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Original article

Antioxidant potential and amino acid analysis of underutilized tropical fruit *Limonia acidissima* L.

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ABSTRACT

Objective: *Limonia acidissima* L. an underutilized edible fruit was evaluated for its antioxidant activity, free radical scavenging ability, proximate and amino acid analysis using established *in vitro* assay models.
Methods: 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay, trolox equivalent antioxidant capacity (TEAC) assay, hydroxyl radical scavenging activity (HRSA), ferric reducing antioxidant power (FRAP) assay, nitric oxide radical (NO$^{\bullet}$) scavenging activity, total antioxidant activity (TAA) were carried out. The total phenolic (TP) and flavonoid contents (TF) of the extracts were determined and expressed as gallic acid and quercetin equivalents.
Results: The highest percentage of phenol and flavonoid contents were observed in methanol and the lowest content was found in chloroform extract. Also, methanol extract recorded higher activity in DPPH$^{\bullet}$, HRSA, FRAP and TAA whereas, ethyl acetate extract of the fruit was found to be active for ABTS$^{\bullet+}$ radical scavenging activity. Further, water extract of the fruit exhibited potentially high nitric oxide radical scavenging activity than other solvent extracts. Moreover, the phenolic and flavonoid contents of the fruit extract significantly correlated with antioxidant capacity. Amino acid analysis revealed that among, all essential amino acids, the concentrations of isoleucine, phenylalanine and tryptophan were found to be present in higher amounts.
Conclusion: Positive correlation was observed between polyphenolic contents and the antioxidant capacities. It is evident from the study that the fruit possess potent antioxidant activity with enormous health benefits and thus may be used in food and pharmaceutical applications.

1. Introduction

Limonia acidissima L. Swingle Syn. *Feronia elephantum Correa*, *Schinus limonia* L. (Rutaceae), is a tropical plant species in the Indian subcontinent. Indigenous to India and was locally known as elephant apple, monkey fruit, curd fruit and katha bel.[1,2] The fruits are woody, rough used in treatment of diarrheoa, dysentery,[3] wounds, cardiac debility, liver tonic hiccough, sore throat, gum disease and hepatitis.[1] The fruits are sour and sweet, has been used in Indian folk medicine, in Ayurveda for the treatment of blood impurities, leucorrhoea and in Yunani medicine as diuretic.[4] Fruit pulp showed anti inflammatory, antipyretic, analgesic activity[5] larvicidal and antimicrobial activity.[6] The fruits of *L. acidissima* contains flavonoids, phytosterols, glycosides, saponins, tannins, coumarins, triterpenoids, carbohydrates, vitamins, amino acids as its chemical constituents[7] and tyramine derivatives have also been isolated.[8] Besides, it is an effective herbal remedy for diabetes, it is experimentally proved for the blood glucose lowering potential.[9] Antioxidant activities and the traditional claims of the *L. acidissima* fruits possessing wound healing property were demonstrated by Ilango et al (2010).[10] Saima et al (2000)[11] isolated the pectic polysaccharide, FL-1a-I from the fruits of *Feronia limonia*, which exhibited *in vivo* antitumour activity of Ehrlich ascites carcinoma in the murine model. The unripe fruit contains 0.015% stigmasterol[12] and seeds contain oil high in saturated fatty acids and lowered the blood glucose levels in streptozotocin-induced diabetic male albino rats.[4] The fruit pulp has also been studied for antiulcer activity.[13] It is recognized that naturally occurring substances in higher plants have antioxidant activity. Reports have shown that presence of natural antioxidants from medicinal and aromatic plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis.[14]

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Plant phenols also exhibit significant antioxidant, antitumour, antiviral and antibiotic properties.[15] Safety and efficacy of the synthetic antioxidants used in the food industry are frequently questioned because such antioxidants are unstable and highly volatile[16] therefore, interest in finding naturally occurring antioxidants that have the potential to protect human beings from damage induced by oxidative stress has intensified.[17] Therefore, present study evaluates the antioxidant activities of the fruit pulp of *L. acidissima.*

2. Materials and methods

2.1. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (trolox), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), 2,4,6-tripyridyl-S-triazine (TPTZ), ferrous ammonium sulphate, ferric chloride, ethylene diamine tetra acetic acid (EDTA) disodium salt, trichloroacetic acid (TCA), Potassium ferricyanide, Folin–Ciocalteu's reagent, aluminium chloride, ascorbic acid, ammonium acetate, glacial acetic acid, acetyl acetone, sodium nitroprusside, sulfanilic acid, naphthyl ethylene diamine dihydrochloride, sodium phosphate and ammonium molybdate were obtained from Himedia (Mumbai, India), Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Plant material and preparation of extracts

The well matured fruits of *L. acidissima* were collected from Vellingiri hills, Coimbatore (T.N.) India was taxonomically identified and authenticated at Botanical Survey of India (BSI), Coimbatore. The rind was cracked and fruit pulp was shade dried, powdered and extracts were prepared by using solvents of varying polarity. A portion (50 gm) of dried fruit material was extracted with 250 ml (1:5) of petroleum ether followed by chloroform, ethyl acetate, methanol and water in a Soxhlet apparatus (8 h for each solvent). Subsequently, the solvent extract was evaporated to dryness at 40 °C in a rotary evaporator (Buchi type, Flawil/Schweiz, Switzerland). The water extract was cooled and lyophilized (Alpha 1-2 LD model, CHRIST, German). The yield of each fruit extract constituents were calculated and stored in the dark at 4 °C prior to use.

2.3. Proximate composition

Chemical analysis to determine proximate composition of sample was carried out using standard procedures of AOAC (1990).[18] The Kjeldahl method was used for total nitrogen determination using a Kjeltec System. Protein was calculated from total nitrogen using a factor of 6.25. The Soxhlet method was used for total fat determination. Total fat was obtained from 6 h hexane extraction. Crude fibre was obtained after samples digestion with boiling diluted acid and alkali. Moisture was determined from sample weight loss after oven drying at 110 °C for 4 h. Ash content was calculated after heating the sample at 550 °C for 2 h. Carbohydrates was determined by difference. All samples were analyzed in triplicate.

2.4. Amino acid composition analysis

The powdered fruit sample of *L. acidissima* was hydrolyzed using 6 N HCl at 110 °C for 24 h. Amino acid analysis was performed on reverse phase-high pressure liquid chromatography (HPLC) (Shimadzu LC-10 AD, Shimadzu Corporation, Kyoto, Japan). Samples were analyzed on Shimpack amino-Na type column (10 cm × 6.0 mm) obtained from Shimadzu Corporation. The post column samples were derivatized with *o*-phthaldialdehyde (OPA) and data were integrated using an integrator model C-R7A (Shimadzu chromatopac data processor).[19] The amount of each amino acid present in the sample was calculated as mg/100 g dry weight.

2.5. Total phenol content (TP)

Total phenolics content was determined by using Folin–Ciocalteu method Singleton et al (1999).[20] An aliquot of sample extract (0.1 ml) was mixed with distilled water (3 ml). To this 0.5 ml of Folin–Ciocalteu reagent was added. After 3 min 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath (100 °C) for exactly 1 min. It was then cooled and the absorbance was measured at 650 nm using spectrophotometer (Shimadzu, UV 2450) against the reagent blank. The results were expressed as milligram (mg) gallic acid equivalent (GAE) per gram sample dry weight.

2.6. Total flavonoid content (TF)

Total flavonoid content in the extract was determined based on the method described by Ordonez et al (2006).[21] A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm with UV–Visible spectrophotometer (Shimadzu, UV 2450). A yellow colour indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as milligram (mg) quercetin (QE) equivalent per gram sample dry weight.

2.7. 2, 2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging assay

The DPPH radical scavenging assay was determined according to Leong and Shui (2002).[22] Briefly, 2 ml of 0.15 mM DPPH (in methanol) was added to the different concentrations of the extract (1 ml). The reaction mixture was incubated for 30 min after which its absorbance was measured at 517 nm, methanol was used as both a blank and negative control. Results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) and defined as mg ascorbic acid equivalents (AA)/100 g of fresh weight basis.

2.8. Trolox equivalent antioxidant capacity (TEAC) assay

The method is based on the reduction of the ABTS radical cations ($ABTS^{\bullet+}$) by antioxidants present in extracts prescribed by Re et al (1999).[23] $ABTS^{\bullet+}$ radical cation was produced by reacting 7 mM aqueous ABTS with 2.45 mM potassium persulfate and kept in the dark at room temperature for 16 h. The blue–green solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. The stock solution of sample extracts was diluted. After the addition of 1 ml of diluted $ABTS^{\bullet+}$ solution to 10 μl of antioxidant compounds or trolox standards in ethanol, it was incubated at 30 °C exactly 30 min. Appropriate solvent blanks were also run in each assay. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The unit of antioxidant activity was defined as the concentration of trolox having the equivalent antioxidant activity expressed as mm trolox equivalent per gram sample dry weight.

2.9. Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain (1996).[24] An aliquot (500 μl) of an extract (appropriate dilution) was added to 3 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3.6H_2O$ solution) and the reaction mixture was incubated in a water bath at 37 °C. The increase in absorbance at 593 nm was measured at 30 min. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as μmol trolox equivalents per gram of plant material on dry weight basis.

2.10. Total antioxidant activity (TAA)

The total antioxidant activities of the extracts were evaluated by the phosphomolybdenum method as described by Prieto et al (1999).[25] Briefly, 0.5 ml of each sample solution or ascorbic acid (200–1000 mM) was combined with 3 ml of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample. All tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples were cooled to room temperature, absorbance of the solution of each sample was measured at 695 nm against the blank using a UV–Vis spectrophotometer (Shimadzu, UV 2450). Total antioxidant activity (TAA) based on the ability to reduce Mo (VI) to Mo (V) by the extracts were expressed as μmol ascorbic acid (AA) equivalents per gram of plant material on dry weight basis.

2.11. Hydroxyl radical scavenging activity (HRSA)

The scavenging activity of hydroxyl radical ($OH^{\bullet}$) was measured according to the method of Klein et al (1991).[26] Various concentrations of extracts and standards (25–150 μg/ml) were added to 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%) and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80–90 °C for 15 min in water bath. After incubation the reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). To about 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured at 412 nm using a spectrophotometer. The % hydroxyl radical scavenging activity (HRSA) was calculated by the following formula: Percentage of HRSA = 1 − (difference in absorbance of sample/difference in absorbance of blank) × 100.

2.12. Nitric oxide radical ($NO^{\bullet}$) scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess–Illosvay reaction.[27] The reaction mixture (3 ml) containing sodium nitroprusside (1 mM, 2 ml), phosphate buffer saline (0.5 ml) and extract or standard solution (25–150 μg/ml) was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25 °C. A pink coloured chromosphere was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Percentage inhibition of nitric oxide scavenging activity = $(A_{control} - A_{sample}/A_{control}) \times 100$.

where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the test.

2.13. Statistical analysis

Three replicates of each sample were used for statistical analysis and data were expressed as mean ± standard deviation (SD). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pair wise comparison test. Differences were considered statistically significant when $p < 0.05$. The INSTAT software program was used in the statistical analysis (INSTAT™, version 3, Graphpad software, San Diego, USA). Pearson's correlation coefficient was performed to study the relationship between phenolic contents and antioxidant assays.

3. Results

3.1. Proximate composition

The proximate analysis of the fruit *L. acidissima* is expressed in percentage (%) and represented Table 1. From the results, it can be inferred that protein and available carbohydrate contents were 2.43% and 42.2% respectively. The fruit contains 85% moisture and 29% lipid. However, results also showed that crude fibre and ash content of the fruit was high (11.52% and 8.5%).

3.2. Amino acid composition analysis

The amino acid compositions of the fruit *L. acidissima* were expressed as mg/100 g presented in Table 2. The highest values among essential amino acids were obtained in isoleucine (2870.41), phenylalanine (984.15) and tryptophan (1154.51). The moderate levels of valine (471.53), histidine (414.95) and leucine (176.06) were recorded. The lowest levels were quantified in methionine, tyrosine, lysine and threonine. Among, all non-essential amino acids, the highest amount was of proline (939.04), the average levels in glycine (152.23), aspartic acid (104.16), alanine (169.82) and negligible amounts were observed in glutamic acid and serine.

3.3. Extract yield

The highest extraction yield of the fruit *L. acidissima* was obtained in methanol extract (7.72%) and lowest yield was observed in chloroform extract (2.6%) as indicated Table 3.

Table 1
Proximate composition of fruit *L. acidissima*.

Proximate composition[b]	Concentration (%)
Moisture	85 ± 0.51[a]
Lipid	29 ± 0.1
Carbohydrates	42.2 ± 0.2
Protein	3.19 ± 0.7
Crude fibre	11.52 ± 0.4
Ash	8.5 ± 0.3

[a] Values are mean ± standard deviation of three replicates.
[b] AOAC.

Table 2
Amino acid composition of fruit *L. acidissima*.

Amino acid composition	Concentration (mg/100 g dry weight)
Aspartic acid	104.16
Glutamic acid	Negligible
Alanine	169.82
Methionine	Negligible
Tyrosine	605.19
Lysine	Negligible
Threonine	Negligible
Proline	939.04
Isoleucine	2870.41
Phenylalanine	984.15
Tryptophan	1154.51
Serine	Negligible
Glycine	152.23
Valine	471.53
Leucine	176.06
Histidine	414.95
Arginine	206.49

3.4. Total phenol content (TPC) and flavonoid contents of extracts (TFC)

Total phenolic content varied significantly among the different fruit extracts (0.75–22 mg GAE/g sample dry weight). Fruit extracts of methanol (22 ± 0.52 GAE/g sample dry weight GAE/g sample dry weight) and water (19.5 ± 1.30 GAE/g sample dry weight) exhibited higher phenolic content. However, a moderate level of phenolics was recorded in ethyl acetate (9 ± 0.53 GAE/g sample dry weight) and low levels were in chloroform extract (0.75 ± 1.5 GAE/g sample dry weight). The data obtained in the quantitative analysis of phenols and flavonoids indicated in Table 3 showed that all samples have considerable amounts of total flavonoid content. However, higher levels of flavonoids were observed in methanol and water extracts (2.6 ± 0.34 and 1.8 ± 0.54 mg QE/g sample dry weight, respectively) followed by ethyl acetate (1.25 ± 0.15 mg QE/g sample dry weight) and chloroform (0.55 ± 0.15 mg QE/g sample dry weight).

3.5. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH˙) radical scavenging assay

The scavenging effect of fruit extracts of *L. acidissima* on DPPH radical is expressed in terms of AEAC under the experimental condition was calculated and data presented in Table 4. All the fruit extracts have noticeable effect on DPPH radical except the chloroform extract. The scavenging effect of fruit extracts on the DPPH radical decreased in the order of methanol > water > ethyl acetate > chloroform. Among, all the fruit extracts especially, methanol extract exhibited stronger radical scavenging activity (1910 ± 20 mg AA/100 g FW). While the AEAC values of water, ethyl acetate and chloroform extracts were found (1819 ± 19, 1910 ± 20, 1528 ± 30 and 1273 ± 30 mg AA/100 g FW respectively) to elicit lesser activity as compared to methanol by DPPH. This may be due to the presence of more amount polyphenols in methanol extract.

Table 3
Yield (%), total phenol and flavonoid contents of different solvent extracts of the fruit *L. acidissima*.

Assay	WE	ME	EE	CE
Yield (%)	6.8 ± 1.1[a]	7.72 ± 0.21[c]	5.92 ± 0.32[c]	2.6 ± 0.34[a]
GA equivalent	19.5 ± 1.30[c]	22.0 ± 0.52[a]	9.0 ± 0.53[a]	0.75 ± 0.15[b]
QE equivalents	1.8 ± 0.54[b]	2.6 ± 0.34[b]	1.25 ± 0.15[a]	0.55 ± 0.15[b]

WE: water extract, ME: methanol extract, EE: ethyl acetate extract, CE: chloroform extract. Values of GA (gallic acid) and QE (quercetin) equivalents are mean ± SD of triplicate determinations (mg GAE/QE/g sample dry weight). Values are mean ± standard deviation of three replicates ($n = 3$). Means followed by different letters in the same column are significantly different ($P < 0.05$).
[a] ($P<0.001$).
[b] ($P<0.01$).
[c] ($P<0.05$).

Table 4
Antioxidant activities of DPPH, TEAC, FRAP and TAA fruit extracts of *L. acidissima*.

Assay	Water	Methanol	Ethyl acetate	Chloroform
AEAC (mg AA/100 g FW)	1819 ± 19[a]	1910 ± 20[a]	1528 ± 30[c]	1273 ± 30[c]
TEAC (μmol TE/g DW)	6625 ± 20[a]	9125 ± 25[a]	8005 ± 40[c]	4050 ± 30[c]
FRAP (μmol $FeSO_4$/g DW)	3400 ± 28[b]	5000 ± 25[a]	2300 ± 30[c]	1180 ± 20[a]
TAA (μmol AA/g DW)	1150 ± 33[c]	2500 ± 33[c]	950 ± 30[c]	650 ± 20[a]

Values are the mean ± standard deviation of three replicate ($n = 3$) determinations. All the values in the table significantly ($p < 0.05$) differ among extracts; different letters in the same column are significantly different.
[a] ($P<0.001$).
[b] ($P<0.01$).
[c] ($P<0.05$).

3.6. Trolox equivalent antioxidant capacity (TEAC)

With regard to fruit extracts, TEAC values ranged between 9125 and 4050 μmol TE/g DW. For the TEAC assay, ethyl acetate (8005 ± 40 μmol TE/g of sample DW) showed the relatively high antioxidant activity next to methanol extract (9125 ± 25 μmol TE/g of sample DW) and then followed by water and chloroform. The TEAC values of water and chloroform extracts showed moderate activity (6625 ± 20, 4050 ± 30 μmol TE/g of sample DW respectively) as indicated in Table 4.

3.7. Ferric reducing antioxidant power (FRAP) assay

Based on the FRAP value obtained, methanolic fruit extract of *L. acidissima* was much found to possess stronger activity than other solvent extracts but, in dose-dependent manner as shown in Table 4. Indicating that methanol extract had more potential antioxidant activity. There were significant ($p < 0.05$) differences in the FRAP values, among the type solvents employed in the fruit extraction. The antioxidant activity for different type of fruit extracts used increased in the order: choloroform < ethyl acetate < water < methanol with the FRAP value of 1180 ± 20, 2300 ± 30, 5000 ± 25, 3400 ± 28 μmol $FeSO_4$/g DW respectively.

3.8. Total antioxidant activity (TAA) by phosphomolybdenum method

The TAA was measured and expressed as ascorbic acid (AA) equivalents/g sample DW. In this assay, methanol (2500 ± 33 μM AA/g sample DW) exhibited 1.2 times greater activity when compared with water extract (1150 ± 33 μM AA/g sample DW). The activities of ethyl acetate (950 ± 30 μM AA/g sample DW) and chloroform (650 ± 20 μM AA/g sample DW) were comparatively lower as shown in Table 4.

3.9. Hydroxyl radical scavenging activity (HRSA)

All extracts showed significant differences in scavenging activity towards hydroxyl radicals in a dose-dependent manner as shown in Table 5. Among, all extracts studied, methanol extract had the highest free radical scavenging activity with an IC_{50} value of 65 ± 2.5 μg/ml. The ability of water extract to scavenge hydroxyl radicals was observed to be moderate (87 ± 4.1 μg/ml) whereas, fruit extracts of ethyl acetate and chloroform exhibited low radical scavenging activity when compared with those of others stated

Table 5
IC_{50} values of fruit extracts of *L. acidissima*.

Sample	Hydroxyl scavenging radical activity (μg/ml)	Nitric oxide scavenging radical activity (μg/ml)
Water	87 ± 4.1[b]	70 ± 3.3[b]
Methanol	65 ± 2.5[C]	90 ± 4.2[a]
Ethyl acetate	125 ± 5.2[a]	120 ± 4.1[b]
Chloroform	132 ± 5.3[a]	125 ± 4.4[a]
BHT	70 ± 2.5[c]	60 ± 4.2[b]
AAC	73 ± 4.0[b]	66 ± 4.3[a]

IC_{50} the inhibitory concentration at which the antioxidant capacity was 50%. BHT – butyl hydroxy toluene, AAC – Ascorbic acid contents. Values are mean ± standard deviation of three replicates ($n = 3$). Means followed by different letters in the same column are significantly different ($P < 0.05$).
[a] ($P<0.001$).
[b] ($P<0.01$).
[c] ($P<0.05$).

above (125 ± 5.2 μg/ml and 132 ± 5.3 μg/ml, respectively). The concentration of the positive controls, ascorbic acid (AA) and Butylated hydroxyanisole (BHA) required to scavenge 50% of the free radical (IC_{50}) was higher than fruit extracts (70 ± 2.5 μg/ml and 73 ± 4.0 μg/ml).

3.10. Nitric oxide ($NO^{\bullet}$) radical scavenging

The fruit extracts of *L. acidissima* exhibited the percentage inhibition values between 75 ± 0.12and 42 ± 0.16% at the concentration of 150 μg/ml. In general, nitric oxide radical inhibition study showed that fruit extracts exhibited $NO^{\bullet}$ scavenging activity at different levels. Interestingly, water extract of the fruit was the potent scavenger of nitric oxide. The concentration needed for 50% inhibition (IC_{50}) was found to be 70 ± 2.5 μg/ml almost near the positive controls BHT and AA (ascorbic acid) whose IC_{50} values were 60 ± 4.2 μg/ml and 66 ± 4.3 μg/ml, respectively. On the other hand, IC_{50} values for the methanol extract (90 ± 4.2 μg/ml) indicates it as a moderate scavenger, ethyl acetate and chloroform extracts had low inhibitory effect (120 ± 4.1 μg/ml and 125 ± 4.4 μg/ml, respectively) as shown in Table 5.

4. Discussion

The fruit pulp of *Carica papaya* (L) morphotypes had relatively same moisture, lower fibre and fat contents as that of *L. acidissima* fruits.[28] The presence of fibre in the diet play a vital role for digestion and for elimination of wastes and contraction of muscular walls of the digestive tract is stimulated by fibre, thus counteracting constipation.[29]

The quality of protein in foods is determined by the content of amino acids especially the essential amino acid.[30] Comparable results were obtained in amino acid profiles of *Prunus spinosa*.[31] Interestingly, similar result with reference to proline content was reported in the aerial parts *Caralluma adscendens* var. fimbriata.[32]

Our results are in agreement with the reports of Chew et al (2009),[33] that methanol showed better recoveries and it is specifically effective in extracting polyphenols. Thus, solvents polarity plays a major role in extraction of the antioxidants. Phenolic compounds are secondary metabolites of plants and naturally present in fruits and vegetables. These compounds are a part of everyday diet and also used as medicines or supplements. Studies have shown that fruits and vegetables contain other antioxidant nutrients, in addition to carotenoids; vitamins E,C and contribute significantly to their total antioxidant capacity.

The major part of those antioxidant nutrients is polyphenolic compounds, which are components of fruits and vegetables having strong antioxidant capacity.[34] Presence of vitamin C, phytosterols, flavonoids and tannins in the fruit pulp was reported by Mahour et al (2008).[35] Loganayaki and Manian (2010)[36] reported the methanolic fruit extract of *Murraya koenigii*, a member of Rutaceae was recorded to have high total phenolics and flavonoid content (11.2 GAE and 39.3 RE g/100 g extract, respectively) than methanolic fruit extract of *L. acidissima*. Further, our results are in agreement with the reports of Ramful et al (2010)[37] that total phenolic content of Mauritian citrus fruit extracts from Rutaceae, widely ranged from high (1882 ± 65–7667 ± 57 μg/g FW), and relatively low flavonoid content (2000–5615 ± 93 μg/g FW). Epidemiological evidence indicates that consumption of foodstuffs containing antioxidant phytochemicals (notably flavonoids and other polyphenols) is advantageous for our health since they can protect the human body from free radicals and retard the progress of many chronic diseases.[38] Moreover, they contribute for many biological functions, such as antimutagenicity, anticarcinogenicity and antiaging originates from this property.[39–42] Thus, development in isolation of natural antioxidants from plant species, especially edible fruits are in progress. DPPH assay evaluates the ability of antioxidants to scavenge free radicals and hydrogen-donating ability is an index of primary antioxidants. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore lead to inhibition of the propagation phase of lipid oxidation.[21,43] The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. In presence of antioxidant compounds, the DPPH is reduced producing a non-colour ethanolic solution. Thus, extraction of phenolic content was strongly dependent on solvents and may contribute directly to antioxidative action.[27] Also, some studies demonstrate, free radical-scavenging activity depends on the structural conformation of phenolic compounds.[44] Thus, free radical-scavenging activity is greatly influenced by phenolic composition of the sample. The polar fractions of methanolic extracts from fruits belonged to the Rutaceae family namely, *Citrus paradise*, *Citrus limon*, *Citrus aurantiifolia*, *Citrus sinensis* (IC_{50} values, 12.78 ± 0.53 mg/ml, 11.15 ± 3.08 mg/ml, 15.92 ± 1.20 mg/ml and 5.55 ± 0.16 mg/ml, respectively) exhibited strong DPPH radical scavenging activity.[45]

TEAC assay measures is the relative ability of antioxidant substances to scavenge 2, 2′-azinobis(3, ethyl benzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), compared with standard amounts of the synthetic antioxidant trolox. TEAC determines the antioxidant activity of hydrogen donating antioxidants (scavenging aqueous phase radicals) and of chain breaking antioxidants (scavenging lipid peroxyl radicals). Higher TEAC values demonstrate higher antioxidant activity. Our results are in accordance with the results obtained in *Citrus grandis* and *Citrus sinensis* of Rutaceae exhibited significant percentage $ABTS^{\bullet+}$ radical scavenging activity in extracts of ethyl acetate than other extracts.[46] It is also, worth noting that high TEAC values of Mauritian fruits of the same family (*Citrus clementina*, *Citrus reticulata*, *C. grandis*, *C. reticulata* × *C. paradise*, *C. reticulata* × *C. sinensis*) exhibited strong antioxidant activity (>35 μmol/g FW) among the group of fruits tested.[37]

FRAP results indicated that methanol extract had significantly ($p < 0.05$) stronger ferric reducing antioxidant power than any other extracts. The FRAP assay treats the antioxidants contained in the samples as reductants in redox-linked colourimetric reaction and the value reflects the reducing power of the antioxidants and has been widely used to directly test “total antioxidant power” of several extracts of foods and plants based on the ability of the analyte to reduce the Fe(III)/Fe(II) couple.[24] Thus, it has been used frequently in the assessment of antioxidant activity of various fruits, vegetables and some biological samples.[47,48]

TAA is a measure that shows the efficiency of antioxidant compounds in scavenging free radicals. The assay is based on the

reduction of the reduction of Mo (VI) to Mo (V) by the extracts and formation of green phosphate/Mo (V) complex at the acidic pH. The high absorbance values indicated that the samples possessed significant ($p < 0.05$) antioxidant activity.[49] Its value depends on the quality and quantity of antioxidants contained in fruits and vegetables.[50] Furthermore, total antioxidant activity (TAA) of fruits and vegetables contribute against cancer, cardiovascular diseases, and other diseases have been attributed to various antioxidants[51,52] Thus, extracts demonstrated electron-donating capacity and may act as radical chain terminators, transforming reactive free radical species into more stable non-reactive products.[53] Certain studies suggested phenolic compounds dominated total antioxidant capacity of citrus fruits belonging Rutaceae.[54] It is evident that, total antioxidant capacity (TAC) varies considerably from one kind of fruit to another.[51]

Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the reactive oxygen species (ROS), which could be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron and cause the ageing of human body and some diseases, interact with purine and pyrimidine bases of DNA as well as abstract hydrogen atoms from biological molecules (e.g. thiol compounds), leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecules.[49] The prevention of such deleterious reactions is highly significant in terms of both human health and the shelf-life of foodstuffs, cosmetics and pharmaceuticals.[55] Thus, hydroxyl radical scavenging ability was attempted in *L. acidissima* fruit extracts using ascorbic acid–iron-EDTA assay model system. Thus, potential hydroxyl radical scavenging activity was observed in methanol extract is similar to the results of DPPH, demonstrating that phenolic compounds in the methanolic fruit extract may contain phenolic hydroxyl group, with the ability to accept electrons, which can combine with free radical competitively to decrease hydroxyl radical.[56]

Nitric oxide ($NO^{\bullet}$) is highly reactive molecule that participates in the signal transduction in cardiovascular and immune systems. It is often characterized by contrasting actions as it can exhibit antioxidant and pro-oxidant functions as well as anti-apoptotic and proapoptotic effect.[57]

The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits the formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide compete with oxygen leading to the reduced production of nitric oxide.[58] The plant products may have the property to counteract the effect of $NO^{\bullet}$ formation and in turn may be of considerable interest in preventing ill effects of excessive $NO^{\bullet}$ generation in human body. The $NO^{\bullet}$ scavenging activity of the extract is of potential health interest, as it has been proposed that $NO^{\bullet}$ plays an important role in the progression of many diseases and pathological conditions such as ischaemia reperfusion, septic shock, atherosclerosis, neurodegenerative disorders like Parkinson's diseases, Alzhemier's, diabetes and cancer.[59,60]

4.1. Correlation analysis between antioxidant activity TPC and TFC contents

Correlations analysis was used to elucidate the relationship of the antioxidative abilities and polyphenolic compounds. The correlations exhibited were shown in Table 6. High correlation between total phenol (TP) and flavonoid (TF) contents were verified ($R^2 = 0.922$). Study states, total phenol content (TPC) was shown to provide the highest association with DPPH ($R^2 = 0.999$). Reports indicate, high positive relationship existed between total phenolics and antioxidant activity in many plant species.[61,62] Similar results were found for hydroxyl radical scavenging ($R^2 = 0.901$), FRAP ($R^2 = 0.708$) and nitric oxide ($R^2 = 0.730$) and moderate association was noted in TEAC ($R^2 = 0.665$) and TAA ($R^2 = 0.633$) scavenging activities. Further, phenolic compounds are attracting considerable interest in the field of food chemistry and medicine due to their promising antioxidant potential.[63] Relatively, similar correlations were obtained for the banana fruit cultivar Pisang Awak between phenolic contents and antioxidant assay.[64] Results of the present study also indicate that phenolics compounds are powerful scavenger of free radicals as demonstrated by a high correlation of DPPH scavenging activity with TPC ($R^2 = 0.999$) and TFC ($R^2 = 0.935$) which is comparable with the results reported by Sultana et al (2007).[65] Sequentially, high correlation existed between total flavonoid content (TFC) and hydroxyl ($R^2 = 0.927$) and TAA ($R^2 = 0.864$). The same trend was observed in, the correlation between hydroxyl and DPPH (0.905); TAA (0.801) and hydroxyl (0.727) scavenging activities, respectively.

Furthermore, weak correlation between FRAP and TFC ($R^2 = 0.267$) was observed in the present study. This weak correlation could be explained by the facts that there are some antioxidative compounds (tocopherols, carotenoids, flavonoids, etc.) that not only exhibit their antioxidant activity by donating hydrogen but also by scavenging oxygen. Consequently, extracts containing such antioxidant compounds might exhibit higher levels inhibition as compared to their reducing power and thus can display a poor correlation.[66] Again, the same was noted in antioxidants assay TEAC and TAA ($R^2 = 0.104$), FRAP and TAA ($R^2 = 0.145$). Over all, data obtained from the correlation showed that DPPH and hydroxyl scavenging activities were well correlated with TPC, TFC and other antioxidant assays.

Table 6
Linear correlation coefficients of phenolic contents with antioxidant activities of fruit extract of *L. acidissima*.

	TPC	TFC	DPPH	TEAC	TAA	FRAP	$OH^{\bullet}$	NO
TPC	1.000							
TFC	0.922	1.000						
DPPH	0.999	0.935	1.000					
TEAC	0.665	0.390	0.641	1.000				
TAA	0.633	0.864	0.654	0.104	1.000			
FRAP	0.410	0.267	0.391	0.630	0.145	1.000		
$OH^{\bullet}$	0.901	0.927	0.905	0.484	0.801	0.514	1.000	
$NO^{\bullet}$	0.578	0.469	0.564	0.604	0.341	0.947	0.727	1.000

TPC, total phenol content; TFC, total flavonoid content; DPPH, Diphenylpicrylhydrazyl; TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant potential; $OH^{\bullet}$, hydroxyl radical scavenging activity; $NO^{\bullet}$, nitric oxide radical scavenging activity.

5. Conclusion

L. acidissima L. an underutilized edible fruit was evaluated for its antioxidant properties. The highest phenol and flavonoid contents were present in methanol and the lowest in chloroform extract. The methanol extract showed higher activity in $DPPH^{\bullet}$, HRSA, FRAP and TAA whereas, ethyl acetate extract of the fruit was found to be active for $ABTS^{\bullet+}$ radical scavenging activity. Also, water extract of the fruit exhibited potentially high nitric oxide radical scavenging activity than other solvent extracts. Moreover, phenolic and flavonoid contents of these fruits extracts strongly correlated with antioxidant capacity. This revealed that remarkable antioxidant activity may be due to polyphenols present in the extracts. Obviously, this fruit may be used as a potential natural antioxidant and in the development of functional food and raw materials of medicine.

Conflicts of interest

All authors have none to declare.

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