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In vitro Antioxidant and Free Radical Scavenging Activity of *Lippia alba* (Verbenaceae)

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Abstract

The research was designated to investigate the polyphenolic contents and antioxidant activity of the aqueous ethanolic extract and various fractions from the plant *Lippia alba* (Mill.) (Family: Verbenaceae). The dried course powder was extracted with 5% aqueous ethanol to get ethanolic extract (LAE) that was partitioned successively with *n*-hexane (LAH), chloroform (LAC), ethyl acetate (LAA), and water (LAQ). All the extracts were tested for polyphenol contents and antioxidant activity. The phenolic contents and the flavonoid contents of LAE, LAH, LAC, LAA, and LAQ were found to be 97.463±0.36, 55.030±0.690, 91.343±0.120, 117.340±1.349 and 81.630±0.550 and 418.470±4.962, 583.847±2.274, 168.623±1.186, 161.970±1.793 and 392.257±4.856 mg of GAE/g of dried extractives, respectively. The data showed that LAA contained highest phenolics and LAH contains highest flavonoids content than that of other fractions. The flavonol contents and the proanthocyanidin contents of LAE, LAH, LAC, LAA, LAQ and standard (at 100 µg/ml each) were 0.611±0.01, 0.144±0.031, 1.938±0.023, 1.944±0.049, 0.385±0.007 and 0.984±0.025 and 0.087±0.005, 0.155±0.004, 0.068±0.002, 0.049±0.002, 0.056 ± 0.003 and 1.533±0.044, respectively, indicating remarkable flavonoid contents of LAC and LAA when compare to standard. However, proanthocyanidin contents of the extractives are less than standard catechin. In total antioxidant activity and reducing power capacity assay LAE, LAH, LAC, LAA, LAQ and standard showed absorbance at 0.667±0.005, 0.753±0.046, 0.444±0.009, 0.409±0.005, 0.235±0.007 and 1.534±0.38 and 1.633±0.055, 0.258±0.033, 3.839±0.051, 2.301±0.004, 0.360±0.042, and 1.046±0.053, respectively. The results demonstrated that all the extractives of *L. alba* had appreciable antioxidant activity when compared to standard. In DPPH radical scavenging assay and hydroxyl radical scavenging assay, the IC_{50} of LAE, LAH, LAC, LAA, LAQ and standard were found to be 15.73±0.51, 58.90±1.86, 15.93±1.24, 3.84±0.18, 7.17±0.36 and 3.48±0.17 µg/ml and 10.95±0.560, 13.25±0.56, 11.69±0.69, 5.68±0.408, 6.36±0.581 and 6.50±0.271 µg/ml, respectively. The significant scavenging activity was found in LAA that was similar to standard BHT and catechin. All these observations demonstrated that the plant *L. alba* specially chloroform and ethyl acetate fractions might be a good source for antioxidative lead.

Key words: *Lippia alba*, Verbenaceae, polyphenols, antioxidant activity, free radicals.

Introduction

Antioxidants are widely used for the prevention of diseases such as diabetes, coronary heart disease, abnormal proliferation of tissues and even altitude sickness (Adwas *et al.*, 2019; Moris *et al.*, 2017). A large variety of dietary constituents have been reported to possess antioxidant activity due to the presence of polyphenols, vitamin C, vitamin E, carotenoids, etc. (García-Closas *et al.*, 2004). High-activity antioxidants were found in berries, citrus, cherries, kiwi fruit, olive oil and also in fruit juices (Kaur and Kapoor, 2001). Several studies have analyzed the antioxidant potential of a wide variety of vegetables, particularly cacao beans, potatoes, tomatoes, spinach, and legumes (Chun *et al.*, 2005; Haminiuk *et al.*, 2012).

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The plant family Verbenaceae contains about 75 genera and 3000 species worldwide. Most of the species are in tropical and subtropical regions and only a few in the temperate regions (Merrett, 2005). *Lippia alba (L. alba)* is one of the important species of the Verbenaceae family. A variety of biological activities have been reported from this plant including cytotoxic, antifungal, antibacterial, antiviral, anti-malarial, and anti-inflammatory activities (Oliveira *et al.*, 2006; Rahmatullah *et al.*, 2011). Traditionally, tea made from *L. alba* leaves has been used as a tranquilizer as well as in gastrointestinal and respiratory disorders throughout Brazil. A decoction of it can be used as a universal antidote. Its roots are anti-cholera and an ingredient in a medicine for chest pain. Root bark extract cures food poisoning. Root bark and leaf pastes together are applied externally in sprains and young bark and leaf are astringent. *L. alba* fruits are pungent, astringent, and remove bile, phlegm, odor, and flatulence. They are also cardiotonic, laxative, and beneficial in mucous colic (Oliveira *et al.*, 2006; Ara and Nur, 2009; Saha *et al.*, 2011; Sena *et al.*, 2006). In addition, *in vivo* sedative and myorelaxant effects were reported for the hydroalcoholic extracts, and anti-hemorrhoidal and antiulcerogenic activity was described for the infusion of leaves (Botelho *et al.*, 2007; Gleiser and Zygadlo, 2007; Hennebelle *et al.*, 2008).

Based on the folkloric reputation and literature review, it was found that extensive antioxidant activities of various fractions were not performed before. Hence, the aim of the study was to evaluate the phenolic, flavonoid, flavonol and proanthocyanidin content and antioxidant activities of the aqueous ethanolic extract and its various fractions from the whole plant of *Lippia alba* (Mill.) (Family: Verbenaceae) using *in vitro* bio-assays.

Materials and Methods

Collection and identification of plant: The whole plant *L. alba* (Mill.) was selected based on its local use and availability. Fresh plant stems were collected from Rajshahi University, Rajshahi, Bangladesh in November 2018 and was identifying by taxonomist A.H.M. Mahbubur Rahman, Professor, Department of Botany, Rajshahi University, Bangladesh. A voucher specimen (PHRU-211) is kept to the Department of Pharmacy, Rajshahi University.

Preparation of plant material: The collected plant was washed with distilled water and then shade dried for 6-7 days with occasional sun drying followed by oven drying at around 45^0 C for 24-36 h to avoid the decomposition of thermolabile phytochemicals. The dried plants were ground by a grinder available at the department of Pharmacy, Rajshahi University.

Extract and fractionation: Around 1.1 kg of powdered sample of *L. alba* was extracted with 95% ethanol (6 litre with 5% water) for 7 days with occasional shaking in extraction bottle. After 7 days, the mixture was filtered through cotton and was concentrated under reduced pressure at 50°C. The residue was extracted twice using same procedure and the whole filtrates were combined and weighted to get concentrated reddish mass (59 g, 5.3%, w/w). An aliquot of the concentrated ethanolic crude extract (LAE) was partitioned to get *n*-hexane (LAH, 6.79 gm), chloroform (LAC, 6.30 gm), ethyl acetate (LAA, 4.78 gm) and aqueous (LAQ, 36.95 gm) fraction.

Determination of total phenolics: The total phenolics of different extractives from the aqueous ethanolic extract of *L. alba* were determined by mixing an aliquot in testube with Na_2CO_3 (75 g/l, 2.0 ml) and 2.0 ml diluted Folin-Ciocalteu reagent. The tube was allowed to stand at room temperature for 20 min (25°C) after vortexed for around 15 sec. At 760 nm absorbance was measured using spectrophotometer. The result was expressed in terms of standard gallic acid (GA) equivalent (mg of GA/gm of whole *L. alba* extract). Each experiment was performed three times (Wolfe *et al.*, 2003).

Determination of total flavonoids: The quantitative estimation of total flavonoids of LAE, LAH, LAC, LAA and LAQ of *L. alba* were performed using $AlCl_3$ colorimetric assay described by Ordonez *et al*. (2006). In brief, 100 µl of 10%

$AlCl_3$, MeOH (1.5 ml), 1M potassium acetate solution (100 µl) and distilled water (2.8 ml) were added to exactly 0.5 ml of sample. 420 nm wavelength was used to measured after 90 minutes of incubation at room temperature (25°C). The result was calculated and expressed as mg of catechin per gram of dry bark extract.

Determination of total flavonols: The content of total flavonols of different extractives were estimated by taking 1.0 ml solutions of different extractives or standard (at final concentration of 0.1 mg/ml) into test tubes followed by adding $AlCl_3$ in ethanol (1.0 ml of 2%) and sodium acetate (50 g/L, 1.5 ml) solution to each of the test tubes. The resulting reaction mixtures were then incubated at 20°C for 2.5 h. After 2.5 h, the absorbance was taken at 440 nm. The content of total flavonols was calculated using ascorbic acid (AA) (mg/ml) as standard compound (Kumaran and Karunakaran, 2007).

Determination of total proanthocyanidins: The content of total proanthocyanidins were determined by using the method reported by Sun *et al.*, (1998) with a modification. 0.5 ml solution of different extractives or catechin (standard) at a concentration of 0.1 mg/ml was taken into the test tubes followed by adding 3.0 ml of 4% vanillin-methanol solution to each of the test tubes. 1.5 ml of hydrochloric acid was then added into each of the test tubes. The reaction mixtures were allowed to stand for 15 mins at RT to complete the reaction. The absorbance of the solutions was measured at 500 nm. The total proanthocyanidins content were expressed as catechin equivalents (µg/ml).

Determination of total antioxidant capacity: Total antioxidant capacity of LAE, LAH, LAC, LAA aqueous LAQ was evaluated by mixing samples (0.5 mL each) at different concentrations with H_2SO_4 (0.6 M, 3 mL), ammonium molybdate (1%) and Na_3PO_4 (28 mM). The samples were incubated at 95°C for 10 min and absorbance was measured at 695 nm. A higher value of absorbance indicates increased total antioxidant capacity (Prieto *et al.*, 1999).

Reducing power capacity assay: Oyaizu *et al.* (1986) described the process to measure the reducing power of various plant extracts. In Oyaizu method, different diluted samples (0.25 ml each) were added to 0.2 M potassium buffer (0.625 ml) and 1% $[K_3Fe(CN)_6]$ solution (0.625 mL). The mixture was incubated at 50° C (20 min). At the end of incubation, TCA (10% solution, 0.625 ml) was added and the solution was centrifuged (3000 rpm, 10 min). Distilled water (1.8 mlL) and 0.1% $FeCl_3$ (0.36 ml) solution was mixed with 1.8 ml supernatant. Finally, 700 nm wavelength was used to measure the absorbance.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay: 2,2-diphenyl-1-picrylhydrazyl abbreviated as DPPH is widely used to determine scavenging potential of free radical. The experiment was performed using Kedare and Singh's (2011) modified method. In brief, DPPH (0.1 mM, 2.4 ml) in methanol solution was mixed with aqueous ethanolic sample (1.6 ml each) of various concentration. The mixture was vortexes, left for 25-30 min in the dark and the absorbance at 517 nm were measured. Formula to calculate % of inhibition = $[(A_0-A_1) A_0]\times100$, where A_0 and A_1 are the absorbance of control and extract, respectively. From the % of inhibition vs conc. curve, IC_{50} called 50% inhibitory concentration in µg/ml was calculated.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity of LAE, LAH, LAC, LAA aqueous LAQ was performed (Klein *et al.*, 1981) by taking 500 µl of extract (1 mg/ml in 70% MeOH) in test tube. A variety of reagents were added to the test tube, these are: H_2O_2 (1.0 mM, 100 µl), 2-deoxy D-ribose (28 mM, 100 µl), $FeCl_3$ (200 µm, 100 µl), EDTA (1.04 mM, 100µL) and ascorbic acid (1mM, 100 µl). The mixed contents were incubated at 37°C (1 hr) and 1 ml (each) 10% TCA and 1% TBA were added in each test tube. The mixtures are incubated finally at 100°C for 20 minutes. The absorbance at 532 nm were taken to calculate % hydroxy radical scavenging activity using the formula = $[(A_0-A_1)/A_0] \times100$, where A_0 and A_1 are the absorbance of control and extractive. From the graph IC_{50} can be calculated.

Results and Discussion

Total phenolic, flavonoid, flavonol and proanthrocianidin content of L. alba: Phenolic content of the LAE of *L. alba* and its four fractions were calculated. Gallic acid with various concentration was used to determine standard curve. The results of phenolic contents were expressed as mg of Gallic acid equivalent (GAE)/gm of extracts. The phenolic contents of LAE, LAH, LAC, LAA, and LAQ were found to be 97.463±0.36, 55.030 ± 0.690, 91.343 ± 0.120, 117.340 ± 1.349 and 81.630 ± 0.550 mg of GAE / gm of dried extract, respectively (Figure 1A). From the data it was shown that the phenolic contents of LAA was higher than phenolic contents of original extract, hence ethyl acetate fraction might serve as a good source for phenolic as well as antioxidants (Alam *et al.*, 2016).

The contents of total flavonoids of LAE, LAH, LAC, LAA, and LAQ (Figure 1B) were found to be 418.470 ± 4.962, 583.847 ± 2.274, 168.623 ± 1.186, 161.970 ± 1.793 and 392.257 ± 4.856 mg of GAE/gm of dried extractives, respectively determined using well known aluminum chloride colorimetric method using gallic acid as standard (Ordonez *et al.*, 2006). Comparing the total flavonoids content of LAE with its four fractions, it was observed that LAH contained highest amount of flavonoids, followed by LAE and LAQ fraction. The results demonstrated that *L. alba* is a significant source of flavonoids.

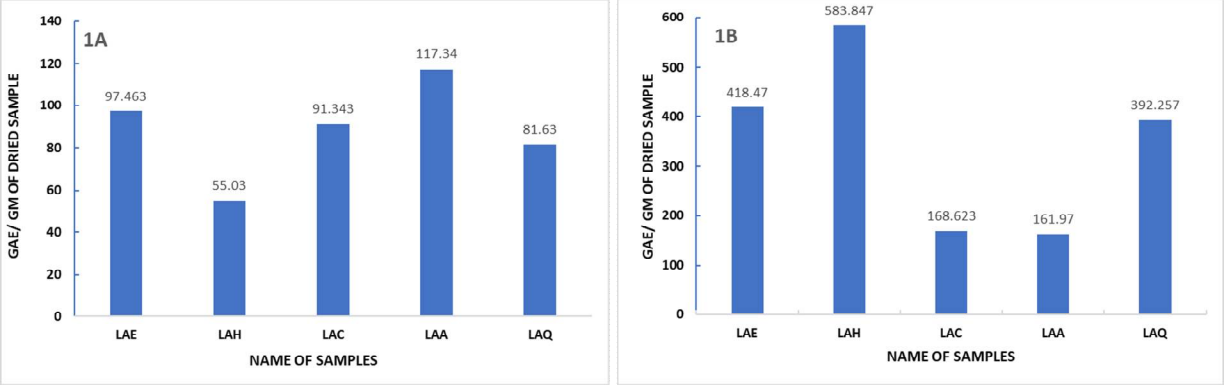

Figure 1. (A) Total phenolic contents and (**B**) Total flavonoid contents of LAE and four fractions of *Lippia alba* (mg/gm plant extract in GAE).

The total flavonols content of LAE, LAH, LAC, LAA, LAQ and standard ascorbic acid were 0.611±0.01, 0.144±0.031, 1.938±0.023, 1.944±0.049, 0.385±0.007 and 0.984±0.025, respectively, at 100 µg/ml (Figure 2A). The results showed that the flavonols content of LAC and LAA (absorbance 1.938 and 1.944) were higher than that of standard ascorbic acid (AA, absorbance 0.984), whereas LAE (absorbance 0.611) showed comparable flavonols content to the standard. The flavonols content of the extractives exhibited the following order: LAA > LAC > AA >LAE> LAQ > LAH.

The content total of proanthocyanidins of LAE and its four fractions were determined using catechin as standard. The results showed that the proanthocyanidins content of LAE, LAH, LAC, LAA, LAQ and standard catechin were 0.087±0.005, 0.155±0.004, 0.068±0.002, 0.049±0.002, 0.056 ± 0.003 and 1.533±0.044, respectively, at 100 µg/ml (Figure 2B). Comparing the total proanthocyanidins content of different fractions, it was observed that the LAH fraction had the highest and LAA had the lowest amount of proanthocyanidins that follows the following order: CA >> LAH > LAE > LAC > LAQ > LAA.

Total antioxidant and ferric-reducing capacity of LAE and various fractions: Prieto *et al.* (1999) described to measure the antioxidant activity of different extractives by phosphomolybdenum method using catechin as standard and the result is shown in

figure 3A. The results showed that LAE (absorbance 0.667) and LAH (absorbance 0.753) had significant antioxidant activity when compared to standard CA (absorbance 1.534) at a concentration of 100 µg/ml. Among the four fractions, LAH showed the highest total antioxidant activity followed by LAE with the following order: CA > LAH > LAE > LAC > LAA > LAQ.

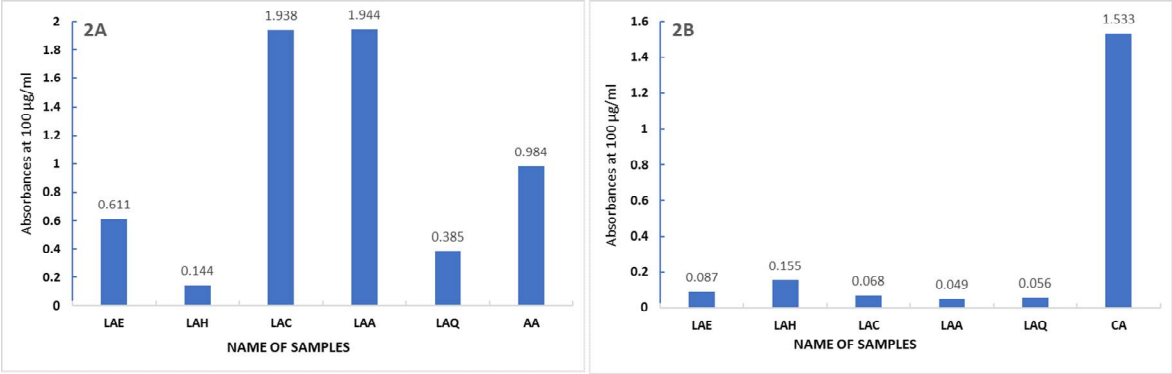

Figure 2. (A) Total flavonols content and (B) Total proanthocyanidins content of *L. alba*, four fractions and standard at 100 µg/ml.

The Fe^{3+} reducing power of the different extractives of *L. alba* was determined by the method described by Oyaizu (1986) with slight modification using ascorbic acid (AA) as standard (Table 1 and Figure 3B). Among the fractions, LAC (absorbance 3.839), LAA (absorbance 2.301), and LAE (absorbance 1.633), showed significant reducing power capacity that are higher than that of standard AA (absorbance 1.046) at a concentration of 100 µg/ml. The results demonstrated that LAC, LAA and LAE had significant iron reducing capacity. The reducing power of different extractives of *L. alba* and AA showed the following order: LAC > LAA > LAE > AA > LAQ > LAH.

DPPH Free radical and hydroxyl radical scavenging activity of various fractions of L. alba: DPPH antioxidant assay is based on the ability of the extractives to scavenge the stable DPPH radical that contains an odd electron. The antioxidant potential of *L. alba* extractives was evaluated by DPPH radical scavenging assay method (Kedare and Singh, 2011). This radical gives absorbance at 517 nm and decolorizes after neutralization by the antioxidants. The activity is increased by increasing the sample concentration. The results of DPPH radical scavenging assay of various fractions and standard BHT (Butylated Hydroxytoluene) are shown in Table 2. The IC_{50} values of LAE, LAH, LAC, LAA, LAQ and standard BHT were found to be 15.73±0.51, 58.90±1.86, 15.93±1.24, 3.84±0.18, 7.17±0.36 and 3.48±0.17 µg/ml. Among the fractions, the highest scavenging activity were found in LAA having IC_{50} value of 3.84 µg/ml, which is similar to that of standard BHT. Other fractions LAE, LAH, LAQ and LAC showed comparable DPPH radical scavenging activity. The activity of different extractives exhibited the following order: BHT > LAA > LAQ > LAE > LAC > LAH

Hydroxyl radical has been implicated as highly active and damaging free-radical induced biological damage found in the living cells (Hochestein *et al.*, 1988). The assay was used to find the quantitative ability of extractives of *L. alba* to remove the hydroxyl radical in solution. The process is a colorimetric method using catechin (CA) as standard and from the data % of scavenging activity was calculated. The activity is increased proportionally by increasing the concentration of the samples. The results of OH radical scavenging assay of various fractions and standard catechin are shown in Figure

4A and 4B. The IC_{50} of LAE, LAH, LAC, LAA, LAQ and standard were found to be 10.95±0.560, 13.25±0.56, 11.69±0.69, 5.68±0.408, 6.36±0.581 and 6.50±0.271 µg/ml, respectively. Among the fractions tested, the highest and significant scavenging activity was found in LAA with IC_{50} value of 5.68 µg/ml. On the other hand, LAQ have similar scavenging activity to that of Catechin. Our observation demonstrated that all the fractions from *L. alba* plant possessed significant OH free radical scavenging activity. The activity of different extractives exhibited the following order: LAA > LAQ > CA > LAE > LAC > LAH.

Table 1. Ferric reducing power capacity of ascorbic acid, LAE and its four fractions at different concentrations

Conc. (µg/ml)	Sample with absorbance (Mean ± STD)*					
	AA	LAE	LAH	LAC	LAA	AQF
100	1.046±0.053	1.633±0.055	0.258±0.033	3.839±0.051	2.301±0.004	0.360± 0.042
80	0.984±0.026	0.627±0.012	0.196±0.006	2.980±0.011	1.988±0.010	0.266±0.009
40	0.717±0.016	0.462±0.006	0.137±0.004	2.020±0.026	1.684±0.045	0.156±0.008
20	0.534±0.039	0.190±0.004	0.093±0.005	1.054±0.082	0.992±0.007	0.113±0.005
10	0.272±0.010	0.082±0.003	0.043±0.005	0.708±0.060	0.621±0.012	0.095±0.004

*Values are (mean ± SD) from triplicate experiment. AA, LAE, LAH, LAC, LAA and AQF represent ascorbic acid, ethanolic extract, hexane fraction, chloroform fraction, ethyl acetate fraction and aqueous fraction, respectively.

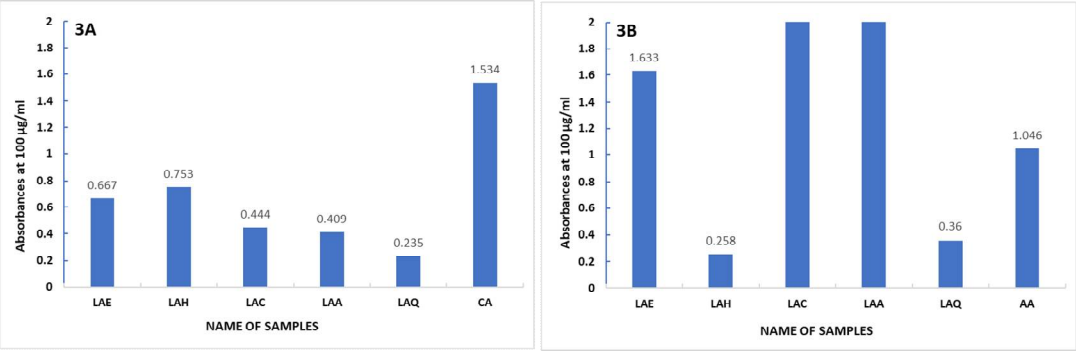

Figure 3. (A) Total antioxidant activity of LAE, extracts and standard catechin and (B) Ferric-reducing antioxidant capacity of LAE, extracts and standard ascorbic acid at 100 µg/ml.

Table 2. DPPH free radical scavenging activity with IC_{50} (µg/ml) of BHT, LAE and various fractions at different concentrations.

Conc. (µg/ml)	Sample with % of scavenging (Mean ± STD)*					
	BHT	LAE	LAH	LAC	LAA	AQF
3.75	53.87±2.67	34.82±2.38	17.64±2.12	33.98±1.15	48.83±2.27	38.17±1.50
7.5	69.12±2.40	40.69±2.17	29.58±1.96	38.60±1.83	67.41±2.63	52.31±2.66
15	80.72±1.01	47.67±1.54	38.95±2.05	47.09±3.60	76.09±3.12	63.95±2.18
30	85.11±3.49	70.67±0.56	45.75±2.76	62.81±1.89	82.42±2.56	76.75±4.59
60	89.76±0.85	84.92±0.48	50.93±1.59	76.93±3.06	87.03±2.61	78.57±3.76
120	91.24±0.42	86.12±0.32	56.23±3.32	79.26±3.86	88.95±1.65	81.28±2.27
IC_{50}	3.48±0.17	15.73±0.51	58.90±1.86	15.93±1.24	3.84±0.18	7.17±0.36

*Values are (mean ± SD) from triplicate experiment. AA, LAE, LAH, LAC, LAA and AQF represent ascorbic acid, ethanolic extract, hexane fraction, chloroform fraction, ethyl acetate fraction and water fraction, respectively.

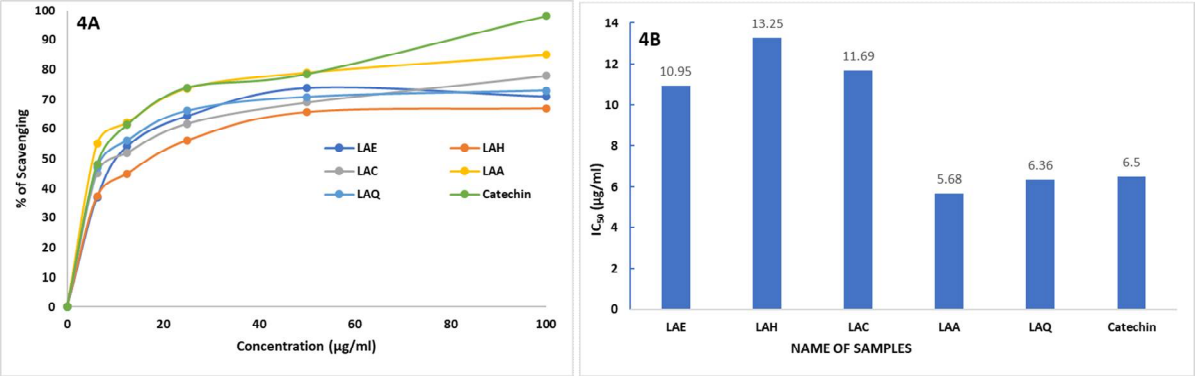

Figure 4. (A) Hydroxyl radical scavenging activity of LAE, various fractions and Catechin at different concentrations and (B) IC_{50} (µg/ml) values of different extractives and standard for hydroxyl radical scavenging activity.

Conclusion

The use of natural antioxidants as well as replacement of synthetic antioxidants with natural one may be advantageous due to availability, less toxicity and economical. In the present study, the crude aqueous ethanolic extract of *L. alba* and its several fractions, especially chloroform fraction and ethyl acetate fraction showed remarkable antioxidant activity by inhibiting DPPH, hydroxyl and reducing power activities when compared with standards. Hence, the whole plant can be a potent source of natural antioxidant due to the presence of high content of total phenolic and flavonoid content.

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