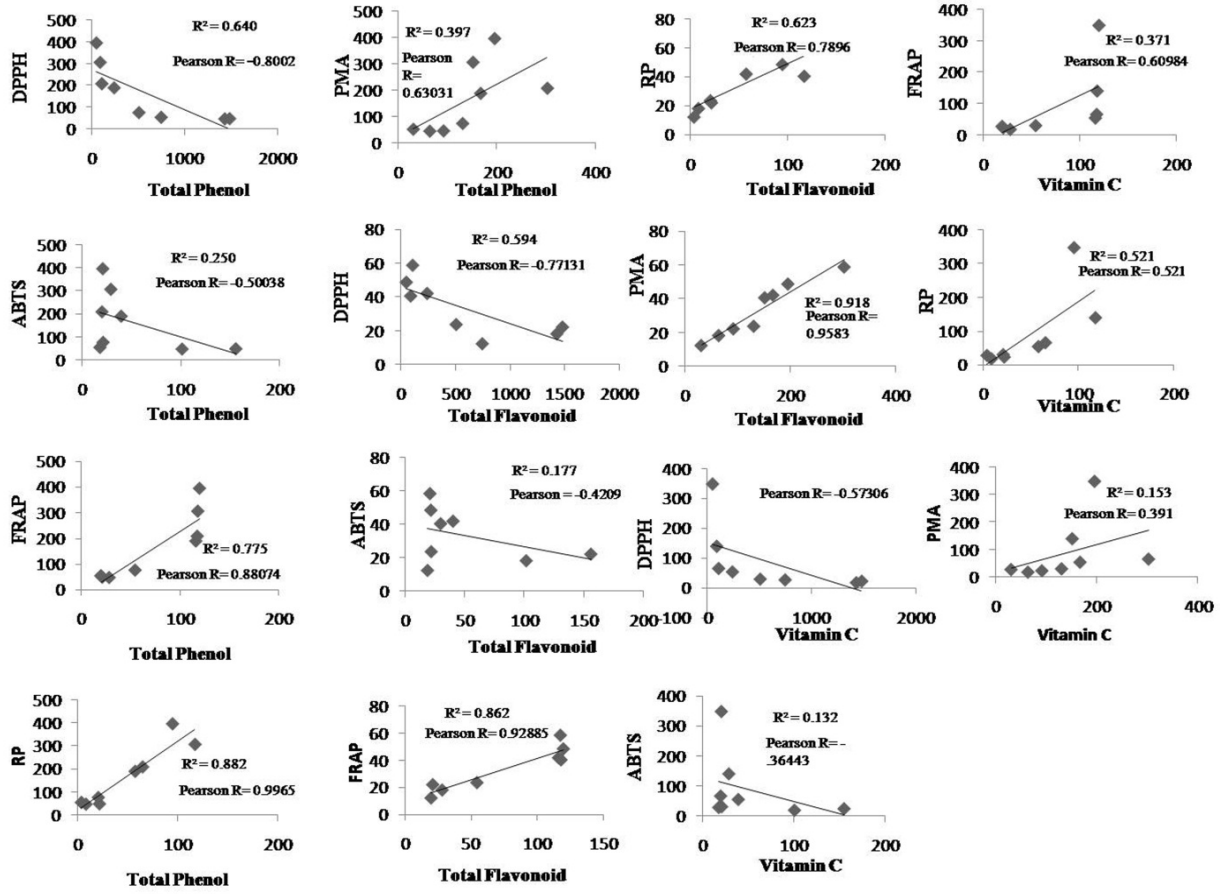


**Figure 6:** Linear regression plots and pearson correlation coefficient of $IC_{50}$ value of DPPH and ABTS, FRAP values, PMA values and RP with respect to total phenols, flavonoids and Vitamin C content of all leave extracts of all four plants.

**ABTS radical cataion scavenging activity**

The free radical scavenging activity was determined using ABTS radical cataion too. In this assay a stable blue green colored ABTS radical cataion was generated by reacting ABTS with potassium persulphate before adding antioxidants, [65]. Adding antioxidants after stable radical formation results in decolorization and decrease in absorbance at 600 nm and this decrease is proportional to antioxidant concentration. The minimum inhibitory concentrations required to scavenge 50% ABTS free radical ($IC_{50}$) of all plant extracts ranges from (18.24 ± 3.9 to 155.88 ± 9.0) and the lowest $IC_{50}$ value was observed in BAQ followed by KET, AET, BET Whose $IC_{50}$ values are not significantly ($P<0.05$) different with standard tannic acid (13.04 ± 0.6) (Table 7). While the $IC_{50}$ value of AAQ, KAQ, MAQ and MET are significantly different from standard tannic acid. The pearson correlation of ABTS with TPC, TFC and Vitamin C was (P=-0.5, P=-0.4 and P=-0.36, respectively) (Figure 6) showing the ABTS radical cataion scavenging activity of antioxidants present in all plant extracts was due to combined effects of phenolics, flavonoids and Vitamin C. These $IC_{50}$ values of above leave extracts are lower with some other leaves [66].

**FRAP assay**

Main mechanisms for free radical scavenging activities of antioxidants involve hydrogen transfer and single electron transfer. In FRAP assay the antioxidants involve single electron transfer and reduces $Fe^{3+}$ -ligand complex to blue colored $Fe^{2+}$ ligand complex in acidic media. [41,67]. The FRAP value for all the leave extract was shown in Table 7 and expressed as µg/mg as gallic acid equivalent. The highest FRAP value was observed in AET (119.61 ± 0.16) followed by AAQ>KET>KAQ>BET>MAQ>MET>BAQ. There is strong Correlation (Pearson's Correlation, P= 0.92) between total flavonoid content and FRAP value in compare to correlation with TPC (P=0.77) and Vitamin C (P=0.371) (Figure 6). It is reported in other study that hydroxyl groups and conjugated double bond of phenolic compound like Vitamin E and flavonoid compound are important for FRAP activity [68,69]. Difference in antioxidant values depending on method used indicates that each method involves different aspects of the antioxidant capacity since FRAP assay involves single electron transfer method, whereas DPPH and ABTS involve both hydrogen transfer and single electron transfer method [70].

**Phosphomolybdate assay**

Phosphomolybdate assay involve reduction of Mo (VI) to Mo (V) *via* single electron transfer method in the presence of antioxidants. Phosphomolybdenum V [Mo (V)] is a green colored complex which measures maximum absorbance at 695 nm [42]. The antioxidant activity was expressed as µg/mg as equivalent as ascorbic acid and shown in Table 7. The values ranges between (302.13 ± 36.8 to 31.46 ± 0.8) and the highest was observed in KET followed by AET>KAQ>AAQ>BET>MET>MAQ>BAQ. Correlation of phosphomolybdate assay with TPC, TFC and Vitamin C showed highest Pearson correlation with TFC (P=0.918) (Figure 6) showing the antioxidant activity in this assay was mainly due to flavonoids, the antioxidant activity in this assay are in correspondence with total flavonoid content (Tables 3 and 7).

**Reducing power assay**

The Reducing power is the significant indicator of antioxidant activity of any compound based on single electron transfer method. In this assay yellow colored $Fe^{3+}$/ferricyanide complex is reduces to blue and green colored $Fe^{2+}$/ ferrous form in the presence of electron donating groups of antioxidants [71]. The total reducing power among all the leave extract was found highest in AAQ and AET followed by KET>KAQ>>MET>BET>MAQ>BAQ (Table 7). Also, the correlation of total reducing power with TPC, TFC and Vitamin C showed highest pearson correlation with TPC (P=0.99) and pearson correlation with TFC was (P=0.78) and Vitamin C was (P=0.521), showing the antioxidant activity of leave extracts was mainly due to phenolics followed by flavonoid and Vitamins (Figure 6). The reducing powers of above leaves are higher in compare to some other leaves [64].

***In vitro* anticancer activity of leave extracts**

*In vitro* anticancer screening of ethanolic/hydro-ethanolic leave extracts of all four plants against three human cancer cell lines (SK-OV-3, A498, and T-24) using SRB assay was depicted in Table 8, Figure 7. The study used Adriamycin (ADR) as positive control. All the hydro-ethanolic crude extracts of leaves showed reduced activity as depicted by high $GI_{50}$ (>80) value (Concentration of drug causing 50 % inhibition of cell growth) (Table 8), showing that these extracts have no significant antiproliferative effects against the above cancer cells. However, among all the extracts AET showed some signs of antiproliferative effect in all the above cell lines but the activity was not significant (Figure 7). Although, the above leaf extracts are not effective against above mentioned cell lines, other study have showed antiproliferative activity of gallic acid from leaves of *Phyllanthus emblica* against human hepatocellular carcinoma

cells BEL-7404 [72], suggesting the leaf extract of *E. officinalis* have selective responses and may show effective anti-cancer activity to other cells.

**Table 8:** *In vitro* Anticancer screening of ethanolic/Hydro-ethanolic leave extracts of all four plants-SRB Assay:

| Samples/Positive Control | SK-OV-3 ($GI_{50}$) | A498 ($GI_{50}$) | T-24 ($GI_{50}$) |
|---|---|---|---|
| **AET** | >80 | >80 | >80 |
| **BET** | >80 | >80 | NE |
| **KET** | >80 | >80 | NE |
| **MET** | >80 | >80 | NE |
| **ADR** | <10 | <10 | <10 |

AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract.
SK-OV-3=Human ovarian cancer cell line; A 498=Human Kidney Carcinoma cell line; T-24=Human Bladder Cancer Cell line.
$GI_{50}$=Concentration of drug causing 50% of growth inhibition.
$GI_{50} \leq 20$ is considered to demonstrate activity.

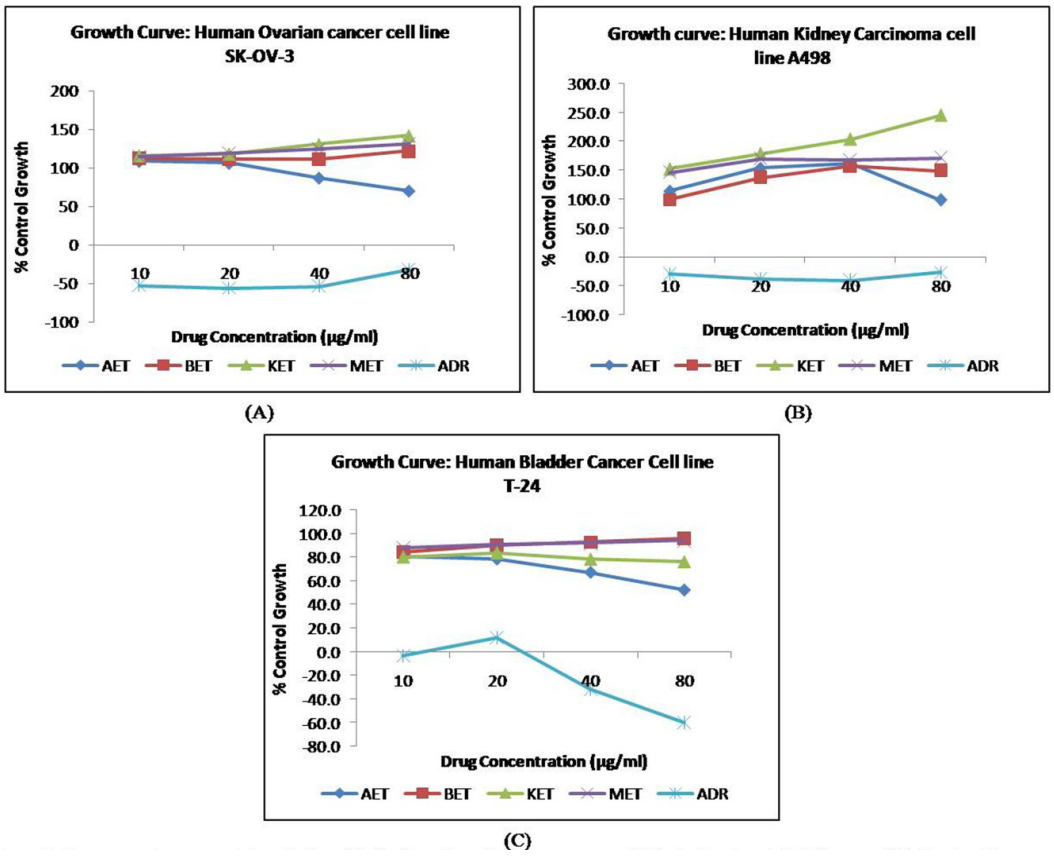


**Figure 7:** *In vitro* anticancer activity of ethanolic/hydro ethanolic leave extracts-sulforhodamine B (SRB) assay: (A) Against Human ovarian cancer cell line SK-OV-3 (B) Against human kidney carcinoma cell line A 498 (C) Against human bladder cancer cell line T-24. AET=Amla Ethanolic Extract, BET=Bael Ethanolic Extract, KET=Kadam Ethanolic Extract, MET=Munga Ethanolic Extract, ADR=Adriamycin(positive control)

## CONCLUSION

The phytochemical characterization revealed that the leaves of all four plants exhibits majorly the phenolics, vitamins and flavonoids in varying concentration and are responsible for their varying antioxidant capacities. *Emblica officinalis* and *Anthocephalus cadamba* ethanolic/hydro-ethanolic and aqueous leave extracts showed highest antioxidant capacity when compare to *Aegle marmelos*, and *Moringa oleifera*, correlating with higher phenolic contents, flavonoids and vitamins. The highest antioxidative capacity of *E. officinalis* hydro-ethanolic leave extract was may be due to pyragallol and vitamin E as reveled by GC-HRMS analysis. However, hydro-ethanolic extract of all the leaves have no significant antiproliferative activity against three human cancer cell lines SK-OV-3, A498 and T-24. Further

isolation of active molecules from crude extracts of above plants and evaluation of its *in vivo* biological activities is needed to identify their medicinal importance.

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**Conflict of Interest**

None.

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