Indian Journal of Natural Products and Resources
Vol. 12(3), September 2021, pp. 463-471

Evaluation of the extraction hours and solvent concentrations on secondary metabolites and antioxidant activity of *Feronia limonia* fruit

Rashmi Srivastava[1], Neha Mishra[2] and Neetu Mishra[1]*

[1]Department of Home Science, University of Allahabad, Prayagraj 211002, Uttar Pradesh, India

[2]Department of Food, Nutrition and Public Health, ECHS, SHUATS, Prayagraj 211010, Uttar Pradesh, India

Received 03 July 2019; Revised 12 July 2021

The study was performed to compare the effect of different solvents and extraction hours on secondary metabolites and antioxidant activity of the unripe fruit of *Feronia limonia.* The investigation was achieved by using two extraction hours (24 and 48 h). Secondary metabolites of unripe fruit of *F. limonia* were extracted in different solvents viz., distilled water, ethanol, methanol, and acetone at 60, 80, and 100% concentrations. Qualitative followed by quantitative analysis of the phytochemical constituents of the fruit was done. Antioxidant activities of the fruit were studied through DPPH free radical scavenging activity, ferric reducing antioxidant power, metal chelating activity, and reducing capacity. The result indicates that 48 h of extraction is more effective for extraction of bioactive compounds than 24 h extraction. The qualitative analysis of phytochemical constitutes indicated the presence of phenols, flavonoids, alkaloids, saponins, and tannins. The highest phenolic content (44.00 mg GAE/g) and DPPH activity were obtained in 60% aqueous acetone while the highest flavonoids content (35.25 mg QCE/g), ferric reducing antioxidant power, metal chelating activity, and reducing capacity were obtained in 100% methanol extract. This study confirmed that the antioxidant activity of unripe *F. limonia* fruit is attributed to both flavonoid and phenols by employing multiple linear regressions. The unripe fruit contains several secondary metabolites and antioxidant activities which could be used to reduce oxidative stress.

Keywords: Antioxidant, Extraction, *Feronia limonia*, Phytochemicals, Secondary metabolites.

IPC code; Int. cl. (2015.01)- A61K 36/00, A61K 36/75, A61P 39/06

Introduction

In recent years, it has become increasingly evident that many natural products have significant therapeutic potential due to the presence of secondary metabolites and antioxidant activity. Secondary metabolites are an array of chemical compounds produced in relatively low quantities by plants[1]. They play an important role in defence against herbivores, microbes, and other species interactions. They constitute important UV absorbing compounds that are efficient in reducing the free radicals formation and thus prevent oxidative damage effectively[2]. Due to these secondary metabolites, plants have been used for therapeutic purposes in traditional medicine[3]. Recent research efforts have been taken to explore the pharmacognosy of medicinal, spicy, and aromatic plants. These encompass the study of secondary metabolites such as polyphenols, flavonoids, terpenoids, and alkaloids, which are positively correlated with lower risks of degenerative diseases, such as diabetes, cardiovascular diseases, and cancer etc[4]. Polyphenols and flavonoids are the most extensively studied metabolites that prevent oxidative damage efficiently in plants. For many years it is of great interest to extract new and safe antioxidants from natural sources. Qualitative analysis of phytochemicals is used to explore antioxidant compounds from natural sources[5]. It has been found that the yield, secondary metabolites, and antioxidant activity depends greatly on the physiological stage, extraction hours, and the concentration of solvents[6]. Several methods have been used to extract antioxidants from plants such as Soxhlet extraction, maceration, supercritical fluid extraction, subcritical water extraction, ultrasound-assisted extraction etc[7]. The Polarity of the solvents affects the solubility of various antioxidant compounds due to different chemical characteristics. Polar solvents are frequently used for recovering polyphenols from natural sources. Aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate are the most suitable solvents for the extraction of polyphenols and other

*Correspondent author
Email: dr.neetu.au@gmail.com
Tel.: 9415347999

antioxidant compounds. Ethanol is safe for human consumption and is known as a suitable solvent for polyphenol extraction. Methanol is used to extract lower molecular weight chemical compounds, whereas aqueous acetone is suitable for the extraction of higher molecular weight chemical compounds[8]. Previous studies mentioned the valuable secondary metabolites of *F. limonia* which governs its antioxidant activity. However, to date, there is a lack of information concerning the bioactive compounds and their functions.

Feronia limonia is a plant in the Rutaceae family that grows in India, Pakistan, China, and Southeast Asia[3]. In India, *F. limonia* is easily cultivated and has been used as murabba (preserve), chutney (sauce) and juices etc[4]. In traditional *Ayurvedic* medicine, they were used for many health remedies such as digestive, stimulant, carminative, astringent, and as an anti-diarrheal over the thousand years[6]. Over the last decades, *F. limonia* has gained significant interest due to its bioactive constituents includes flavonoids (including luteolin), phenols, alkaloids, sterols, glycosides, saponins, and triterpenoids which have several therapeutic potentials includes anti-diabetic[9], anti-cancer, and hepatoprotective[10,11,12], anti-bacterial[13], anti-fertility[14], neuroprotective and wound healing[15,16]. It has been shown to have negligible toxicity and possesses diuretic, muscle relaxant, and antispasmodic activities too. The previous studies confirmed the presence of secondary metabolites and antioxidant activity of *F. limonia* both *in vitro* and *in vivo* studies[17]. Therefore, the objective of this work was to investigate the effects of extraction hours and different solvents concentrations on the yield, secondary metabolites from unripe fruit of *F. limonia* and their antioxidant activity. This study also investigated the correlation of secondary metabolites (phenols and flavonoids) with the antioxidant activity of *F. limonia* fruit.

Materials and Methods

Unripe *F. limonia* fruits were purchased from the local market of Prayagraj, India, in the month of August 2017. The fruit was identified by Dr. Satya Narain (Professor), Department of Botany, University of Allahabad, India. All chemicals used for the study were purchased from Sigma-Aldrich GmbH (Sternheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

The unripe fresh fruit was thoroughly washed with tap water. The hard rind was broken with the help of a hammer. The raw pulp of the fruit was sliced with the help of a slicer and oven-dried at 40 ^{o}C[18]. After complete drying, the pulp was ground into powder using a kitchen milling machine and passed through a 60-mesh sieve. There was 750 g of powder formed from the 2 kg of pulp. The sieved powder was mixed in aqueous and all three solvents, including ethanol, methanol, and acetone (at 60, 80 and 100%) and left for 24 and 48 h at room temperature for extraction[7]. The mixture was centrifuged using a tabletop centrifuge (Remi) for 10 minutes at 1000 rpm after 24 and 48 h. The supernatant of the mixture was collected within the amber-coloured glass bottle and stored in the refrigerator (4 ^{o}C) for further analysis[18].

Qualitative analysis of phytochemicals

Qualitative analysis of phytochemical compounds such as alkaloids, phenolics, flavonoids, tannins, triterpenoids, saponins, glycosides, gums and mucilage, was performed in four different solvents (aqueous, ethanol, methanol, and acetone).

Exactly 2 mL of each extract was added to concentrated hydrochloric acid followed by a few drops of Mayer's reagent. The presence of green colour or white precipitate indicated the presence of alkaloids[19].

Exactly 2 mL of 5% ferric chloride was mixed with 1 mL of extract. The formation of dark blue or greenish-black indicated the presence of tannins[20].

Exactly 2 mL of each extract was added to 3 mL chloroform and 10% ammonia solution. The presence of pink colour indicated the presence of glycosides[21].

Exactly 2 mL of fruit extract was shaken with 2 mL of distilled water in test tubes; the formation of foam layer indicated the presence of saponins[22].

Exactly 0.5 mL of each extract was treated with chloroform and conc. sulphuric acid. The formation of red-brown colour at the interface indicated the presence of triterpenoids.

Gums and mucilage of fruit were estimated based on solubility in different solvents[20].

Determination of total phenol content

The total phenol content (TPC) of the sample was determined spectrophotometrically according to the Folin–Ciocalteau method[23]. Ten test tubes were arranged and 0.2 mL of each extract was taken including control. Then, 10% diluted Folin–Ciocalteau phenol reagent (5 mL) was added. All test tubes were shaken with added reagents and 7.5% sodium carbonate solution (4 mL) was added within

5-10 minutes. The mixture was incubated for 60 minutes in dark. The absorbance was read at 765 nm using a spectrophotometer (Model Evolution 600, Thermo Scientific, US) and compared with the standard curve prepared by gallic acid. The amount of TPCs was expressed as gallic acid equivalent on the dry weight.

Determination of Total flavonoid content

The total flavonoid content (TFC) was measured using aluminium chloride colourimetric assay. Exactly 2 mL of each extract was taken, to which 150 µL of 5 % $NaNO_2$ followed by 150 µL of 10 % $AlCl_3$ was added. After 10 minutes, 1 mL of 1 M sodium hydroxide was added to the mixture and the total volume was made up to 10 mL with distilled water. The mixture was incubated for 10 minutes. The absorbance was recorded against a blank at 510 nm and compared with the calibration curve of quercetin solution (20, 40, 60, 80, and 100 mg/L). The results were expressed as mg quercetin equivalent (QCE)/ g of sample on a dry basis[24].

Antioxidant activity

DPPH free radical scavenging activity

100 µL of fruit extract was mixed with 150 µL of 0.1 mmol DPPH methanol solution. The mixture was incubated for 15 min in the dark at room temperature. Methanol used as blank and absorbance was measured at 515 nm. The radical scavenging activity was expressed as % of inhibition of the DPPH radical[25] and calculated using the following equation:

Radical scavenging activity (%) = (1-absorption of sample / absorption of control) × 100.

Ferric-reducing antioxidant power assay

Ferric-reducing antioxidant power (FRAP) reagent was prepared from 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. Exactly 200 µL of the diluted sample was added to 1.3 mL of the FRAP reagent. After 30 minutes of incubation at 37 ^oC, absorbance was measured at 593 nm using a spectrophotometer. 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 a dry basis[26].

Metal chelating activity

The metal chelating activity of the extracts was evaluated by the ferrozine method[27]. An aliquot (0.5 mL) of fruit extract was mixed with 50 µL of ferrous sulphate. After 5 minutes, 100 µL ferrozine was added and the absorbance was measured at 562 nm after 10 minutes. The metal chelating activity was expressed as % of inhibition of the ferrous sulphate of sample on a dry basis and calculated using the following equation:

Metal chelating activity (%) = (1- absorption of sample / absorption of control) × 100.

Reducing capacity

To 1 mL of extract, 2.5 mL (200 mmol) of sodium phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide (1% w/v) was added and the mixture was incubated for 20 minutes at 50 ^oC. Then, 2.5 mL of TCA (10% v/v) was added and the samples were centrifuged at 10,000 rpm for 10 minutes. The upper layer (2.5 mL) of supernatant was mixed with 2.5 mL of deionised water and 0.5 mL of ferric chloride (0.1% v/v). The absorbance was subsequently measured at 700 nm in the spectrophotometer[25]. The reducing power was related to ascorbic acid solution and expressed as µmol of ascorbic acid equivalents (AAE)/ g of dry weight.

Statistical analyses

The data obtained were presented as mean± standard deviation. One way analysis of variance (ANOVA) test was performed followed by Duncan's multiple range method to compare the significant differences using SPSS version 16.0 for Window. The values of $P < 0.05$ were considered statistically significant. Pearson's correlation coefficients and multiple linear regression ($P < 0.01$) were also performed.

Results and Discussion

Qualitative analysis of phytochemicals

The qualitative analysis of phytochemical compounds in different solvents such as aqueous, methanol, ethanol and acetone are summarized in Table 1. The study reported the presence of phytochemical compounds includes alkaloid, tannin, phenol, flavonoid, glycosides, saponins and triterpenoids in all solvents while gum and mucilage are present only in aqueous extract. This is attributed to the non-suitability of gums and resins in alcohol and non-polar solvents[11,20].

Effect of extraction hours on yield and secondary metabolites content

Extraction yield

The findings depict the significant (P <0.05) effect of extraction hours on extraction yield content and found that 48 h of extraction hours was more effective for maximum extraction of secondary metabolites of unripe *F. limonia* fruit. The results indicated that the extraction yield increases with the increasing polarity of the solvent. As shown in Table 2, extraction yields in 100% pure acetone to 60% aqueous acetone ranged from 11.20 to 52.09% and 10.42 to 33.65% at 48 and 24 h respectively. The result of the present study suggested that higher yield extracted in the aqueous mixture of solvents than that of pure solvent[28] and is in agreement with the extraction yields of rice bran, *Limnophila aromatica* and some medicinal plant[29,30]. This is reasoned as the combined use of water and organic solvent may facilitate the extraction of chemicals that are soluble in water or organic solvents. Table 2 shows that yield in diluted solvents at different concentrations was higher than yield of pure solvent extracts and decreases in the following order: 60 % aqueous acetone > 60 % aqueous ethanol > 80 % aqueous methanol > 60 % aqueous methanol > 80 % aqueous acetone > 80 % aqueous ethanol > 100 % methanol > aqueous > 100 % ethanol > 100 % acetone.

Table 1 — Qualitative analysis of phytochemicals of unripe *F. limonia* fruit extracts

Metabolites	Extracts			
	AE	ME	EE	AcE
Alkaloid	+	+	+	+
Tannin	+	+	+	+
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Triterpenoids	+	+	+	+
Gum and mucilage	+	-	-	-

"+" = Presence of compound; "-"= Absence of compound

AE- Aqueous Extract, ME- Methanolic Extract, EE- Ethanolic Extract, AcE- Acetonic Extract.

Total phenol and total flavonoid content

Table 2 shows the effect of extraction hour and solvent concentration on the extraction of the TPC and TFC of the fruit. The present study found that 48 hours extraction shows the highest phenol and flavonoid content of the fruit. The TPC varied from 4.41 to 44.00 and 4.00 to 32.65 mg GAE/ g at 48 and 24 h respectively. The highest quantity of TPC (44.00 mg GAE/g) was found in 60% aqueous acetone followed by 80 % aqueous ethanol and 100 % methanol at both extraction hours. The 60% aqueous acetone showed the highest TPC content at 48 h extraction which is higher than 24 h, and it is significantly different (P <0.05) from other concentrations. The 80% aqueous ethanol and 100% methanol used were the second and third best solvents for TPC extraction respectively. Table 2 shows 100% methanol showed the highest total flavonoid content followed by 80% aqueous ethanol, 60% aqueous acetone, 100% ethanol, 80% aqueous methanol, 60% aqueous ethanol, 80% aqueous acetone, aqueous and 100% acetone at 48 h extraction. The TFC of different solvents at varied concentrations ranged from 5.56 to

Table 2 — Effect of solvents at different concentrations on yield and secondary metabolites content of unripe *F. limonia* fruit

Extraction hour	24 h			48 h		
Solvent system	Extraction yield (%)	TPC (mg GAE/g)	TFC (mg QCE/g)	Extraction yield (%)	TPC (mg GAE/g)	TFC (mg QCE/g)
Aqueous (Aq)	20.08±1.04^{f}	07.60±0.51^{h}	04.40±0.10^{h}	25.64±1.90^{de}	08.73±0.30^{h}	06.50±0.20^{i}
100% Methanol (M1)	25.06±1.32^{e}	28.12±2.33^{c}	24.90±1.21^{a}	28.09±2.51^{de}	30.94±2.10^{c}	35.25±1.51^{a}
80% Aqueous methanol (M2)	29.92±1.86^{c}	09.78±1.91^{g}	10.90±0.90^{e}	44.97± 2.20^{b}	10.22±0.30^{g}	14.56±0.80^{e}
60% Aqueous methanol (M3)	29.00±1.61^{c}	05.07±0.92^{i}	06.70±0.71^{g}	41.91±1.25^{b}	06.51±0.21^{i}	07.32±0.41^{h}
100% Ethanol (E1)	18.03±2.66^{g}	17.71±1.21^{f}	12.48±1.11^{d}	22.00±2.10^{f}	18.87±0.40^{f}	16.35±0.81^{d}
80% Aqueous ethanol (E2)	28.12±1.58^{d}	29.69±1.90^{b}	16.90±1.21^{c}	30.72±1.00^{cd}	35.32±2.30^{b}	21.65±1.20^{b}
60% Aqueous ethanol (E3)	30.92±1.64^{b}	22.94±2.00^{d}	10.00±1.10^{e}	50.11±2.21^{a}	25.99±0.50^{d}	12.45±1.21^{f}
100% Acetone (A1)	10.42±1.53^{h}	04.00±2.10^{j}	03.48±0.91^{i}	11.20±0.90^{g}	04.41±0.31^{j}	05.56±0.80^{j}
80% Aqueous acetone (A2)	28.69±1.07^{d}	18.14±1.81^{e}	08.31±1.30^{f}	35.08±1.05^{c}	20.68±0.40^{e}	10.35±0.91^{g}
60% Aqueous acetone (A3)	33.65±1.35^{a}	32.65±2.50^{a}	18.64±1.51^{b}	52.09±1.59^{a}	44.00±1.09^{a}	19.95±1.20^{c}

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

19.95 mg QCE/ g at 48 h extraction. The 100% aqueous acetone showed the highest TFC content at 48 h extraction which is higher than 24 h, and it is significantly different (P <0.05) from other concentrations. The 80% aqueous ethanol and 60% aqueous acetone used were the second and third best solvents for TFC extraction respectively. The results of the present study were similar to previous studies that reported higher phenol content in acetone fraction[31-33]. In support of the present study, several previous studies have found that extraction hours have a significant effect on extraction efficiency[34].

DPPH free radical scavenging activity

Fig. 1a shows the DPPH free radical scavenging activities of the different extracts in a concentration-dependent manner. The extract obtained by 60% aqueous acetone shows the highest DPPH radical scavenging activity followed by 80% aqueous ethanol, 100% methanol, 60% aqueous ethanol, 80% aqueous acetone, 100% ethanol, 80% aqueous methanol, 60% aqueous methanol, aqueous and 100% acetone at both 24 and 48 h extraction. The result of the DPPH assay showed that the values from various polarity solvents were significantly different (P <0.05) and the highest DPPH (60.70%) activity was attained by 60% aqueous acetone at 48 h. The range of the DPPH free radical scavenging activity is 13.65 to 60.70% at 48 h extraction. This indicates that the 60% aqueous acetone extract at 48 h is the most suitable solvent among all the three solvents at different concentrations for DPPH free radical activity of unripe *F. limonia* fruit. Extracts obtained by using different concentrations of solvents of ethanol and acetone have stronger radical scavenging capacity than pure while pure methanol extract shows stronger radical scavenging capacity than other extracts of methanol. A similar trend was observed in the study of DPPH radical scavenging activity of pineapple crude extract[34] and defatted wheat germ[30] both showed higher DPPH free radical scavenging activity in the fractions of solvents than 100% distilled water.

Ferric reducing antioxidant power

Fig. 1b shows the FRAP activity in the different solvents at different concentrations. The highest FRAP activity was obtained by 100% methanol followed by 80% aqueous ethanol, 60% aqueous acetone, 100% ethanol, 80% aqueous methanol, 80% aqueous ethanol, 60% aqueous ethanol, 80% aqueous acetone, aqueous and 100% acetone at both 24 and 48 h extraction. The result reveals that FRAP values were significantly different (P <0.05) in all extracts and the highest FRAP (18.56 mmol of Fe (II) E /g) was attained in 100% methanol at 48 h.

Metal chelating activity

It was reported that the metal chelating activity of all extracts was significantly different (P <0.05). Among the three solvents at different concentrations, pure methanol extract has the highest metal chelating activity followed by 80% aqueous ethanol, 60% aqueous acetone, 100% ethanol, 80% aqueous methanol, 80% aqueous ethanol, 60% aqueous ethanol, 80% aqueous acetone, aqueous and 100% acetone at both 24 and 48 h extraction. Fig. 1c elicited the highest metal chelating activity (96%) for methanol extract at 48 h. This indicates that the 100% methanol extract at 48 hours is the most suitable solvent for the metal chelating activity of unripe *F. limonia* fruit.

Reducing power

The study found that all extracts at different concentrations exhibited degrees of electron-donating capacity in a concentration-dependent manner. The 100% methanol extract (70.59 µmol AAE/g) attained the highest reducing power and the lowest reducing power was found in the 100% acetone extract. The reducing power of all extracts was significantly different (P <0.05) at all concentrations studied. Fig. 1d illustrated the trend of reducing power in the different extracts as 100% methanol followed by 80% aqueous ethanol, 60% aqueous acetone, 100% ethanol, 80% aqueous methanol, 80% aqueous ethanol, 60% aqueous ethanol, 80% aqueous acetone, aqueous and 100% acetone at both 24 and 48 h extraction.

Correlation between the secondary metabolites and antioxidant activities

Pearson's correlation coefficient was applied to evaluate the relationship between the antioxidant activity and secondary metabolite contents, including TPC and TFC based on DPPH, FRAP, metal chelating activity, and reducing capacity as shown in Table 3.

The TPC showed a significantly stronger correlation to DPPH with Pearson's correlation coefficient of 0.98 and good correlation to FRAP, metal chelating activity and reducing capacity with Pearson's correlation coefficient of 0.76, 0.71, and 0.81 respectively. A

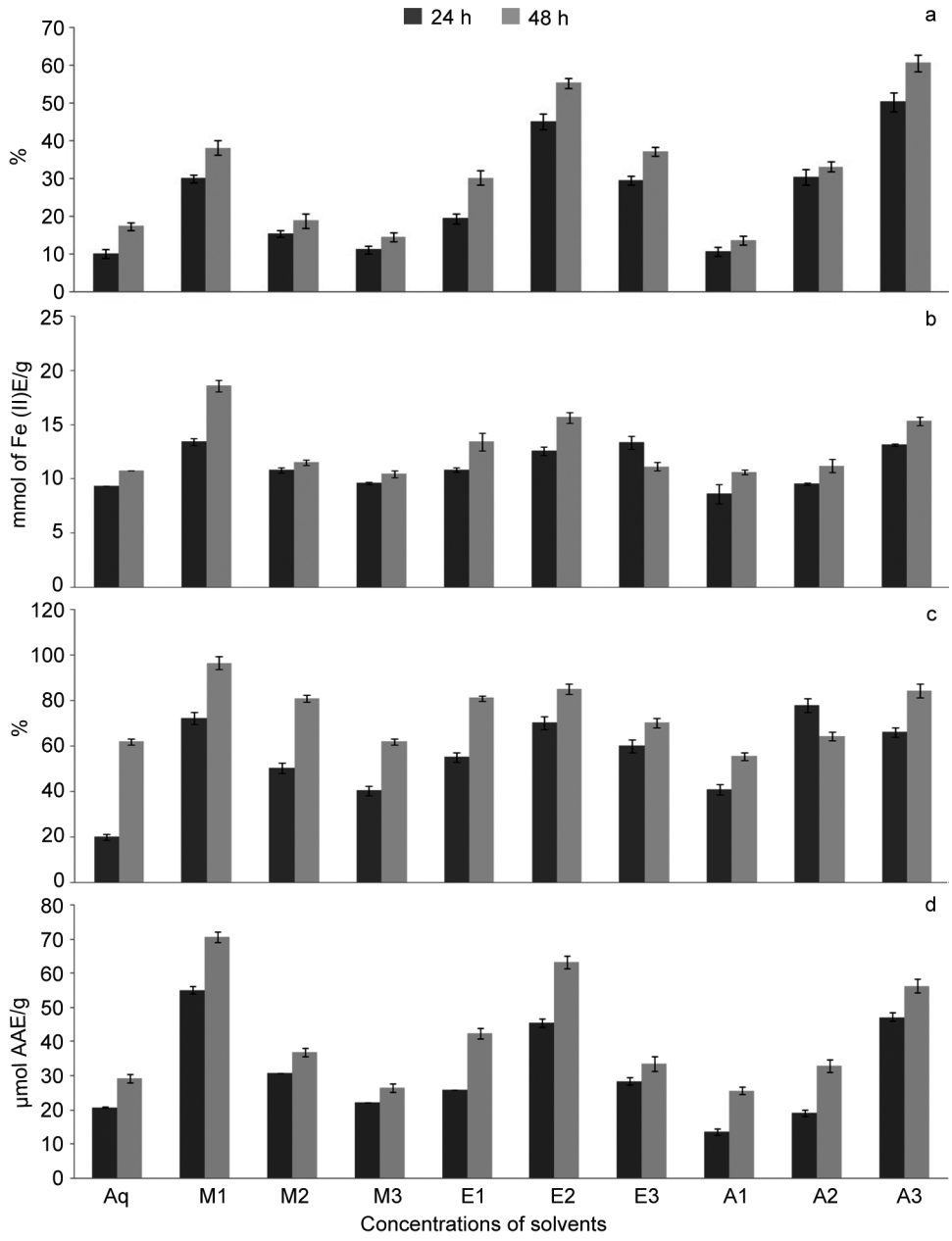

Fig. 1 — Antioxidant activity of unripe *F. limonia* fruit in different extracts of solvents for both extraction hours, a) DPPH free radical scavenging activity of unripe *F. limonia* fruit, b) FRAP values of unripe *F. limonia* fruit, c) Metal chelating activity of unripe *F. limonia* fruit and, d) Reducing capacity of unripe *F. limonia* fruit. Aq- Aqueous, M1-100% Methanol, M2- 80% Aqueous methanol, M3- 60% Aqueous methanol, E1- 100% Ethanol, E2- 80% Aqueous eth

significant stronger correlation between the TFC versus FRAP, metal chelating activity and reducing capacity was found with Pearson's correlation coefficient of 0.96, 0.94, and 0.95 respectively while a good correlation was found between TFC and DPPH with Pearson's correlation coefficient of 0.62. Total flavonoid content seemed to have a higher correlation with all antioxidants except DPPH than phenols.

To further analyze the contribution of phenols and flavonoids to the antioxidant activity of unripe *F. limonia* fruit, multiple linear regressions were employed. The antioxidant activity (DPPH, FRAP, metal chelating activity and reducing capacity) as the dependant variable (Y) and TPC and TFC as an independent variable (X) are considered to establish the best fit model that could exactly represent the

Table 3 — 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	0.98	0.76	0.71	0.81
TFC	0.62	0.96	0.94	0.95

Correlation is significant at P <0.01.

Table 4 — Linear regression models of DPPH, FRAP, Metal chelating activity and reducing capacity with TPC and TFC (P <0.01)

Model	Dependant variable (Y)	Independent variable (X)	Standardized Coefficients	Regression equation	R^2	Significant (P <0.01)
1	DPPH	TPC(X1)	0.95	Y=1.35X1+0.29X2+8.30	0.98	0.00
		TFC(X2)	0.29			
2	FRAP	TPC(X1)	0.05			
		TFC(X2)	0.85	Y=0.03X1+0.26X2+8.22	0.94	0.00
3	Metal chelating activity	TPC(X1)	0.09			
		TFC(X2)	0.87	Y=0.09X1+1.29X2+52.82	0.89	0.00
4	Reducing capacity	TPC(X1)	0.22			
		TFC(X2)	0.75	Y=0.34X1+1.34X2+14.64	0.94	0.00

influence of TPC and TFC on the antioxidant activity of unripe *F. limonia* fruit. As indicated in Table 4 model (1) was the best fit model for DPPH, indicating that DPPH was significantly linearly correlated with TPC (R^2= 0.98, P <0.05), of which 98% of DPPH variation was dependant on this factor. The effect of TPC with a standardized coefficient of 0.95 was higher than that of TFC with a standardized coefficient of 0.29. The best fit model for FRAP (model (2) in Table 4) contains TPC and TFC suggesting that TPC and TFC (Standardized coefficient of TPC and TFC are 0.85 and 0.05 respectively) significantly linearly correlated with FRAP (R^2= 0.94, P <0.05), of which 94% of FRAP variation depends on these factors while the effect of TFC on FRAP was higher than that of TPC due to the standardized coefficient. Similarly to FRAP, model (3) showed that metal chelating activity was also significantly linearly correlated with both TPC and TFC (R^2= 0.89 P <0.05). Thus TPC and TFC may be responsible for 89% variation of metal chelating activity. However, the effect of TFC with a standardized coefficient of 0.87 was higher than that of TPC with a standardized coefficient of 0.09. Model (4) showed that reducing capacity was also significantly linearly correlated with TPC and TFC (R^2= 0.94, P <0.05), thus TPC and TFC may be responsible for the 94% variation of reducing capacity. The effect of TFC with a standardized coefficient of 0.75 was higher than that of TPC with a standardized coefficient of 0.22.

This study confirmed that the antioxidant activity of unripe *F. limonia* fruit is attributed to both flavonoid and phenols by employing multiple linear regressions which is inconsistent with the previous studies[23,24,35]. The extract exhibited strong antioxidant activity with a significant correlation between phenol content and DPPH free radical scavenging antioxidant activity[36,37]. These results may be explained by the chemical structural differences between phenol compounds[38]. Phenols are the key class of antioxidant agents that can quench and neutralize the free radicals[39]. TFC had a high influence on antioxidant activity because its linear correlation with FRAP, metal chelating activity and reducing capacity were statistically significant with a standardized coefficient of 0.85, 0.87, and 0.75 respectively. Based on the above finding it can be concluded that antioxidant activity is exhibited by both phenol and flavonoid content of the fruit. The flavonoid content of the fruit shows a higher linear correlation with all four antioxidant activities than phenol content. Based on the above results the specific flavonoid and phenolic compounds that correlated with the antioxidant activity of unripe *F. limonia* fruit need to be further investigated.

Conclusion

The present study found that 48 h extraction was better for maximum extraction of secondary metabolites and antioxidant activity of unripe *F. limonia* fruit than 24 h. Qualitative analysis of the phytochemicals of unripe *F. limonia* fruit revealed that alkaloids, phenolics, flavonoids, glycosides, saponins, tannins, and triterpenoids were found in all

four solvents named aqueous, methanol, ethanol, and acetone extract. The results showed that 60% aqueous acetone is the best solvent for TPC and DPPH free radical scavenging activity while 100% methanol was the most suitable solvent for TFC as well as FRAP, metal chelating activity and reducing the capacity of unripe *F. limonia* fruit. The correlation of major secondary metabolites includes TPC and TFC with antioxidant activity elicited that flavonoids have a strong influence on all antioxidant activity except DPPH than phenols. Further studies need to be conducted to identify flavonoid and phenolic compounds that are responsible for the antioxidant activity of unripe *F. limonia* fruit. These additional results would provide new insight for drug development from unripe *F. limonia* fruit.

Acknowledgement

Rashmi Srivastava acknowledges the Junior Research Fellowship from University Grant Commission, New Delhi, India.

References

1 Darsini D T P, Maheshu V, Vishnupriya M, Nisha S and Sasikumar J M, Antioxidant potential and amino acid analysis of underutilized tropical fruit *Limonia acidissima L*, *Free Radic Antioxidants*, 2013, **9**(6), 62-69

2 Pagare S, Bhatia M, Tripathi N, Pagare S and Bansal Y K, Secondary metabolites of plants and their Role: Overview, *Curr Trends Biotechnol Pharm*, 2015, **9**(3), 293-304.

3 Pang Z, Chen J, Wang T, Gao C, Li1 Z, *et al.*, Linking plant secondary metabolites and plant microbiomes: A review, *Front Plant Sci*, 2021, **12**, 300 .

4 King A and Young G, Characteristics and occurrence of phenolic phytochemicals, *J Am Diet Assoc*, 1999, **9**(9), 213-218.

5 Turkmen N, Sari F and Velioglu Y S, Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Foline Ciocalteu methods, *Food Chem*, 2006, **9**(9), 835-841.

6 Dai J and Mumper R J, Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties, *Molecules*, 2010, **15**(3), 13-20.

7 Bonoli M, Verardo V, Marconi E and Caboni M F, Antioxidant phenols in barley (*Hordeum vulgare L.*) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds, *J Agric Food Chem*, 2004, **52**(5), 195-200.

8 Do Q D, Angkawijaya A E, Tran-Nguyen P L, Huynh L H, Soetaredjo F E, *et al*., Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatic*, *J Food Drug Anal*, 2014, **22**(2), 296-302.

9 Priya E M, Gothandam K M and Karthikeyan S, Antidiabetic activity of *Feronia limonia* and *Artocarpus heterophyllus* in streptozotocin induced diabetic rats, *Am J Food Technol*, 2012, **7**(1), 43-49.

10 Phapale R and Thakur S M, Antioxidant activity and antimutagenic effect of phenolic compounds in *Feronia limonia* swingle fruit, *Int J Pharm Pharm Sci*, 2010, **2**(4), 68-73.

11 Jayashree V, Hanchinalmath and Londonkar R, Cytotoxic and apoptosis-inducing effect of luteolin isolated from *Feronia limonia* on hepg2 cells, *Biolife*, 2014, **2**(4), 1287-1292.

12 Vasant R A and Narasimhacharya A, Alleviation of fluoride-induced hepatic and renal oxidative stress in rats by the fruit of *Limonia Acidissima*, *Res Rep Fluoride*, 2011, **44**(1), 14–20.

13 Kumar A S, Venkatesalu V, Kannathasan K and Chandrasekaran M, Chemical constituents and antibacterial activity of the leaf essential oil of *Feronia limonia*, *Indian J Microbiol*, 2010, **50**(1), 70–73

14 Dhanapal R, Ratna J V, Sarathchandaran I and Gupta M, Reversible antispermatogenic and antisteroidogenic activities of *Feronia limonia* fruit pulp in adult male rats, *Asian Pac J Trop Biomed*, 2012, **2**(9), 684-690.

15 Rakhunde P B, Sahar S, and Ali S A, Neuroprotective effect of *Feronia limonia* on ischemia reperfusion induced brain injury in rats, *Indian J Pharmacol*, 2014, **46**(6), 617-621.

16 Ilango K and Chitra V, Wound healing and anti-oxidant activities of the fruit pulp of *Limonia Acidissima* Linn (Rutaceae) in rats, *Trop J Pharm Res*, 2010, **9**(3), 223-230.

17 Bhat R and Karim A A, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, *Food Chem*, 2009, **115**(7), 857-888.

18 Sagar V R and Kumar P S, Recent advances in drying and dehydration of fruits and vegetables: A review, *J Food Sci Technol*, 2010, **47**(1), 15–26.,

19 Yin W Q, Duan S Q, Zhang Y, Zeng L and Song X M, Antioxidant activities of different solvents extracts and alkaloids of *Uncaria rhynchophylla* (Miq.) Jacks, *J Guangxi Normal Univ*, 2010, **28**(1), 31–34.

20 Pandey S, Satpathy G and Gupta R K, Evaluation of nutritional, phytochemical, antioxidant and antibacterial activity of exotic fruit *"Limonia acidissima"*, *J Pharmacogn Phytochem*, 2014, **3**(2), 81-88.

21 Harborne J B, Classes and functions of secondary products from plants, In: *Chemicals from plants perspectives on plant secondary products. London*, edited by J N Walton & D E Brown, (UK: Imperial College Press), 1999, 1-25.

22 King A and Young G, Characteristics and occurrence of phenolic phytochemicals, *J Am Diet Assoc*, 1999, **9**(9), 213-218.

23 Ghasemzadeh A, Jaafar H Z and Rahmat A, Effects of solvent type on phenolics and flavonoids content and antioxidant activities in two varieties of young ginger (*Zingiber officinale Roscoe*) extract, *J Med Plant Res*, 2011, **5**(1), 1147-1154.

24 Do Q D, Angkawijaya A E, Tran-Nguyen P L, Huynh L H, Soetaredjo FE *et al*., Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatic*, *J Food Drug Anal*, 2014, **22**(2), 296-302.

25 Pe´rez-Jime´nez J, Arranz S and Tabernero M, Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: Extraction, measurement and expression of results, *Food Res Int*, 2008, **41**(2), 74-85.

26 Benzie I F F and Strain J J, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power". The FRAP Assay, *Anal Biochem*, 1996, **23**(9), 70-76.

27 Antolovich M, Prenzler P D, Patsalides E, McDonald S and Robards K, Methods for testing antioxidant activity, *Analyst*, 2002, **127**(1), 183–198.

28 Sultana B, Anwar F and Ashraf M, Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extract, *Molecules*, 2009, **14**(2), 167-180.

29 Chatha S A S, Anwar F and Manzoor M, Evaluation of the antioxidant activity of rice bran extracts using different antioxidant assays, *Grasas Aceites*, 2006, **57**(5), 328-35.

30 Zhu K X, Lian C X, Guo X N, Peng W and Zhou H M, Antioxidant activities and total phenolic contents of various extracts from defatted wheat germ, *Food Chem*, 2011, **12**(6), 1122-1126.

31 Su D, Zhang R, Hou F, Zhang M, Guo J, *et al*., Comparison of the free and bound phenolic profiles and cellular antioxidant activities of litchi pulp extract from different solvents, *BMC Complement Altern Med*, 2014, **14**(9), 1-10.

32 Liu S C, Lin J T, Wang C K, Chen H Y and Yang D J, Antioxidant properties of various solvent extracts from lychee, *Food Chem*, 2009, **11**(4), 577-581.

33 Chigayo K, Mojapelo P E L, Moleele S M and Misihairabgwi J M, Phytochemical and antioxidant properties of different solvent extracts of Kirkia wilmsii Tubers, *Asian Pac J Trop Biomed*, 2016, **6**(12), 1037–1043.

34 Antolovich M, Prenzler P D, Patsalides E, McDonald S and Robards K, Methods for testing antioxidant activity, *Analyst*, 2002, **127**(1), 183–198.

35 Alothman M, Bhat R and Karim A A, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, *Food Chem*, 2009, **11**(5), 785-788.

36 Bors W and Michel C, Chemistry of the antioxidant effect of polyphenols, *Ann N Y Acad Sci*, 2002, **95**(1), 57–69.

37 Bhat R and Karim A A, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, *Food Chem*, 2009, **115**(7), 857-888.

38 Pu F, Ren X L and Zhang X P, Phenolic compounds and antioxidant activity in fruits of six *Diospyros kaki* genotypes," *European Food Res Technol*, 2013, **237**(6), 923– 932.

39 Pandey S, Satpathy G and Gupta R K, Evaluation of nutritional, phytochemical, antioxidant and antibacterial activity of exotic fruit *"Limonia acidissima"*, *J Pharmacogn Phytochem*, 2014, **3**(2),81-88.