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Research Article

ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITY OF *AVERRHOA BILIMBI* EXTRACT

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ABSTRACT

Objectives: The present investigation attempts to study the antioxidant and antihemolytic activity of ethyl acetate fraction of the bilimbi extract (BE) and determine the contributory phytochemicals.

Methods: Fresh fruits of *Averrhoa bilimbi* were dried and subjected to 60% aqueous methanol extraction followed by biphasic extraction with ethyl acetate and water. The ethyl acetate fraction BE underwent phytochemical screening, analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical scavenging activity, hydroxyl radical scavenging activity, red blood cell (RBC) protection activity, and DNA protection activity.

Results: In phytochemical screening analysis, we detected lipids, while alkaloids, flavonoids, saponins, terpenoids, cardiac glycosides, reducing sugars, and amino acids were not detected. HPLC analysis showed prominent peaks at 23, 27, and 37 min under 310 nm. The fraction expressed $ABTS^{\cdot+}$ radical scavenging activity, hydroxyl radical scavenging activity, RBC protection activity, and DNA protection activity, wherein TEAC value was 11.5 µM, and IC_{50} value of hydroxyl RSA and antihemolytic activity were 49 and 47 µg/ml, respectively.

Conclusion: The ethyl acetate fraction of bilimbi predominantly comprised lipids which exhibited significant antioxidant and protective properties.

Keywords: Antioxidant, DNA protection activity, Antihemolytic activity.

INTRODUCTION

The human body hosts a complex system of natural enzymatic and non-enzymatic antioxidant defenses which render protection from the harmful effects of free radicals and other oxidants. Free radicals include hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxyl ($ROO^{\cdot}$), and lipidperoxyl ($LOO^{\cdot}$) radicals while oxidants which are non free radicals but can easily lead to free radical reactions in living organisms include hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen (O_2), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and lipid peroxide(LOOH) [1]. ROS and RNS are required at low or moderate concentration for the maturation process of cellular structures. They also function as weapons for the host defense system, wherein phagocytes (neutrophils, macrophages, and monocytes) release free radicals to destroy invading pathogenic microbes as a part of the body's defense mechanism against disease [2]. However, if produced in excess, free radicals tend to react with various organic substrates such as carbohydrates, lipids, proteins, and DNA [3]. The resultant has contributed to a large number of diseases including atherosclerosis, cancer, cardiovascular disease, neural disorders, Alzheimer's disease, Parkinson's disease, alcohol-induced liver disease, ulcerative colitis, and aging [4-6]. Protection against free radicals can be enhanced by ample intake of dietary antioxidants. Substantial evidence indicates that foods containing antioxidants and possibly in particular the antioxidant nutrients may be of major importance in disease prevention and postponing the onset of degenerative disorders [7]. Following up the harmful tendencies of free radicals, indigenous fruits such as that of *Averrhoa bilimbi* are targets of potential sources of antioxidants and therapeutic components. *A. bilimbi* L. (Oxalidaceae), a native of Malaysia and Indonesia, is a widely cultivated tree in southern India, particularly in Mangalore and Udupi. Commonly known as bilimbi, the edible fresh oblong very sour fruits undergo production of vinegar, wine, pickles, jams, and jellies. Bilimbi is also medicinally used as a folk remedy for many symptoms and diseases such as fever, mumps, pimples, inflammation of the rectum and diabetes, itches, boils, rheumatism, syphilis, bilious colic, whooping cough, hypertension, stomach ache, and ulcer and as a cooling drink [8]. Experimental pharmacological studies have shown that the fruit alleviates hypertension while aqueous extract of fresh bilimbi shows low antioxidant activities and low nitric oxide inhibition activity. Interestingly, the ethyl acetate extract of the fruit has expressed significant antioxidant activity [9]. Our earlier findings using aqueous methanol extract of bilimbi have also shown significant antioxidant activity and expressed proapoptotic and antiangiogenic activity against Ehrlich ascites carcinoma cells *in vivo* [10,11]. The following investigation is thus the first report to study the antioxidant and antihemolytic activity of ethyl acetate fraction of the aqueous methanol bilimbi extract (BE) after its subjection to biphasic extraction and determining its contributory phytochemicals.

MATERIALS AND METHODS

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), sodium dihydrogen phosphate, disodium hydrogen orthophosphate, Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid), Tris, 2-deoxy-D-ribose, potassium dihydrogen orthophosphate, potassium hydroxide, ferric chloride, ascorbic acid, thiobarbituric acid, trichloroacetic acid, potassium ferricyanide, sodium chloride, agarose, and ethidium bromide were obtained from Himedia, India; hydrogen peroxide, glacial acetic acid, methanol, and lambda phage DNA were purchased from Merck, India.

Plant extraction preparation

A. bilimbi fruits were collected from Mangalore, Karnataka. Dr. Krishna Kumar H.N., Department of Botany, Pooja Bhagavat Memorial Mahajana Education Centre, Mysuru (Voucher No. 2017/11/01), authenticated the fruits deposited as voucher specimens at the Herbarium Repository, Department of Botany, PBMMEC, Mysuru. 1 kg of diced fruits dried at 50°C after thoroughly washing it. The dried fruits were ground to a fine powder. 10 g of the dried fruit powder underwent extraction under continuous agitation for 3 h at 40°C using 60% methanol in water followed by filtration using muslin cloth and drying of filtrate at 50°C. The aqueous reconstituted dried crude extract was centrifuged at

3000 rpm for 20 min. The supernatant underwent biphasic extraction using equal volumes of ethyl acetate at room temperature for 12 h. The collected ethyl acetate phase (BE) dried at room temperature and used for the following analysis.

Phytochemical screening

Test for the presence of phytochemicals such as sugars, lipids, flavonoids, alkaloids, cardiac glycosides, saponins, and terpenoids was conducted using standard protocols [12,13].

$ABTS^{\cdot+}$ radical scavenging activity

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical scavenging activity of the BEA extract was conducted using Trolox as a standard [1] and TEAC of the extract calculated using the standard curve produced by percentage inhibition of Trolox from the following formula:

$$\%\text{Inhibition} = \left[1 - \frac{A_{(\text{Test})}}{A_{(\text{Control})}}\right] x100$$

Hydroxyl radical scavenging activity

The measured scavenging ability of hydroxyl radicals by the method of Kunchandy and Rao [13] expressed as IC_{50} value using the following formula:

$$\%\text{Inhibition} = \left[1 - \frac{A_{(\text{Test})}}{A_{(\text{Control})}}\right] x100$$

Inhibition of DNA damage

DNA protection assay assessed the potential of the extract to protect DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent [14].

Antihemolytic activity

The method described by Mosbah *et al*. [15] determined the antihemolytic activity of BE fraction in the form of IC_{50} value using the following equation:

$$\%\text{Inhibition} = \left[1 - \frac{A_{(\text{Test})}}{A_{(\text{Control})}}\right] x100$$

High-performance liquid chromatography (HPLC)

A BDS HYPERSIL C-18 column (150×4.6 mm, 5 µm particle size) equipped with PDA/ultaviolet detector with 310 nm as the detecting wavelength in room temperature (27°C) enabled chromatic separations under the following conditions: 1 ml min^{-1}; solvent A, 10% acetic acid in water; and solvent B, 15% methanol in water starting from 0 to 20 min (40–52% A), 20–40 min (52–80% A), and 40–60 min (80% A).

Statistical analysis

All experiments performed in three replicates expressed as mean±standard deviation (SD) underwent statistical analysis using Origin 5.0.

Table 1: Phytochemical analysis of BEA extract

Phytochemical	Present (+)/Absent (-)
Alkaloids	-
Flavonoids	-
Saponins	-
Terpenoids	-
Cardiac glycosides	-
Reducing sugars	-
Amino acids	-
Steroids	-
Lipids	+

RESULTS

Phytochemical screening

Phytochemical screening revealed the presence of lipids but the absence of reducing sugars, cardiac glycosides, alkaloids, flavonoids, saponins, terpenoids and amino acids (Table 1).

Results are expressed after the experiment was conducted in triplicates.

$ABTS^{\cdot+}$ radical scavenging activity

The percentage inhibition of BE was dose dependent, wherein as the concentration of extract increased, the percentage inhibition increased. The $ABTS^{\cdot+}$ Radical scavenging activity of BE was found to be 11.5 µM TEAC (Table 2).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity measured the inhibiting of degradation of deoxyribose by the free radicals generated by the Fenton reaction. The BE inhibited to produce hydroxyl radicals wherein the IC_{50} value of the extract and Trolox was 69.42 µg/ml and 45.58 µg/ml, respectively (Table 2).

Antihemolytic activity

The red blood cell (RBC) protection activity of BE and etodolac was dose dependent. As the concentration of compounds/drug increased, the percentage inhibition rose. The IC_{50} value of etodolac was 45.22 µg/ml while that of BEA 47.43 µg/ml (Table 2).

Experiments were performed in triplicates with a sample size of n=3. Results are expressed as mean±SD.

Inhibition of DNA damage

The DNA protection assay bases the ability of the compounds to protect the lambda phage DNA against damage caused by hydroxyl (•OH) radicals. At a concentration of 100 µg/ml, the extract protected the DNA from fragmentation even after exposure to $OH^{\cdot}$ radicals generated through Fenton reactions (Fig. 1).

HPLC

After running the extract in HPLC, chromatographic separations showed prominent peaks at 23, 27, and 37 min under 310 nm (Fig. 2).

DISCUSSION

Several *in vitro* test procedures are carried out for evaluating antioxidant activities with the sample of interest. Antioxidants generally function on the principle of terminating oxidation reactions paving its way to importance as a protective factor against quantitative damage due to oxidative stress. Among free radical scavenging methods, ABTS decolorization assay is applicable for both hydrophilic and lipophilic antioxidants [1]. BE extract expressed significant TEAC values although Trolox was a better antioxidant than the extract in the hydroxyl radical scavenging activity. Hydroxyl radical is one of the potent reactive oxygen species in the biological system that reacts with polyunsaturated fatty acid moieties of cell

Table 2: TEAC, hydroxyl RSA, and RBC protection assay of Trolox, etodolac, and BE extract

TEAC (µM TEAC)	IC_{50} of OH. Radical Scavenging Activity (µg/ml)		IC_{50} of RBC protection activity (µg/ml)	
	Trolox	BEA Extract	Etodolac	BEA Extract
11.5±0.757	45.58±0.980	69.42±2.03	45.22±1.04	47.43±0.72

RBC: Red blood cell, BE: Bilimbi extract

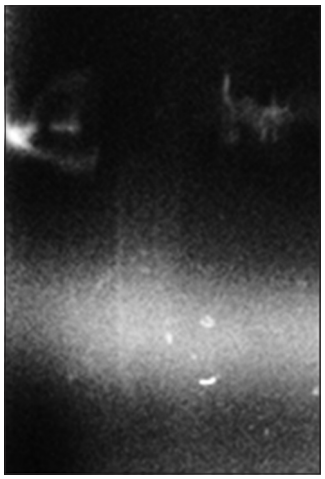

Fig. 1: DNA Protection of bilimbi extract. Lane 1 - Control DNA, Lane 2 - DNA + Fenton Reagent, Lane 3 - DNA + BEA + Fenton Reagent

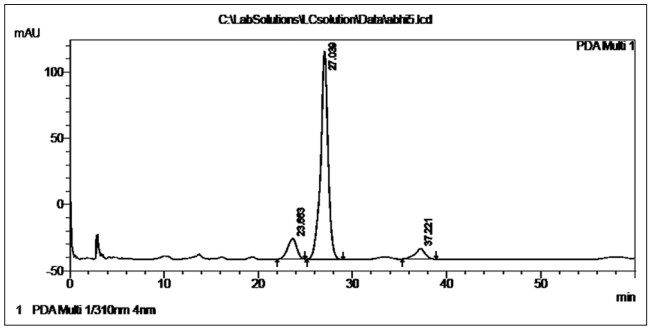

Fig. 2: High-performance liquid chromatography profile of *Averrhoa bilimbi* extract at maximum absorption of 310 nm

membrane phospholipids and causes damage to cell [7]. This damage observed predominantly in RBC cells exposed to oxygen, and hence, its free radicals are the most. In addition, the hemoglobin in RBCs is a strong catalyst which can initiate lipid peroxidation resulting in affecting the –SH proteins. Reduced levels of GSH result in membrane instability [16]. One of the distinguished inflammatory responses is the rupturing of RBC cells. Etodolac is a commercially available anti-inflammatory drug, and BE extract expressed an anti-inflammatory potential equivalent to etodolac. Hydroxyl radicals generated by the Fenton reaction are also known to cause oxidative-induced breaks in DNA strands to yield its open circular or relaxed forms, wherein it reacts with the sugar moiety causing breakage of sugar phosphate backbone of nucleic acid, resulting in strand break [14]. Fig. 1 shows the DNA exposed to Fenton's reagent results in fragments while DNA treated with BE extract protected from fragmentation. The extract was able to protect the DNA from damage. Our study has shown that the ethyl acetate fraction expresses significant antioxidant activity. Reports by Kurup and Mini [9] have shown that ethyl acetate extract of bilimbi fruits expressed highest antioxidant capacity. Phytochemical screening of the fraction revealed predominantly lipids. Reports have suggested that during HPLC on lipids, a stationary phase similar in chain length to the fatty acyl chains maximizes the interactions and should give the highest efficiency [17]. Combination of methanol/water in gradient elution served as an efficient modifier solvent exhibiting efficient separation of the sample. Studies have shown that the retention time in HPLC is dependent on the total carbon atoms present, especially in triacylglycerols, and a double bond in the fatty acid reduces the retention by two carbon atoms [17]. The retention time of the BE fraction could suggest unsaturation of the lipids which can further be studied and confirmed using other analytical tools. Lipid antioxidants such as tocopherol are more efficient than water-soluble antioxidants during conditions of reducing lipid soluble peroxy radicals [18,19]. Chain-carrying peroxyl radicals are also one of the factors that damage the cell membranes of cells including erythrocytes, resulting in further uptake of oxygen radicals. Lipid antioxidants thus prevent such conditions by scavenging these radicals. The ethyl acetate fraction of bilimbi fruit extract expressed significant antioxidant and anti-hemolytic activities which were on par with standard antioxidants and drugs. The lipid content could be responsible for the therapeutic potential of the fraction, and further, characterization and molecular mechanism need elucidation. This is the first report of the collected ethyl acetate fraction through biphasic extraction expressing significant therapeutic potential.

CONCLUSION

The ethyl acetate fraction of bilimbi predominantly composed of lipids which exhibited significant antioxidant and protective properties. Isolation of the active principle of the extract is underway and its therapeutic potentials under study.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest.

REFERENCES

1. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: Properties, sources, targets, and their implication in various diseases. Ind J Clin Biochem 2015;30:11-26.
2. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci 2008;4:89-96.
3. Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine. 4th ed. Oxford, UK: Clarendon Press; 2007.
4. Singh U, Jialal I. Oxidative stress and atherosclerosis. Pathophysiology 2006;13:129-42.
5. Butterfield DA. Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: Implications for neurodegeneration in Alzheimer's disease brain. A review. Free Radic Res 2002;36:1307-13.
6. Bahorun T, Soobrattee MA, Luximon-Ramma V, Aruoma OI. Free radicals and antioxidants in cardiovascular health and disease. Internat J Med Update 2006;1:1-17.
7. Meyer AS, Isaksen A. Application of enzymes as food antioxidants. Trends Food Sci Technol 1995;6:300-4.
8. Kumar AS, Kavimani S, Jayaveera KN. A review on medicinal plants with potential antidiabetic activit. Internat J Phytopharmacol 2011;2:53-60.
9. Kurup SB, Mini S. *In vitro* evaluation of free radical scavenging and antioxidant activities of *Averrhoa bilimbi* fruit extracts. J Plant Chem Ecophysiol 2017;2:1014-20.
10. Chauhan JB, Kapfo W. Effect of traditional sun-drying on phenolic antioxidants of *Averrhoa bilimbi* L. Internat J Appl Biol Pharma Technol 2013;4:26-34.
11. Chauhan JB, Jayarama S, Kyathegowdanadoddi BS, Kapfo W. Proapoptotic potential of *Averrhoa bilimbi* extraction Ehrlich ascites carcinoma induced mice. Asian J Clin Pharmaceut Res 2016;9:348-52.
12. Zaman R, Parvez M, Jakaria MD, Ali MS, Hoque SA. Phytochemical screening, *in vitro* cytotoxic and antimicrobial activity of methanolic extract of leaves of *Ficus sagittata* vahl. Innov J Life Sci 2016;4:12-5.
13. Kunchandy E, Rao MN. Oxygen radical scavenging activity of curcumin. Int J Pharm 1990;58:237-40.
14. Jiang Y, Han W, Shen T, Wang MH. Antioxidant activity and protection from DNA damage by water extract from pine (*Pinus densiflora*) Bark. Prev Nutr Food Sci 2012;17:116-21.
15. Mosbah A, Sobhi W, Benboubetra M. Antioxidant and anti-hemolitic activities of Algerian *Nigella sativa* total oil and its neutral lipid fraction. Internat J Pharm Pharm Sci 2015;7:50-4.
16. Asgary S, Naderi GH, Askari N. Protective effect of flavonoids against red blood cell hemolysis by free radicals. Exp Clin Cardiol 2005;10:88-90.
17. Nikolova-Damyanova B. Reversed-phase high-performance liquid chromatography: General principles and applications to the analysis of fatty acids and triacylglycerols. Adv Lipid Methodol 1997;4:193-251.
18. Niki E. Lipid antioxidants: How they may act in biological systems. Br J Cancer 1987;55:153-7.
19. Shaban S, El-Husseny MW, Abushouk AI, Salem AM, Mamdouh M, Abdel-Daim MM. Effects of antioxidant supplements on the survival and differentiation of stem cells. Oxid Med Cell Longev 2017;2017:1-16.