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EFFECT OF SOLVENTS ON ANTIOXIDANT ACTIVITIES OF *FERONIA LIMONIA* FRUIT

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Keywords:

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ABSTRACT: *Feronia limonia* (*F. limonia*) is an underutilized edible fruit commonly known as kaitha. The study was performed to compare the effect of three solvents at different concentrations on antioxidant activities of *F. limonia* fruit. Fruits were extracted in different solvents *viz*., ethanol, methanol, and acetone at concentrations of 50%, 70%, and 90%. Each extract was analyzed for total phenol and flavonoid content and their antioxidant activities such as Free radical scavenging activity (DPPH), Ferric reducing antioxidant power (FRAP), Metal chelating activity and reducing capacity (RC). The results indicate that 70% aqueous acetone works best for the extraction of TPC (35.60 mg GAE/g), TFC (31.69 mg QCE/g), and other antioxidant activities. The phenol and flavonoid content showed a significant correlation with antioxidant activities. The findings of the study suggested that *F. limonia* fruit is a good and cheap source of natural antioxidant which can serve as a functional food. The fruit possesses potent enormous health benefits and thus may be used in food and pharmaceutical applications.

INTRODUCTION: *Feronia limonia (F. limonia.)* plant, which belongs to the Rutaceae family is a native plant of India. It grows in India, Pakistan, China, and other Southeast Asian countries. The shape of the fruit is spherical, with 5-12.5 cm in diameter. The rind of the fruit is hard, woody and very difficult to crack [1]. In India, the fruit is consumed as syrup, drink, murabba, chutney, and juices. Fruit posses enough therapeutic potentials including anti-hypercholesterolemic, antidiabetic, anticarcinogenic, antimutagenic, anti-inflammatory and antibacterial activities due to the presence of bioactive and antioxidant compounds.

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The antioxidant properties of *F. limonia* fruit could be attributed to phenolic compounds, phenolic glycosides, flavonoids, tannins, coumarin, terpenoid, marmesin and luteolin [2-3]. These compounds help to decrease the level of Glutathione (GSH), Glutathione peroxidase (GPX), Superoxide dismutase (SOD) and Catalase (CAT). Therefore it is used in the treatment of various diseases such as diarrhea, dysentery, chest pain, kidney stones, painful cramps, and ulcer [4].

Antioxidant activity of many plants and their therapeutic effects were studied in recent years. This is frequently involved in various mechanisms such as inhibition of free radical generation as well as enhancement of scavenging capacity against free radicals and so on. Antioxidants were studied to develop natural antioxidant-rich food, cosmetics, and other products [5]. The regular consumption of natural antioxidants has long been associated with the prevention of several diseases such as diabetes,

cardiovascular diseases and cancer [6]. Therefore, finding new and safe antioxidants from natural sources is of great interest now a day. Since biologically active compounds occur naturally in very small concentrations and presence of various antioxidant compounds with different chemical characteristics may or may not be soluble in a particular solvent; therefore, the choice of a suitable concentration of solvent is an important step in antioxidant studies [7]. The polarity of the solvents plays an important role in the extraction of bioactive compounds. Several previous studies reveal that mixture of solvent with water is an effective method of extraction of bioactive compounds from natural sources.

The compounds such as phenols and flavonoids showed a preventive effect against degenerative diseases such as diabetes, cardiovascular disease, cancer, obesity, and inflammations. Phenol shows their redox abilities, which are a class of antioxidant agents that can quench and neutralize the free radicals [8]. The objective of this work was to investigate the effect of three solvents at different concentrations on the extraction of phenols and flavonoids content of *F. limonia* fruit and to their antioxidant activity of the extracts by *in-vitro* method. The study also investigated the correlation of TPC and TFC with antioxidant activities including DPPH free radical scavenging activity, FRAP, metal chelating activity and reducing capacity.

MATERIALS AND METHODS: Unripe *F. limonia* fruit used in this study was purchased from the local market of Prayagraj (Allahabad), India. All the chemical used for the study includes foline ciocalteu reagent, methanol, ethanol and acetone, gallic acid, sodium nitrite, sodium hydroxide, trichloroacetic acid, ferric chloride anhydrous, ascorbic acid, sulphuric acid, and potassium-hexacyanoferrate, anhydrous monobasic potassium phosphate, sodium carbonate anhydrous, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and aluminum chloride anhydrous, were purchased from Sigma-Aldrich GmbH (Sternheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation: Fruits of *F. limonia* were washed with water, and their rinds were removed. Fresh *F. limonia* pulp was oven-dried at 40 ºC. The dried samples were ground into powder and passed through a 60-mesh sieve. Then 1 g powder was mixed in 100 ml solvents at the different concentrations and kept a room temperature for 24 h. The solid-liquid mixture was obtained which was centrifuged using centrifuged for 10 min at 1000 rpm. The supernatant of the solid-liquid mixture was collected within the amber-colored glass bottle and the extracts were stored in the refrigerator at 4 ºC for further analysis.

Determination of Total Phenol Content (TPC): Folin-ciocalteu method was used to determine the TPC content of sample [9]. 0.2 ml of each extract was taken in test tubes and added 5 ml of 10% diluted folin-ciocalteu phenol reagent. 4 ml of 7.5% sodium carbonate solution was added within 5-10 min. The mixture is allowed to stand for 60 min in dark. TPC was determined using spectro-photometric method (Model Evolution 600, Thermoscientific, US) by comparison of standard plots at 765 nm. A standard curve was prepared using gallic acid solutions (20, 40, 60, 80, 100 mg/L). The absorbencies were recorded at 765 nm. The results were expressed as mg of gallic acid equivalents / g of samples on a dry basis.

Determination of Total Flavonoid Content (TFC): Aluminum chloride colorimetric assay was used to measure the TFC content of the sample. 2 ml of sample extracts were taken in test tubes added 150 µl of 5% $NaNO_2$ shake the mixture and left it for 5 min, added 150 µl of 10% $AlCl_3$ in the test tubes. After 10 min, 1 ml of 1 mol sodium hydroxide (NaOH) was added and 10 ml total volume was made with the help of distilled water. The mixture was vortex and after keeping the mixture in incubation for 10 min and the absorbance was recorded against a blank at 510 nm[10]. A calibration curve was prepared using a standard solution of quercetin (20, 40, 60, 80 and 100 mg/L). The results were expressed as mg quercetin equivalent (QCE)/g of sample on a dry basis.

Antioxidant Activity:

DPPH Free Radical Scavenging Activity: DPPH free radical scavenging activity was evaluated by 1, 1-diphenyl 2-picryl-hydrazil (DPPH). 100 µl of fruit extract was mixed with 150 µl of 0.1 mmol

DPPH methanol solution and incubated for 15 min. Absorbance was measured using the spectrophotometer (Model Evolution 600, Thermoscientific, US) at 515 nm [11].

DPPH activity was calculated using the formula:

(A control – A sample / A control) × 100

Where A control denotes the absorption of the DPPH solution and A sample, which is the absorption of the DPPH solution after the addition of the sample. Results were expressed as % of inhibition of the DPPH radical.

Ferric-Reducing Antioxidant Power (FRAP) Assay: Sodium acetate buffer (300 mmol, pH 3.6), 10 mmol TPTZ solution (40 mmol HCl as solvent) and 20 mmol iron (III) chloride solution in a volume ratio of 10:1:1 respectively were used for preparing FRAP reagent. 200 µl extract of the three solvents at different concentrations was taken in test tubes and 1.3 ml of FRAP reagent added. The mixture was incubated for 30 min at 37 ºC. Absorbance was measured using spectrophotometer (Model Evolution 600, Thermoscientific, US) at 593 nm. The standard curve was prepared using $FeSO_4.7H_2O$ solution (200, 400, 600, 800, 1000 µmol), and the results were expressed as µmol of ferrous equivalent Fe (II)/g of sample on the dry basis [12-13].

Metal Chelating Activity: An aliquot (0.5 ml) of fruit extracts was mixed with 50 µl of ferrous sulfate. Add 1.6 ml of 80% ethanol and 100 µl ferrozine after 5 min. The mixture was vortexes for 1 min. After 10 min, absorbance was measured at 562 nm [14]. The metal chelating activity was calculated using the ratio:

(1-absorption of sample /absorption of control) × 100

Results were expressed as % of inhibition of the ferrous sulfate of the sample on a dry basis.

Reducing Capacity (RC): 1 ml of different concentrations of extracts was mixed with 2.5 ml sodium phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1% w/v). The mixtures were incubated for 20 min at 50 ºC. After that, 2.5 ml of TCA (10% v/v) was added and the samples were centrifuged at 10,000 rpm by 10 minutes. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1% v/v). The absorbance was subsequently measured at 700 nm with spectrophotometer[15]. The reducing power was related to ascorbic acid solution and expressed as µmol of ascorbic acid equivalents (AAE)/ g of dry weight.

Statistical Analysis: The analysis was carried out in triplicate and values were expressed as mean ± standard deviation. SPSS version 16.0 for Windows software (IBM corp.) was used for data analysis. Analysis of variance and Duncan's multiple range method were used to compare any significant difference between the solvents. Differences were considered significant at $P<0.05$ and $P< 0.01$. All the figures are prepared using Microsoft excel. Correlations analysis between the antioxidant activities of the four independent tests (DPPH, FRAP, Metal Chelating Activity, and Reducing Capacity) with TPC and TFC were conducted using statistical package for social sciences (SPSS) 16.0.

RESULTS AND DISCUSSION:

TABLE 1: EFFECT OF SOLVENTS AT DIFFERENT CONCENTRATIONS ON TOTAL PHENOL AND FLAVONOID CONTENT OF *F. LIMONIA* FRUIT

S. no.	Solvent system	TPC (mg GAE/g)	TFC (mg QCE/g)
1	50 % aqueous ethanol (E1)	10.86 ± 1.21^{g}	15.33 ± 1.21^{e}
2	70 % aqueous ethanol (E2)	22.71 ± 1.90^{d}	20.79 ± 1.71^{f}
3	90 % aqueous ethanol (E3)	20.53 ± 1.91^{e}	18.52 ± 1.11^{g}
4	50 % aqueous methanol(M1)	14.72 ± 1.92^{f}	10.68 ± 1.00^{g}
5	70% aqueous methanol (M2)	25.46 ± 2.50^{c}	19.59 ± 2.10^{d}
6	90% aqueous methanol (M3)	28.57 ± 2.00^{b}	22.42 ± 1.10^{c}
7	50 % aqueous acetone (A1)	05.26 ± 1.30^{h}	25.43 ± 2.91^{b}
8	70% aqueous acetone (A2)	35.60 ± 3.81^{a}	31.69 ± 1.20^{a}
9	90% aqueous acetone (A3)	02.20 ± 2.10^{i}	04.56 ± 0.51^{h}

GAE =Gallic acid equivalent; QCE =Quercetin equivalent, TFC =Total Flavonoid Content; TPC = Total Phenol Content, Analysis of variance $P<0.05$. Means carrying the same letter in superscript in a column do not differ significantly ($P<0.05$)

Total Phenol and Total Flavonoid Content: **Table 1** shows the TPC value of the three solvents at different concentrations, ranging from 2.20 to 35.60 mg GAE/g. TPC value is decreasing in the following order: 70% aqueous acetone > 90% aqueous methanol > 70% aqueous methanol > 70% aqueous ethanol > 90% aqueous ethanol > 50% aqueous methanol > 50% aqueous ethanol > 50% aqueous acetone > 90% aqueous acetone. The highest TPC content shown by 70% aqueous acetone (35.60 mg GAE/g) and it is significantly different (P<0.05) from other concentrations while 90% aqueous methanol (28.57 mg GAE/g) and 70% aqueous methanol (25.46 mg GAE/g) used as second and third best solvent for TPC extraction respectively. The results of the present study were similar to previous reported studies [16, 17, 18]. **Table 1** shows Total Flavonoid Content (TFC) of *F. limonia* fruit. The TFC of different solvents at varied concentration ranges from 4.56 to 31.69 mg QCE/ g. TFC is decreasing in the following order: 70% aqueous acetone > 50% aqueous acetone > 90% aqueous methanol > 70% aqueous ethanol > 70% aqueous methanol > 90% aqueous ethanol > 50% aqueous ethanol > 50% aqueous methanol > 90% aqueous acetone. The highest TFC content shown by 70% aqueous acetone (31.69 mg QCE/g) and the value is significantly different (P<0.05) from other concentrations while 90% aqueous acetone (4.56 mg QCE/g) shows the least TFC content.

Antioxidant Activities of *F. limonia* Fruit:

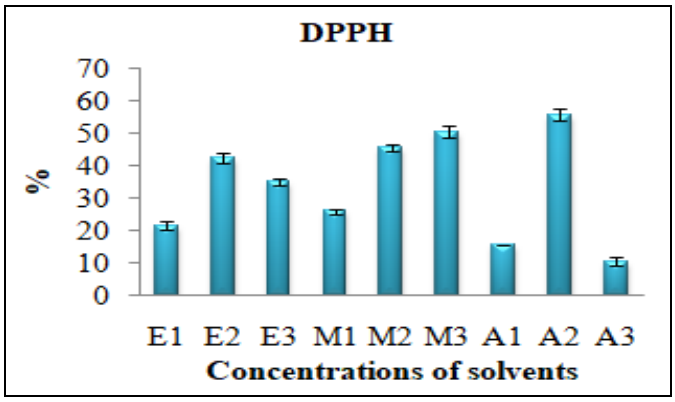

FIG. 1: DPPH FREE RADICAL SCAVENGING ACTIVITY OF *F. LIMONIA* FRUIT

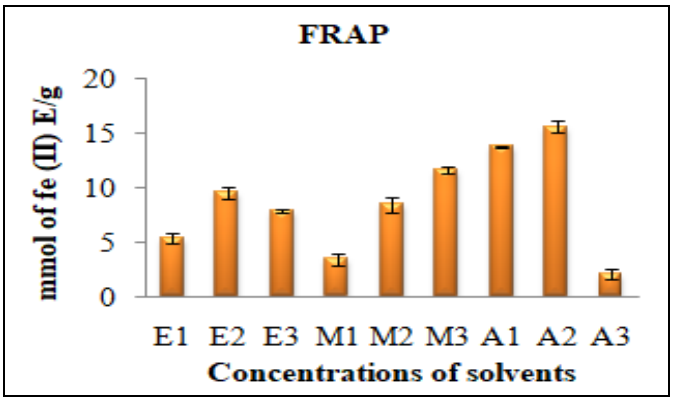

FIG. 2: FRAP VALUES OF *F. LIMONIA* FRUIT

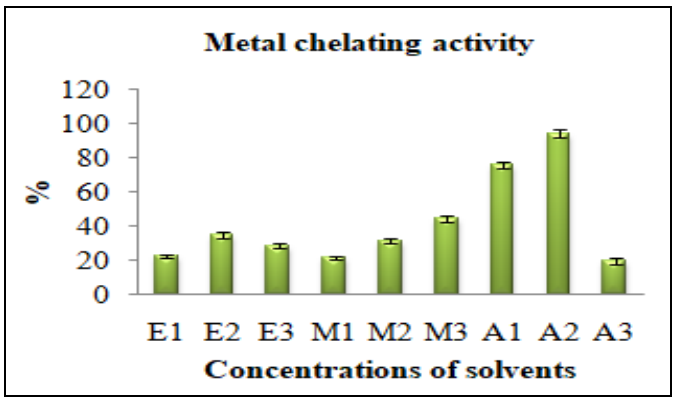

FIG. 3: METAL CHELATING ACTIVITY OF *F. LIMONIA* FRUIT

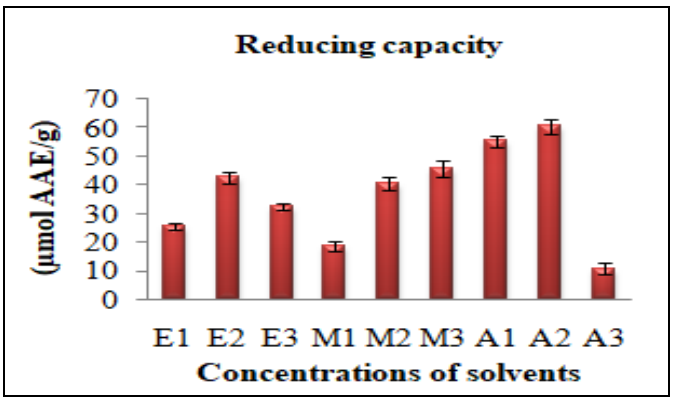

FIG. 4: REDUCING CAPACITY OF *F. LIMONIA* FRUIT

E1-50% aqueous ethanol, E2- 70% aqueous ethanol, E3- 90% aqueous ethanol, M1- 50% aqueous methanol, M2- 70% aqueous methanol, M3- 90% aqueous methanol, A1- 50% aqueous acetone, A2- 70% aqueous acetone, A3- 90% aqueous acetone

DPPH Free Radical Scavenging Activity: **Fig. 1** shows the DPPH free radical scavenging activities of three solvents at different concentrations. The DPPH free radical scavenging activity by various solvents decreased in the following order: 70% aqueous acetone > 90% aqueous methanol > 70% aqueous methanol > 70% aqueous ethanol > 90% aqueous ethanol > 50% aqueous methanol > 50% aqueous ethanol > 50% aqueous acetone and 90% aqueous acetone. The results show that values

obtained from the various polarity solvents were significantly different (P<0.05), and 70% aqueous acetone shows the highest (55.63%) value for DPPH free radical activity. The range of the DPPH free radical scavenging activity is varied from 10.63 to 55.63%. This indicates that 70% aqueous acetone extract is the most suitable solvent among all the three solvents at different concentrations for DPPH free radical activity of *F. limonia* fruit. The results indicate that 70% aqueous acetone extract shows the highest DPPH free radical scavenging activity (55.63%) and it is significantly different (p<0.05) from other concentrations of solvents. The extraction pattern of DPPH free radical scavenging activity in different concentrations of solvents followed the TPC extraction pattern. The present study shows that both TPC and DPPH are following the same pattern of decrease in respective solvents. This could be due to high quenching and neutralizing power of phenol against free radicals [8]. A similar trend was also observed in the study of DPPH radical scavenging activity of pineapple crude extract [19].

Ferric Reducing Antioxidant Power (FRAP): Fig. 2 shows that the FRAP value of various solvents decreased in following order: 70% aqueous acetone > 50% aqueous acetone > 90% aqueous methanol > 70% aqueous ethanol > 70% aqueous methanol > 90% aqueous ethanol > 50% aqueous ethanol > 50% aqueous methanol and 90% aqueous acetone. This study reveals both TFC and FRAP are following the same pattern of decrease in respective solvents. Findings of the FRAP analysis are shown that values obtained from various polarity solvents were significantly different (P<0.05) and 70% aqueous acetone reveals the highest (15.57 mmol of Fe (II) E /g) FRAP value suggested as the most suitable solvent among all the three solvents at different concentrations.

Metal Chelating Activity: In **Fig. 3**, among the three solvents at different concentrations, 70% aqueous acetone shows the highest metal chelating activity by various solvents decreased in the following order: 70% aqueous acetone > 50% aqueous acetone > 90% aqueous methanol > 70% aqueous ethanol > 70% aqueous methanol > 90% aqueous ethanol > 50% aqueous ethanol > 50% aqueous methanol and 90% aqueous acetone. It is significantly different (P<0.05) from other concentrations. Metal chelating activity for 70% aqueous acetone extract was the highest (94.54%). Both TFC and metal chelating is following the same pattern of decreasing in respective solvents. This indicates that the 70% aqueous acetone extract is the most suitable solvent among all the three solvents at different concentrations for the metal chelating activity of *F. limonia* fruit.

Reducing Power: In **Fig. 4**, all extracts of different solvents at different concentrations show some degrees of electron-donating capacity in a concentration-dependent manner. The highest reducing capacity of *F. limonia* fruit is shown by 70% aqueous acetone extract and is significantly higher (p<0.05) than that of the other extracts at all concentrations studied, followed by that of the 50% aqueous acetone, 90% aqueous methanol, 70% aqueous ethanol, 70% aqueous methanol, 90% aqueous ethanol, 50% aqueous ethanol, 50% aqueous methanol and 90% aqueous acetone. The present study shown both TFC and reducing capacity is following the same extraction pattern of decrease in respective solvents. The lowest reducing power was found in the 90% aqueous acetone extract. The value is also significantly lower than that of the other extracts at all concentrations studied. The analysis of results indicated that the reducing capacity of the 70% aqueous acetone was the highest (60.56 µmol AAE/g) and is significantly different (P<0.05) from other solvents.

Correlation between the Total Phenol, Flavonoid Content and Antioxidant Activities of *F. limonia* Fruit: Pearson's correlation coefficient was applied to determine the relationship between the antioxidant activities include DPPH, FRAP, metal chelating activity and reducing capacity and TPC, TFC shown in **Table 2**.

TABLE 2: PEARSON'S CORRELATION COEFFICIENTS OF DPPH, FRAP, METAL CHELATING ACTIVITY AND REDUCING CAPACITY VERSUS TPC AND TFC

	DPPH	FRAP	Metal chelating activity	Reducing capacity
TPC	0.88	0.25	0.40	0.32
TFC	0.24	0.96	0.94	0.98

*Correlation is significant at $P < 0.01$.

The TPC showed a strong correlation to DPPH with Pearson's correlation coefficient of 0.88 and also revealed correlation to metal chelating activity and reducing capacity with Pearson's correlation coefficient of 0.40 and 0.32 respectively while TPC shows a negligible correlation to FRAP with Pearson's correlation coefficient of 0.25. A very strong correlation between the TFC versus FRAP, metal chelating activity, and reducing capacity was seen with Pearson's correlation coefficient of 0.96, 0.94 and 0.98 respectively. The TFC shows a negligible correlation to DPPH with Pearson's correlation of 0.24. The correlation coefficient between TFC versus FRAP, metal chelating activity, and reducing capacity were statistically higher than those between TPC versus FRAP, metal chelating activity and reducing capacity. Flavonoid seemed to have a higher correlation with various antioxidant activities than phenols. Several previous studies demonstrated that both phenols and flavonoids which are major antioxidants found in natural products possess antioxidant activity[20-22].

The present study confirmed that the antioxidant activities of selected fruit are attributed to both flavonoids and phenols. TFC shows the high influence on antioxidant activities as it is strongly correlated with FRAP, metal chelating activity, and reducing capacity with Pearson's correlation coefficient of 0.96, 0.94, and 0.98, respectively while TPC shows a good correlation with Pearson's correlation coefficient of 0.88. TPC was strongly correlated with DPPH free radical scavenging activity. These results may be explained by the interaction of chemical structural differences between phenol compounds and concentrations of the solvents used. TPC was highly correlated with DPPH in comparison to FRAP, metal chelating activity and reducing capacity while TFC was highly correlated with FRAP, metal chelating activity and reducing capacity. Hanchinalmath *et al*., [5] suggested that luteolin is a primary active ingredient of *F. limonia* fruit, which is a flavonoid in nature [23-24]. Thus, this specific flavonoid compound may effectively contribute to the antioxidant activities of *F. limonia* fruit. The findings of the present study reveal the particular flavonoid and phenolic compounds that correlated with the antioxidant activities of *F. limonia* fruit need to be further investigated. Our study supports to Jin Gan *et al*. [25]

CONCLUSION: It can be concluded that 70% aqueous acetone is the best solvents for the extraction of TPC, TFC, and antioxidant activity of *F. limonia* fruit. The correlation results and statically analysis of this study is suggested that the antioxidant activity of *F. limonia* fruit is not only because of phenols but also flavonoids. Thus, flavonoids and phenols both are the major substances that contribute to the antioxidant activity of *F. limonia* fruit. Flavonoids exposed to have a stronger correlation with antioxidants than phenols. There is a need to conduct more studies to identify flavonoid and phenolic compounds that are correlated with the antioxidant activity of *F. limonia* fruit. The results of the present study would provide a new approach for further studies to the mechanisms which attribute the special bioactive of *F. limonia* fruit.

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