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Research Article

ISOLATION AND *IN VITRO* ANTIOXIDANT ACTIVITY OF FLAVONOID FROM *LINDERNIA CRUSTACEA* (L) F. MUELL

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ABSTRACT

Objective: The objective of the study was to investigate the antioxidant property of different extracts of *Lindernia crustacea* (L) F. Muell and isolate flavonoid from the potent extract and characterize it.

Methods: Isolation was carried out by flash chromatography using Toluene:acetic acid (4:1) as eluent. The isolated compound was characterized using spectroscopic methods. 2, 2′- diphenyl-1-picrylhydrazyl, ferric thiocyanate, thiobarbituric acid, and reducing power assay methods were followed for the antioxidant study.

Results: Characterization of the isolated compound confirms it as the flavonoid. Results of the antioxidant study showed that benzene extract has the highest antioxidant activity with a less IC_{50} value in comparison to ethyl acetate and ethanol extracts. The isolated compound showed significant antioxidant activity when compared with aspirin.

Conclusion: The results of the study suggest that *L. crustacea* (L) F. Muell is a source of flavonoid which has potent antioxidant activity.

Keywords: *Lindernia crustacea* (L) F. Muell, Flash chromatography, Flavonoid, Antioxidant activity, IC_{50} value.

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INTRODUCTION

Oxidation of an oxidizable substrate is significantly inhibited by the antioxidant substances when reacting concentration is less in comparison with that of the substrates [1]. Free radicals are reactive molecules and associated with aging, cancer, strokes, cardiac, DNA destruction, artery obstruction, and central nervous system disorders. There is an increased effort in research on the substances which can prevent the reactive oxygen species and thus can prevent such diseases [2,3]. Many research showed the positive role of flavonoids in the enzymatic action on the brain receptors, and effects on the central nervous system, which included neurodegenerative preventive action associated with Parkinson's and Alzheimer's diseases. Free radical scavenging and/or antioxidant activity of flavonoid is already proved by research works. Other pharmacological activities are also possessed by different types of flavonoids [4].

Globally, plants have been used traditionally as medicine to treat diseases, since ancient times [5]. As the natural sources have proved to be a resource of various potent chemical compounds, which are also pharmacologically active, so the global interest has grown to commercialize therapeutic drugs from the natural sources [6]. The potentiality of many such plants remains unexplored and unrevealed. One such plant is *Lindernia crustacea* (L) F. Muell, which belongs to family Linderniaceae. It is found throughout India in moist places such as river beds, rice fields, and open grassy places [7]. *L. crustacea* is also a popular and useful ethnomedicinal plant has been traditionally used throughout the world [8].

Previous research work showed the presence of flavonoid in the benzene, ethyl acetate, and ethanol extract of *L. crustacea* (L) F. Muell with comparatively potent pharmacological activities of benzene extract [9]. There is no research report found on the isolation of flavonoid from *L. crustacea* and its antioxidant property. Therefore, it was aimed to investigate *in vitro* antioxidant activity of the extracts and to isolate flavonoid from the potent extract followed by characterization and evaluation of *in vitro* antioxidant activity of the isolated flavonoid.

MATERIALS AND METHODS

Materials

Analytical grade reagents and solvents used in the study. Silica Gel-G (Merck, India), benzene (Merck, India), ethyl acetate (Merck, India), ethanol (Merck, India), 2, 2′- diphenyl-1-picrylhydrazyl (DPPH, SRL India), thiobarbituric acid (TBA) (Sigma-Aldrich, India), trichloroacetic acid (Merck, India), linoleic acid (Sigma-Aldrich, India), ammonium thiocyanate (SRL India), ascorbic acid (Merck, India), potassium ferricyanide (Merck, India), and ferric chloride (Merck, India) were used during the experimental protocol.

Isolation was carried out using flash chromatography (Teledyne ISCO Combi Flash R_f 150), and the melting point was determined using DSC (Perkin Elmer, DSC 4000). Instruments used for characterization of the isolated compound are: CHN Analyzer (Perkin Elmer, series II 2400), ultraviolet (UV) spectrophotometer (UV-1800, Shimadzu), infrared spectrophotometer (Alpha-E, Bruker), and 1H NMR and 13C NMR (Bruker Avance II 400 NMR spectrometer) where Tetramethylsilane (TMS) was used as internal reference standard, mass spectrometer (Waters, Q-TOF Micromass, ESI-MS, and mass spectrometer).

Preparation of extract and phytochemical analysis

The aerial parts of *L. crustacea* (L) F. Muell were collected from the paddy field of Dharapur, Guwahati, Assam, in the month of April and May and were authenticated by Dr. P.P. Baruah, HOD, Department of Botany, Gauhati University, Guwahati, Assam, as *L. crustacea* (L) F. Muell with family Linderniaceae and accession number was given for the specimen is 18063. Shade-dried and coarsely powdered aerial parts of *L. crustacea* were subjected to successive extraction for 72 h by cold maceration in benzene, ethyl acetate, and ethanol. The solvents were filtered and evaporated using rotary evaporator (Buchi,

Switzerland). Phytochemical screening was carried out as per the standard method [10].

Isolation of flavonoid

Phytochemical screening of all extracts showed the presence of flavonoid whereas benzene extract showed the highest antioxidant activity in comparison to ethyl acetate and ethanol extracts. Hence, it was aimed to isolate and characterize flavonoid from the benzene extract of *L. crustacea.* Thin-layer chromatography (TLC) was performed to optimize the solvent system for the separation of flavonoid [11]. Approximate 10 g of benzene extract was chromatographed in flash liquid chromatography with the TLC optimized eluent. The fractions which showed the same R_f value were collected. The isolate was then dried using rotary evaporator and crystallized. The compound was further characterized using UV-visible spectrophotometry, DSC, Infra-red spectroscopy, mass spectroscopy, CHN analyzer, and NMR spectroscopy.

Antioxidant assay

Preparation of test sample

Extract samples were dissolved in DMSO to prepare a stock solution of 10 mg/ml concentration. For different concentration series of extracts, the required amount of stock solution was diluted with 95% methanol. For measuring antioxidant activity 10, 20, 35, 60, 125, 250, and 500 µg/ml, concentrations of the extracts were studied. The isolated compound was dissolved in 95% methanol to give the concentrations of 10, 20, 35, 60, and 125 µg/ml.

Preparation of reference standard solution

The reference standard ascorbic acid were dissolved in 95% methanol to prepare the concentrations of 10, 20, 35, 60, and 125 µg/ml.

DPPH assay

DPPH assay was performed as per the method described by Brand-Williams, [12] to measure *in vitro* radical scavenging activity. DPPH (24 mg) was dissolved in 100 ml methanol to prepare the stock solution, and it was kept at 20°C [13]. To prepare a working solution, the DPPH stock solution was diluted with methanol and adjusted to an absorbance at 517 nm of about 0.98±0.02. To 100 µl of each sample, 3 ml of the DPPH working solution was added. The content was shaken and then incubated in the dark for 15 min at room temperature. The absorbance of the resulting solutions was measured at 517 nm [14]. The control solution was prepared as mentioned above but without the addition of extract or isolated compound. The percentage of scavenging effect was calculated as per the following equation:

$$\text{Scavenging effect } (\%) = \frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{contol}}} \times 100$$

Where, "A" denotes as the absorbance

IC_{50} value (sample concentration that produced 50% inhibition) for each sample was calculated.

Ferric thiocyanate (FTC) method

Test and reference samples in different concentrations (1 ml) were mixed with of 4 ml of absolute ethanol, 4.1ml linoleic acid (2.51%) in absolute ethanol, 8.0mL 0.02M phosphate buffer (pH 7.0), and 3.9ml distilled water. The mixture was kept in an oven which was maintained at 40°C. From this, 0.1ml was transferred to a tube and 9.7ml aqueous ethanol (75% v/v), followed by 0.1ml aqueous ammonium thiocyanate (30% v/v) and 0.1ml of 0.02M ferrous chloride (prepared in 3.5% hydrochloric acid) were added [15]. The absorbance was measured after 3 min at 500nm and after every 24 h, until reached its maximum value. Mixture without the extract or isolated compound was used as the control.

$$\text{Percentage } (\%) \text{ of Inhibition} = \frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{contol}}} \times 100$$

Where, "A" denotes as the absorbance

IC_{50} value for each sample was calculated.

TBA method

The method of Kikuzaki and Nakatani [15] was followed for this assay method. 2 ml (20% v/v) of trichloroacetic acid and 1 ml (0.67% w/v) of TBA were added to 2 ml of reactions mixture, which was prepared and incubated as described in the FTC method. The final mixture was kept for 10 min on a boiling water bath. After cooling, the mixture was centrifuged for 20 min at 3000rpm. On every 24 h, the absorbance was measured at 552nm and recorded when it has reached its maximum value. Mixture without the extract or isolated compound was used as the control.

Antioxidant activity was described by percentage inhibition and calculated using the following equation:

$$\text{Percentage } (\%) \text{ ofInhibition} = \frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{contol}}} \times 100$$

Where, "A" denotes as the absorbance

IC_{50} value for each sample was calculated.

Reducing power assay

A substance with reduction potential reacts with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+). This reacts with ferric chloride to form a ferric ferrous complex which shows maximum absorption at 700 nm. This assay was performed as per the method described by Oyaizu [16]. Different concentrations of the samples (1 ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1% w/v). The mixture was incubated for 20 min at 50°C and after cooling 2.5 ml trichloroacetic acid (10 % v/v) was added [17]. It was centrifuged for 10 min at 3000 rpm. Then, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and freshly prepared 0.5 ml ferric chloride solution (0.1% w/v). After 10 min, the absorbance of the resulting mixture was measured at 700 nm.

Data analysis

Experiments were performed in triplicate. Result data were calculated as the means with standard deviation. The IC_{50} values were calculated by regression analysis using MS Excel.

RESULTS

Benzene extract showed a maximum yield of 12.43%, whereas ethyl acetate and ethanol extract showed 10.24% and 6.40%, respectively. The presence of flavonoid, phenolic compound, and tannins in all three extracts was indicated by the phytochemical analysis. Toluene:acetic acid (4:1) was optimized as the solvent system to separate flavonoid which confirmed by aluminum chloride spraying reagent. Similar R_f value of 0.31 was shown by the fraction no. 27–38 obtained from flash chromatography which was pulled together and crystallized.

Characterization of isolated compound

Isolated compound, sample code given as LCF-I, obtained as fine yellow crystals; melting point 314.32; yield 2.12 % w/w; UV- visible (Methanol) λ_{max} = 371.80, 255.40 nm (Table 1); IR absorption peaks: 3263 cm^{-1}, 1664 cm^{-1}, 1609 cm^{-1}, 1435 cm^{-1}, 1352 cm^{-1}, 1310 cm^{-1},1206$cm^{-1,}$ 1238 cm^{-1},1160 cm^{-1}; CHN analysis: carbon 82.608%, hydrogen 2.248%, oxygen 15.144%; *m/z* 302.24 [M+H]+ and *m/z* 301.22 [M-H]-; 1H-NMR (400 MHz, DMSO), δ (ppm), 9.34 (1H, s, H-3), 12.48(1H, s, OH-5), 6.19 (1H, d, *J*=2.44 Hz, H-6), 10.78 (1H, s, OH-7), 6.41(1 H, d,

J=1.56 Hz, H-8), 7.68 (1 H, d, *J*=2.04 Hz, H-2′), 9.34 (1H, s, H3′), 9.57 (1H, s, OH-4′), 6.90 (1H, d, *J*=8.44 Hz, H-5′), 7.55 (1H, dd, *J*= 1.32/9.68 Hz, H-6′); 13C-NMR (400 MHz, DMSO) δ (ppm), 147.64 (C-2), 135.69 (C-3), 175.79 (C-4), 160.68 (C-5), 98.15 (C-6), 163.84 (C-7), 93.32 (C-8), 156.09 (C-9), 102.97 (C-10), 121.93 (C-1′), 115.03 (C-2′),145.00 (C-3′),146.74 (C-4′), 115.56 (C-5′),119.95(C-6′). The findings of NMR spectrum confirmed by comparison with literature data [18,19]. LCF-I was identified as 2-(3′,4′-dihydroxy phenyl)-3,5,7-trihydroxy chromen- 4-one.

Antioxidant assay

DPPH assay

Fig. 1 shows the DPPH radical scavenging activity of different extracts and the isolated compound (LCF-I) of *L. crustacea* compared with aspirin. It was observed that benzene extract produced the highest activity, followed by ethyl acetate and ethanol extracts, respectively (Table 2). At the concentration of 500 µg/ml, the scavenging activity of benzene extract reached 70.31±0.02%, but at the same concentration, that of ethyl acetate and ethanol extracts were 53.21±0.09% and 37.45±0.10%, respectively. LCF-I showed 79.66±0.06% scavenging activity at the concentration of 125 µg/ml which is comparable with the 88.77±0.10% scavenging activity of aspirin at the same concentration. The result also showed that LCF-I has less IC_{50} value which was comparable with the aspirin (Table 3).

FTC method and TBA method

The antioxidant activity of extracts and LCF-I was measured to study lipid peroxidation by FTC and TBA methods and results represented in Tables 2 and 3. Benzene extract showed the highest antioxidant activity at 500 µg/ml concentration in both FTC and TBA methods, among all three extracts, whereas LCF-I at the concentration of 125 µg/ml showed 82.11±0.06% and 85.20±0.03% inhibition, respectively, in FTC and TBA methods (Figs. 2 and 3). Ascorbic acid showed 93.33±0.02% inhibition in FTC and 90.42±0.08% inhibition in TBA method at the concentration of 125 µg/ml.

Reducing power assay

All three extracts of *L. crustacea* showed increased reducing power activity with the increase in the concentrations (Table 2). Benzene extract showed the highest reducing power at 500µg/ml concentration

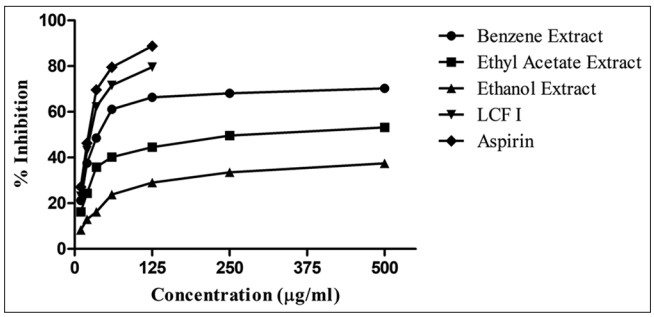

Fig. 1: Antioxidant activities by DPPH radical scavenging method

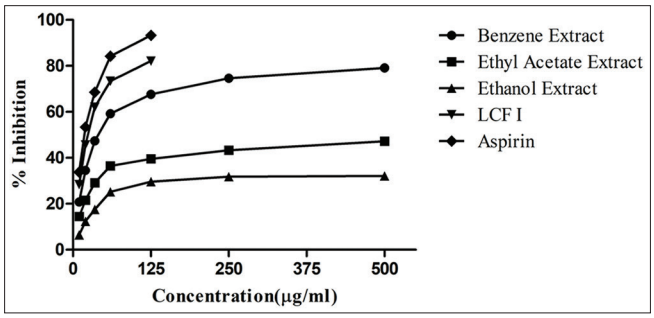

Fig. 2: Antioxidant activities by ferric thiocyanate method

Table 1: UV-visible absorption peaks of LCF-I in methanol and after addition of shifting reagents

Absorption peak (nm)					
CH_3OH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
371.80	327.50	443.60	432.00	394.90	388.04
255.40		269.20	261.40	383.40	270.12
				273.40	

UV: Ultraviolet, $AlCl_3$: Aluminum chloride, NaOAc: Sodium acetate, NaOMc: Sodium methoxide

Table 2: *In vitro* antioxidant activities of different extracts and isolated compound of *Lindernia crustacea* (L) F. Muell

Sl. No	Extracts	Study Model	Antioxidant activity						
			10 µg/ml	20 µg/ml	35 µg/ml	60 µg/ml	125 µg/ml	250 µg/ml	500 µg/ml
1.	Benzene	DPPH	21.17±0.05	37.55±0.11	48.51±0.07	61.15±0.09	66.35±0.05	68.10±0.12	70.31±0.02
		TBA	23.42±0.18	32.33±0.04	45.22±0.12	58.92±0.08	70.55±0.11	75.33±0.06	81.23±0.10
		FTC	20.76±0.04	34.57±0.04	47.41±0.07	59.22±0.08	67.66±0.10	74.59±0.11	79.10±0.13
		RP	0.124±0.05	0.278±0.03	0.361±0.07	0.592±0.07	0.777±0.06	0.892±0.05	1.186±0.04
2.	Ethyl Acetate	DPPH	16.21±0.08	24.38±0.12	35.78±0.08	40.17±0.10	44.51±0.06	49.6±0.05	53.21±0.09
		TBA	13.52±0.05	19.11±0.04	24.22±0.04	29.74±0.14	32.11±0.11	35.81±0.12	37.27±0.09
		FTC	14.46±0.12	21.55±0.03	29.12±0.07	36.47±0.06	39.53±0.13	43.33±0.06	47.20±0.08
		RP	0.097±0.10	0.133±0.02	0.209±0.09	0.366±0.11	0.482±0.07	0.532±0.06	0.601±0.06
3.	Ethanol	DPPH	8.25±0.14	12.96±0.05	16.22±0.10	23.78±0.15	29.08±0.07	33.55±0.06	37.45±0.10
		TBA	10.25±0.04	15.41±0.10	19.16±0.13	26.50±0.05	33.23±0.03	37.14±0.08	41.81±0.08
		FTC	6.44±0.08	12.36±0.04	17.52±0.14	25.2±0.06	29.61±0.12	31.77±0.07	32.11±0.06
		RP	0.045±0.05	0.089±0.10	0.152±0.02	0.220±0.08	0.316±0.05	0.379±0.08	0.430±0.04
4.	LCF-I	DPPH	23.14±0.06	43.15±0.08	62.23±0.11	71.62±0.04	79.66±0.06	-	-
		TBA	28.32±0.05	46.95±0.07	63.14±0.09	78.63±0.08	85.20±0.03	-	-
		FTC	28.41±0.12	45.65±0.05	62.06±0.09	73.38±0.07	82.11±0.06	-	-
		RP	0.577±0.06	0.741±0.06	0.858±0.03	1.091±0.06	1.155±0.05	-	-
5.	Ascorbic acid	DPPH	27.09±0.08	46.27±0.09	69.65±0.03	79.51±0.04	88.77±0.10	-	-
		TBA	28.03±0.05	52.22±0.05	71.87±0.07	81.03±0.06	90.42±0.08	-	-
		FTC	33.77±0.07	53.36±0.05	68.54±0.04	84.23±0.09	93.33±0.02	-	-
		RP	0.723±0.03	0.925±0.04	1.286±0.07	1.412±0.06	1.732±0.08	-	-

Values are mean±SEM of 3 replicates. FTC: Ferric thiocyanate, TBA: Thiobarbituric acid

Table 3: IC_{50} values from DPPH, TBA, and FTC method

Sl. No.	Activity	Extracts/Compound	IC_{50} (µg/ml)
1	DPPH	Ascorbic acid	23.69
		LCF-I	35.76
		Benzene Extract	96.2
		Ethyl Acetate Extract	352.48
		Ethanol Extract	654.38
2	TBA	Ascorbic acid	17.5
		LCF-I	26.11
		Benzene Extract	89.76
		Ethyl Acetate Extract	728.37
		Ethanol Extract	562.28
3	FTC	Ascorbic acid	13.58
		LCF-I	29.65
		Benzene Extract	92.48
		Ethyl Acetate Extract	463.03
		Ethanol Extract	807.76

FTC: Ferric thiocyanate, TBA: Thiobarbituric acid

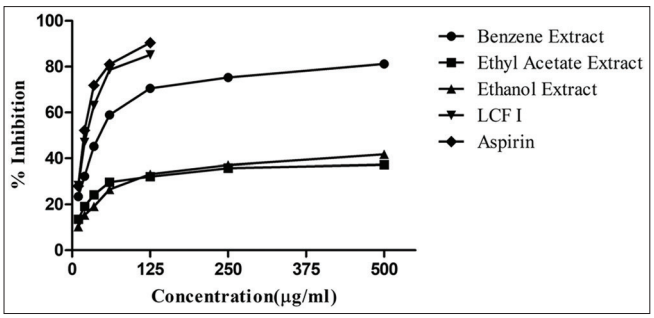

Fig. 3: Antioxidant activities by thiobarbituric acid method

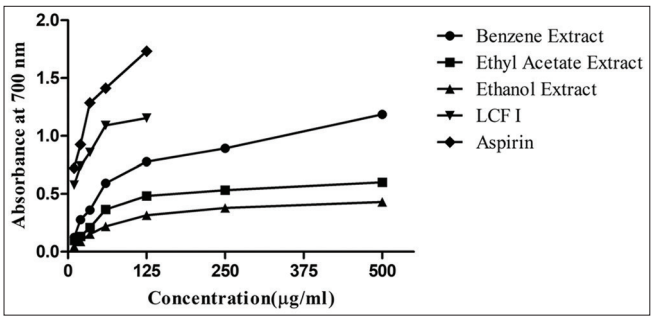

Fig. 4: Antioxidant activities by reducing power assay method

than the ethyl acetate and ethanol extracts (Fig. 4). Although ascorbic acid showed the highest reducing power, LCF-I showed a comparable result at the same concentration (125 µg/ml).

DISCUSSION

Infrared spectra of LCF-I showed OH stretching vibration of phenol, which was indicated by the absorption peak at around 3263 cm^{-1}. The absorption peak at 1664 cm^{-1} was observed due to C=O aryl ketonic stretching vibrations. The peak positioned at 1609 cm^{-1} due to C-C and 1435 cm^{-1} due to the presence of the aromatic group [20]. The absorption peak at 1352 cm^{-1} was observed due to OH bending vibrations of phenols. Absorption peaks at 1310cm^{-1} and the peaks at the lower frequencies between 950 cm^{-1} and 634 cm^{-1} were observed due to the presence of C-H bending vibrations of aromatic hydrocarbons. C-O stretching vibrations of phenols and aryl ether were indicated by the presence of peaks at 1206cm^{-1} and 1238 cm^{-1}, respectively. Due to the presence of C-CO-C stretching and bending vibrations of ketones, the absorption peak at 1160 cm^{-1} was observed [21].

The UV-visible spectrum of methanolic (CH_3OH) solution of LCF-I showed two major absorption bands at 371.80 nm (band-I) due to cinnamoyl system and 255.40 nm (band-II) due to benzoyl system. This confirmed the flavonol structure in LCF-I. From the results of the addition of different shifting reagents in a methanolic solution of LCF-I (Table 1), this is revealed that a complex was formed by aluminum chloride ($AlCl_3$) with the hydroxyl group at C-3 or/and C-5 and also with the ketone group at C-4 of the flavonol structure. The flavonol-aluminum complex produces a bathochromic shift. An acid-labile complex was also formed with the catechol functional group which was dissociated on the addition of hydrochloric acid, and produced a hypsochromic shift, compared to the aluminum chloride spectrum. Sodium acetate (NaOAc) ionizes the flavonols with a free 7- hydroxyl and exhibiting a Band II shift. Addition of sodium methoxide degenerated of the band I which indicates free hydroxyl groups at C-3 and C-4′. Hypsochromic shifts with $AlCl_3$/HCl and a bathochromic shift in band-I with NaOAc and boric acid ($NaOAc/H_3BO_3$) indicated the presence of 3, 3′, 4′ trihydroxy system in LCF-I [22].

It is found that due to the hydrogen-donating ability of the antioxidants, they exert an effect on DPPH [23-25]. Although the DPPH radical scavenging abilities of LCF-I were found to be lower than that of the aspirin, it was evident that LCF-I showed remarkable hydrogen-donating ability. This property of LCF-I served as free radical scavengers which acted possibly as primary antioxidants. Thus, the isolated compound from the benzene extract of *L. crustacea* found to be scavenged DPPH radical [26,27]. The amount of peroxide produced at the initial stages of lipid oxidation is measured in the FTC method [28], whereas the amount of peroxide produced in the secondary stages of lipid peroxidation is measured by the TBA method [29,30]. The higher antioxidant activity of showed by LCF-I in the TBA method than in FTC method indicated that, the peroxide amount produced at the initial stages of lipid oxidation, is lower than the second stage [31]. The less IC_{50} values (Table 3) of 29.65 and 26.11 for FTC and TBA methods, respectively, suggested better effect against lipid peroxidation which was comparable with ascorbic acid [26].

In reducing power assay, ferric/ferricyanide complex is reduced to the ferrous form in the presence of antioxidants. This reducing capacity of the sample is the indicator of antioxidant property, and an increase in reducing the power of the sample is indicated by the increase in absorbance [32]. This is used to find out the ability of an antioxidant sample to donate electron [33,34]. Many research reports have shown that there is a correlation between reducing power and antioxidant activity [35,36]. The result revealed that LCF-I donates an electron to react with free radicals which convert to a stable product and thus terminates radical chain reaction [37]. This fact indicates the strong antioxidant activity of LCF-I.

CONCLUSION

The results of the study showed that benzene extract of *L. crustacea* (L) F. Muell and the isolated flavonoid has potent antioxidant activity whereas ethyl acetate and ethanol extracts showed weak antioxidant activity. The study results suggest that benzene extract of *L. crustacea* plant is a potential natural antioxidant source. Although the isolated compound is characterized and identified, the mechanisms of antioxidant activity need to be further studied.

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AUTHOR'S CONTRIBUTIONS

Smriti Rekha Chanda Das has contributed to the concept and design of the research work, experimental studies, data acquisition, data analysis, and manuscript writing; Abdul Baquee Ahmed has supervised and

involved in the concept and design of the research work, experimental studies and manuscript revision; Dibyendu Shil has helped in experimental studies, data acquisition, and data analysis; Indranil Chanda has contributed in data analysis, interpretation of spectral data and manuscript revision.

CONFLICTS OF INTEREST

No conflicts of interest are associated with this work.

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