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Comparative *In Vitro* Antioxidant Activity and HPTLC Fingerprint of *Averrhoa bilmbi* linn. and *Averrhoa carambola* linn. Fruit Extracts

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Abstract : There is a growing interest in the food industry and in preventive health care for the evaluation and development of natural antioxidants from medicinal plant materials. In the present work, aqueous extracts of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruits were screened for their phenolic profiles and antioxidant properties. The antioxidant activity of the extracts was determined by Total Antioxidant Activity (TAA), Ferric Reducing Power (FRP), Nitric oxide radical scavenging activity and Nitro Blue Tetrazolium scavenging activity (NBT). HPTLC fingerprint of methanolic extract of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. with Tannic acid and Ascorbic acid was developed. The results indicate that aqueous extracts of *Averrhoa bilmbi* Linn. had the highest antioxidant capacity as Ferric Reducing power and Nitric oxide scavenging activity. While aqueous extract of *Averrhoa carambola* Linn. showed higher percent inhibition than *Averrhoa bilmbi* Linn. in Nitro Blue Tetrazolium method. The less phenolic contents and antioxidant capacities were observed in *Averrhoa carambola* Linn as compared to *Averrhoa bilmbi* Linn. fruits. The results obtained in the present study indicate that aqueous fruit extracts of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. are a potential source of natural antioxidants. Further investigations are needed to verify this antioxidant effect *in vivo*.

Key words : *Averrhoa bilmbi* Linn., *Averrhoa carambola* Linn., *in vitro* Antioxidant activity, HPTLC.

Introduction

Oxidative stress is involved in the pathogenesis of various chronic diseases, such as cardiovascular disease and cancer (Lau *et al*., 2005; Neuhouser, 2004). Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen, including superoxide radicals, hydroxyl radicals and hydrogen peroxide are often generated as by products of biological reactions or from exogenous factors (Cerutti, 1991; Harman, 1994; Ames, 1998, Finkel, 2000). The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA (Farber, 1994). This oxidative damage is a decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing course (Hogg, 1998). Antioxidants protect against free radicals and they are therefore essential in obtaining and preserving good health. Much attention has been given to polyphenols with strong antioxidant activities, which are ubiquitously present in a broad range of medicinal plants and dietary products. Furthermore, as reported by many investigators (Vaya *et al*., 2003; Kris-Etherton *et al*., 2002; Fuhrman and Aviram, 2001), polyphenol from medicinal and aromatic plant possess a high anti-oxidant potential due to their hydroxyl groups and protect more efficiently against free radical-related diseases such as atherosclerosis. Epidemiological studies have brought into being that the intake of antioxidants such as Vitamin C reduces the risk of coronary heart disease and cancer (Finkel and Holbrook, 2000). The antioxidants may reconcile their upshot by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions (Robak and Marcinkiewicz, 1995).

Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially accessible but are quite perilous and their toxicity is a problem of disquiet (Madhavi and Salunkhe, 1995). The use of these synthetic antioxidants, have been restricted in foods as they are suspected to be carcinogenic. Therefore, the importance of search of natural antioxidants has greatly increased in the recent years (Jayaprakasha *et al*., 2003).

Recently special attention has been paid towards edible plants, which are rich in phytochemicals and there is now increasing interest in antioxidant activity of such phytochemicals present in the diet. Antioxidants are

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important in prevention of pollution damage of plants, disease prevention in both plants and animals and play a very important role in the body defense system and reactive oxygen species (Ou Huang *et al*., 2002). The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins**, lignans, catechin and isocatechins. In additions to above compounds found in natural foods, Vitamin C and E, â-carotene and á- toc**opherol are known to posses antioxidant potentials (Prior, 2003; Cai *et al*., 2004; Kaur and Kapoor, 2002).

Averrhoa bilimbi Linn. (Oxalidaceae) is locally known as bilimbi. It is used to make jam or jelly other than act as preservative in food (Diliman, 1971). The ascorbic acid content of ripe bilmbi fruits was reported to be 60.95 mg/100 g (Vera *et al.*, 2001). *Averrhoa bilimbi* Linn. fruits are used in various ways such as to treat skin disorders and fever (Anon, 2007). The fruit are good remedy for scurvy and beneficial in diarrhea, hepatitis and in inflammatory condition (Goh *et al.*, 1995). Other than that in Indonesia, the local use of *A. bilimbi* is to treat goiter, cough, sore throat and rheumatism (Nurul *et al.*, 2009).

Averrhoa carambola Linn. (Oxalidaceae) also known as star fruit is cultivated extensively in India for its edible fruits (Almeida, 1996; Cooke, 1967). A wide range of Ascorbic acid content (0.3- 23.0 mg/100 g) is recorded from different places in India (Khare, 2007). In India, the ripe fruit or its juice may be taken to counteract fever. A salve made of the fruit is employed to relieve eye afflictions. In Brazil, the carambola is recommended as diuretic in kidney and bladder complaints. In Chinese Materia Medica it is used to quench thirst, increase the salivary secretion and in fever. In Ayurveda, the ripe fruit is considered as digestive, tonic and causes biliousness. The dried fruit is also used in fever; it is cooling and possesses antiscorbutic properties. It is considered as one of the best Indian cooling medicines (Kirtikar and Basu, 1989; Parrotta, 2001).

Therefore, the purpose of the present study is to evaluate the antioxidant activity of aqueous fruit extracts of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. to elucidate antioxidative capacity. HPTLC fingerprint with tannic acid and ascorbic acid as standard was also developed.

Materials and Methods

Chemicals

Potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium nitroprusside, sulphanilamide, napthylethylenediamine dihydrochloride, Ascorbic acid, sulphuric acid, ammonium molybdate, nitro blue tetrazolium, riboflavin, gallic acid and tannic acid were purchased from HiMedia, Mumbai. All other chemicals and reagents were of analytical grade and used without further purification.

Plant Materials and Extraction

Fresh fruits of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. were collected from Colaba, Mumbai and Badlapur, District- Thane, Maharashtra, India respectively. Authentication was done from Blatter Herbarium, St. Xavier's College, Mumbai. The specimen of plant has been submitted to the Department of Botany-Herbal Sciences, Birla College, Kalyan for future reference. The collected fruits were dried under shade and powdered with a mechanical grinder and stored in an air tight container.

1000 mg fruit powder of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. was extracted separately in 100 ml of distilled-water for 12 hours. The content was filtered through Whatman filter paper No. 1. The filtrate was evaporated on boiling water bath until dry. The dry residue obtained was reconstituted in distilled water to acquire different concentrations used for *in vitro* antioxidant studies and was abbreviated as ABAE (*Averrhoa bilmbi* Linn. aqueous extract) and ACAE (*Averrhoa carambola* aqueous extract).

For HPTLC fingerprint 1000 mg of fruit powder was extracted in 10 ml of methanol and extracted for 12 hours. The content of test tube was filtered through Whatman filter paper No. 1, and it was used for developing HPTLC fingerprint of tannic acid and ascorbic acid with *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts.

Total Phenolic Content

The total soluble phenolic content in the extract was determined using Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977). 1 ml of ABAE and ACAE **(1 ìg ml^{-1} to 10 ìg ml^{-1})** was added to 1 ml of Folin-Ciocalteu reagent and the content was mixed thoroughly. 3 minute later 3 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 2 hours with intermittent shaking. The absorbance of **the blue color that developed was read at 760 nm. Gallic acid was used as a standard. The concentration of total phenols in the extract was expressed as ìg** g^{-1} of prepared extract. The concentration of total phenolic compounds in the extracts was determin**ed as ìg of gallic acid equivalent using an equation obtained from the standard gallic acid graph.**

Total Antioxidant Activity

0.1 ml of ABAE and ACAE was mixed with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank (Shirwaikar *et al.,* 2006).

Reducing power assay

The reducing power of aqueous extracts was determined according to the method of Oyaizu (1986). Different concentrations of ABAE and ACAE (50 - 1000 **ìg ml**$^{-1}$) in 1ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm and compared with standard Ascorbic acid. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. During the reaction sodium nitroprusside in aqueous solution at physiological *p*H spontaneously generates nitric oxide (Green *et al*., 1982), which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide (Sreejayan, 1997). Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0 ml of different concentrations (1000-5000 **ì g ml**$^{-1}$) of the ABAE and ACAE prepared in the distilled water and incubated at 25°C for 150 min. Above mixture was reacted with Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance at standard solutions of potassium nitrite, treated in the same way with Griess reagent. The percentage scavenging of nitric oxide of ABAE, ACAE and standard Ascorbic acid was calculated using the following formula:

NO Scavenged (%) = (A cont–A test)/A cont×100

Where A cont is the absorbance of the control reaction and A test is the absorbance in the presence of the sample of the extracts.

Nitro Blue Tetrazolium (NBT) Method

The method was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radical generated in riboflavin-light-NBT system. The reaction mixture contains EDTA, riboflavin, nitro blue tetrazolium (NBT), various concentrations (250 – 2500 **ìg ml**$^{-1}$) of ABAE and ACAE and phosphate buffer (pH 7.6) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min and absorbance was measured at 560 nm (Raju *et al*., 2005). The percentage inhibition was calculated by following formula:

% Inhibition = (A cont–A test)/Acont×100

Where A cont is the absorbance of the control reaction and A test is the absorbance in the presence of the sample of the extracts.

HPTLC fingerprint of Averrhoa bilmbi Linn. and Averrhoa carambola Linn. with Tannic acid and Ascorbic acid

Chromatography was performed on aluminium HPTLC plates coated with silica gel 60 F_{254} (Merck # 5554). Samples **(10 ìl**) were spotted using Camag Linomat V sample applicator. The plates were then developed in glass twin trough chamber (10 × 10 cm) pre-saturated with mobile phase. The mobile phase consisted of toluene: ethyl acetate: glacial acetic acid, 7: 2: 1 (v/v) for tannic acid and ethyl acetate: methanol: distilled water, 4: 2: 1 (v/v). The densitometric evaluation of the plate was performed at 254 nm in reflectance-absorbance mode using deuterium lamp with a Camag Scanner III in conjunction with Cats 4 Version Software. The wavelength used for densitometry was selected after acquiring spectra of the standard. The identity of the band of Tannic acid in the sample was confirmed by overlaying the chromatogram of sample with that of the Tannic acid and by comparing their R*f* (0.09) and (0.30) for Ascorbic acid.

Result and Discussion

The present study was conducted to investigate the antioxidant potential of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts.

Total phenolic content

Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived

from plant sources, and they have been shown to possess significant antioxidant activities (Van-Acker *et al*., 1996). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables (Tanaka *et al*., 1998). Total phenolic compounds are reported as gallic acid equivalents. The total phenolic contents of ABAE and ACAE were 4.29 ± 0.23 and 0.70 ± 0.38 mg gallic acid equivalent respectively. Results are tabulated in table 1 and graph of concentration of standard gallic acid versus absorbance are shown in figure 1.

Total antioxidant activity

Total antioxidant activity of ABAE and ACAE is shown in table 1. The phosphomolybdenum method was based on reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic *p*H. In this assay ABAE was found to have higher activity, ACAE showed lower activity. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transformating reactive free radical species into stable non reactive products (Dorman *et al*., 2003).

Reducing power ability

Figure 2 shows the reductive capabilities of the extract when compared to the standard, Ascorbic acid. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The reducing power increased with increasing amount of the extract. Increased absorbance of the reaction mixture indicated increased reducing power (Gupta *et al*., 2007). The ABAE and ACAE showed the highest reducing ability. However, the activity was less than the standard Ascorbic acid.

Nitric oxide scavenging method

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons,

Table 1: Total Antioxidant Activity and Total Phenolic content of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Extracts	Total Antioxidant Activity ($\mu g\ ml^{-1}$)	Total Phenolic content (µg ml-1)
Averrhoa bilmbi Linn.	0.52 ± 0.025	0.34 ± 0.001528
Averrhoa carambola Linn	0.426 ± 0.020	0.32± 0.002517

Values are mean ± S.D (n=3)

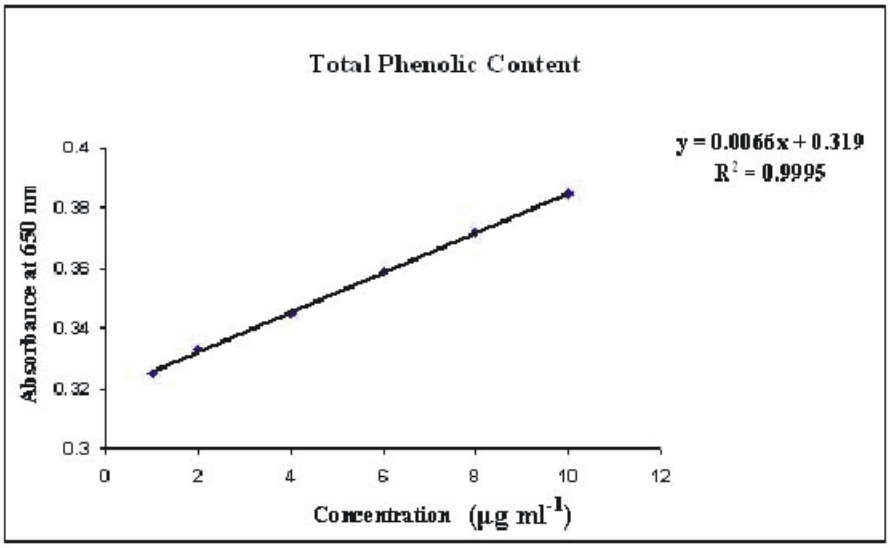

Figure 1: Total Phenolic content of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

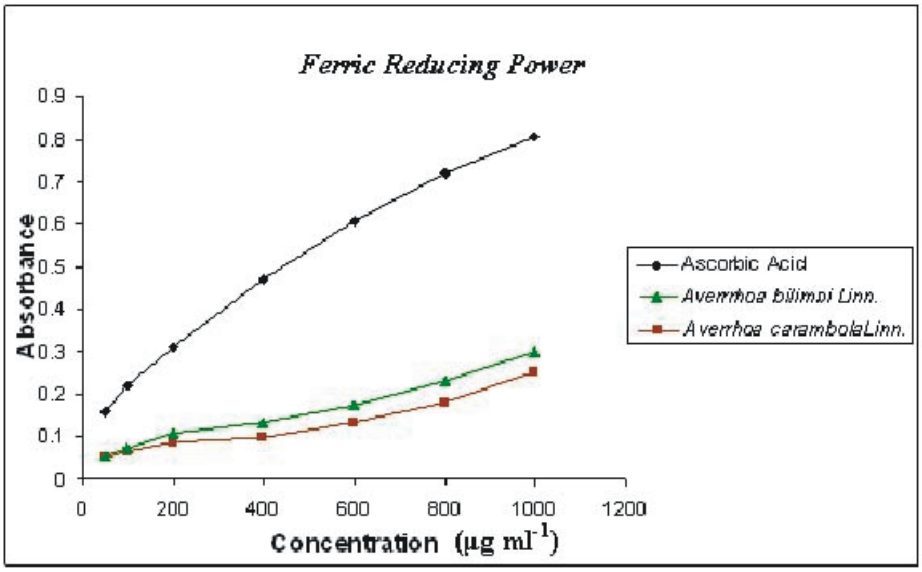

Figure 2: Ferric Reducing Power of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Table 2: Reducing power of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Concentration (µg ml^{-1})	Absorbance at 700 nm		
	Ascorbic Acid	*Averrhoa bilmbi* Linn.	*Averrhoa carambola* Linn.
50	0.16 ± 0.015	0.053 ± 0.0026	0.053 ± 0.0025
100	0.24 ± 0.025	0.064 ± 0.002	0.073 ± 0.0040
200	0.34 ± 0.035	0.084 ± 0.0025	0.106 ± 0.0015
400	0.47 ± 0.018	0.095 ± 0.0025	0.132 ± 0.0025
600	0.63 ± 0.025	0.134 ± 0.0035	0.174 ± 0.0025
800	0.74 ± 0.025	0.180 ± 0.0020	0.24 ± 0.023
1000	0.82 ± 0.022	0.254 ± 0.0040	0.32 ± 0.02

Values are mean ± S.D (n=3)

etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals (Kumaran, 2007). The nitric oxide scavenging method showed moderate scavenging activity compare to standard ascorbic acid. Table 3 shows the Nitric oxide scavenging activity of ABAE and ACAE. The IC_{50} values of ABAE and ACAE extract were found to be 1.6 **ì** g ml^{-1} and 2.0 **ì** g ml^{-1} respectively.

Nitro Blue Tetrazolium method

Superoxides are produced from molecular oxygen due to oxidative enzymes (Sainani *et al*., 1997) of body as well as via non-enzymatic reaction such as autoxidation by catecholamines (Hemmani, 1998).

Table 4 shows the Nitro Blue Tetrazolium scavenging activity of ABAE and ACAE on the NBT system. The increase of percentage scavenging activity thus indicates the consumption of superoxide anion in the reaction mixture by the plant extracts. The IC_{50} values of ABAE and ACAE extract were found to be 1.25 **ì** g ml^{-1} and 1.0 **ì** g ml^{-1} respectively.

HPTLC Fingerprint

HPTLC fingerprint of ABAE and ACAE show presence of Tannic acid when the plate was eluted with mobile phase toluene: ethyl acetate: glacial acetic acid

Table 3: *Nitric oxide* scavenging activity of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Concentration ($\mu g\ ml^{-1}$)	% Scavenging activity		
	Ascorbic Acid	*Averrhoa bilmbi* Linn.	*Averrhoa carambola* Linn.
1000	54.901	42.6	42.2
2000	57.51	55.2	49.5
3000	59.21	56.3	59.2
4000	69.203	66.7	70
5000	99.084	87.4	75
	IC_{50} value	**1.6 $\mu g\ ml^{-1}$**	**2.0 $\mu g\ ml^{-1}$**

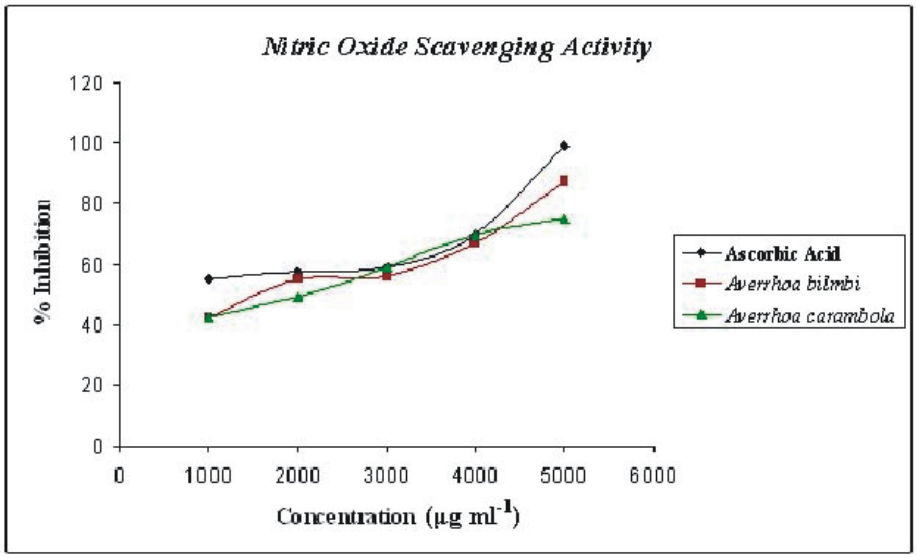

Figure 3: Percentage inhibition of nitric oxide radical of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Table 4: *Nitro Blue Tetrazolium* scavenging activity of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

Concentration ($\mu g\ ml^{-1}$)	% Scavenging activity		
	Ascorbic Acid	*Averrhoa bilmbi* Linn.	*Averrhoa carambola* Linn.
250	25.9	-	-
500	39.3	25.9	33
1000	56.5	50	60
1500	63.6	66.6	77.7
2000	75	71.4	81.8
2500	77.7	75	84.6
	IC_{50} value	**1.25 $\mu g\ ml^{-1}$**	**1.0 $\mu g\ ml^{-1}$**

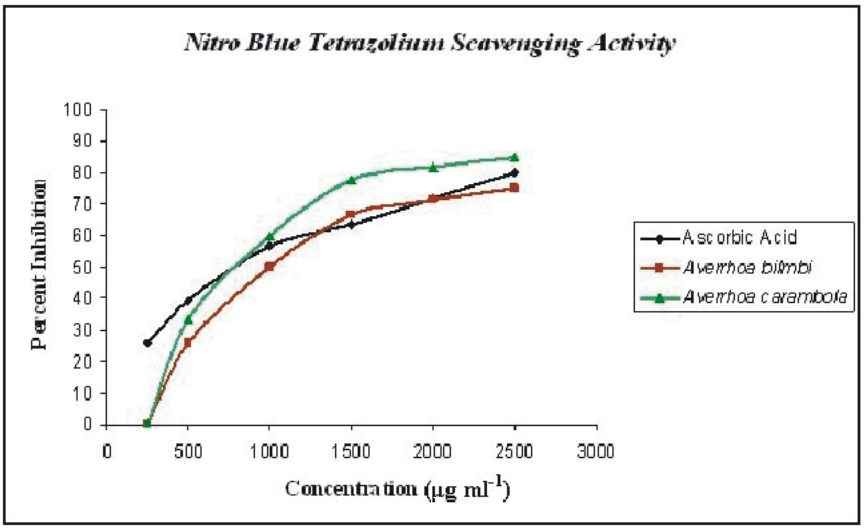

Figure 4: Percentage inhibition of nitro blue tetrazolium radical by *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruit extracts

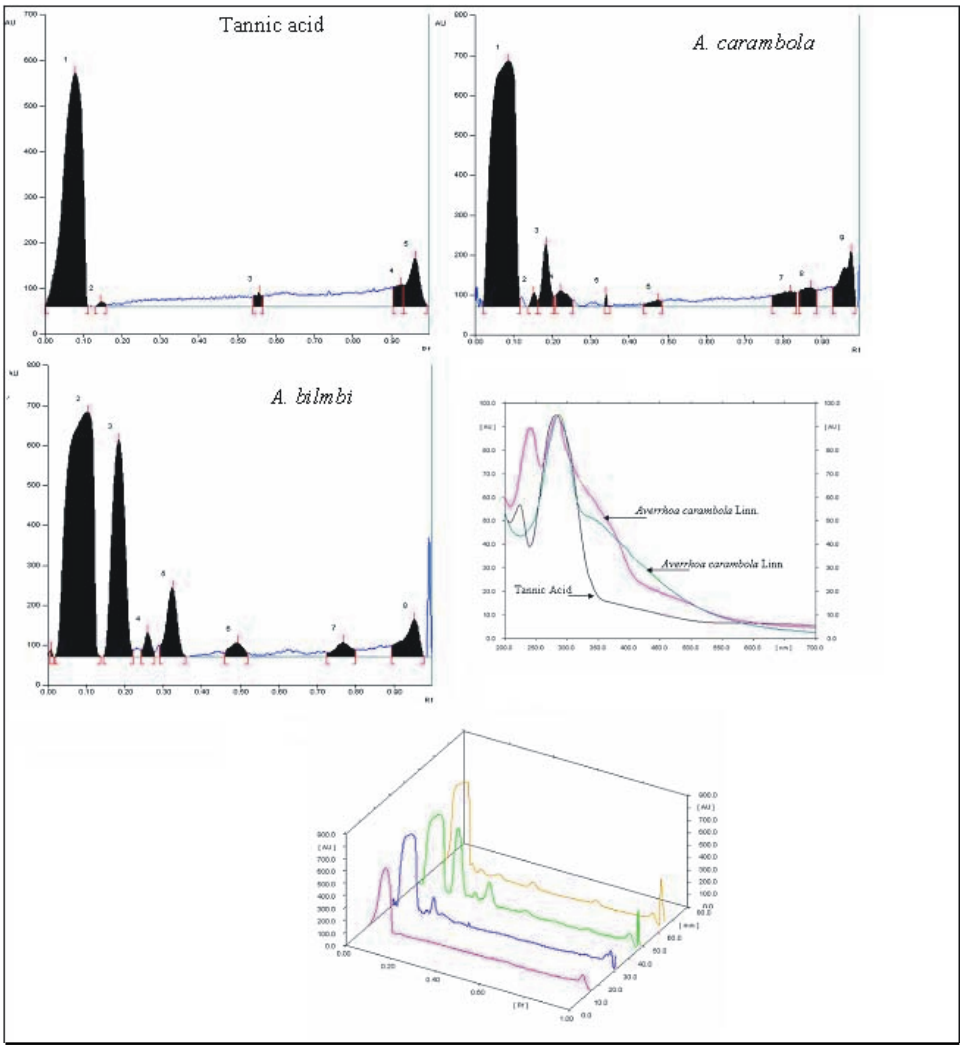

Figure 5: HPTLC fingerprint profile of Tannic Acid with *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. methanolic extract at 254 nm.

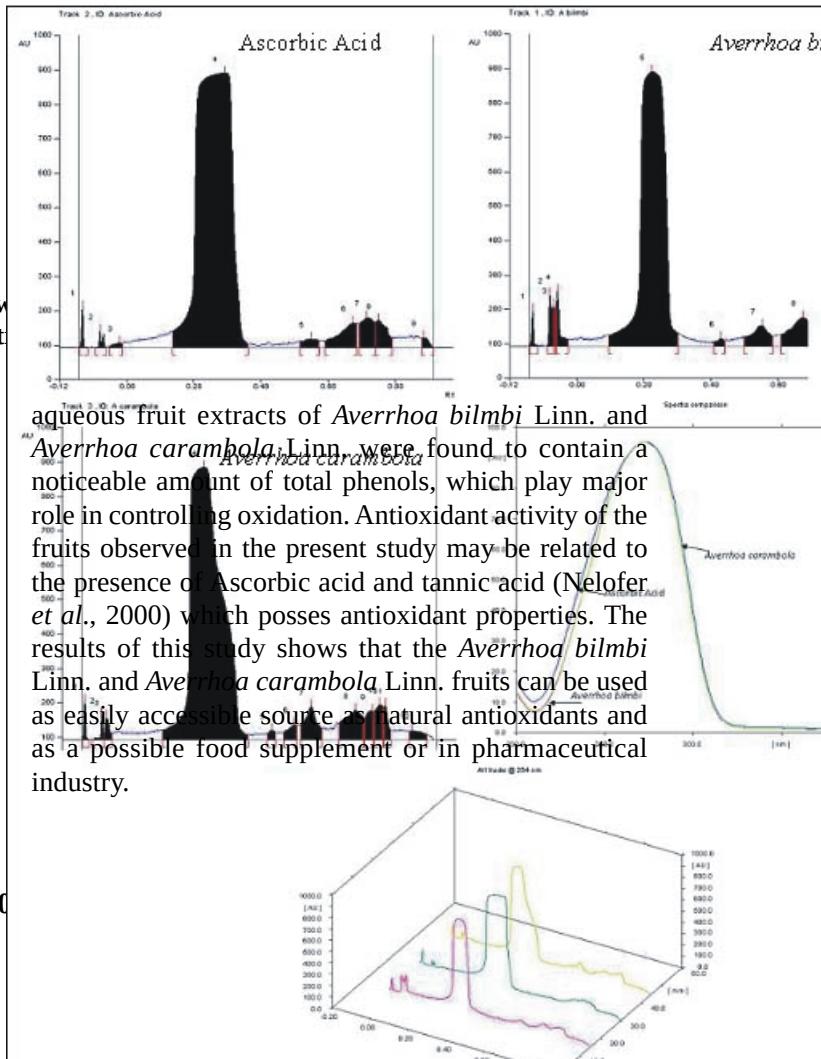

Figure 6: HPTLC fingerprint profile of Ascorbic Acid w
methanolic ext

[7:2:1 (v/v)] and ethyl acetate: methanol: distilled water [4: 2: 1 (v/v)] for ascorbic acid. Spectrum analysis confirms the presence of tannic acid and Ascorbic acid in fruit extract of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. The densitograms and spectrum of standard Tannic acid and Ascorbic acid with ABAE and ACAE are shown in figure 5.

Conclusion

Averrhoa bilmbi Linn. and *Averrhoa carambola* Linn. fruit extracts showed potent antioxidant activity, nitric oxide radicals and reducing power activities when compared with standard Ascorbic acid. In addition, the aqueous fruit extracts of *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. were found to contain a noticeable amount of total phenols, which play major role in controlling oxidation. Antioxidant activity of the fruits observed in the present study may be related to the presence of Ascorbic acid and tannic acid (Nelofer *et al.*, 2000) which posses antioxidant properties. The results of this study shows that the *Averrhoa bilmbi* Linn. and *Averrhoa carambola* Linn. fruits can be used as easily accessible source as natural antioxidants and as a possible food supplement or in pharmaceutical industry.

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