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In vitro Evaluation of Antioxidant Activity of Ripe Fruits of *Averrhoa carambola* L.

Nilofar Yasmin[1, 3] and Kaushal Sood[2*]

[1]Centre for Biotechnology and Bioinformatics, Dibrugarh University, Dibrugarh, Assam- 786004
[2] Department of Zoology, Tinsukia College, Tinsukia, Assam- 786125
[3]National Institute of Pharmaceutical Education & Research (NIPER)- Guwahati, Assam- 781101
[*]Corresponding author: kaushalsood@gmail.com

Abstract

The study evaluated the antioxidant activities of petroleum ether and ethyl acetate extracts of ripe fruits of *A. carambola* by multiple *in vitro* assays- DPPH and ABTS free radical scavenging, Nitric oxide scavenging (NO), Total Antioxidant Capacity (TAC), Iron chelating activity and ferric reducing antioxidant power (FRAP) along with the determination of total phenolic and flavonoid contents. The extracts exhibited scavenging of DPPH and ABTS free radicals and of Nitric Oxide in addition to significant levels of iron chelating activity, ferric reducing power and total antioxidant capacity. The total phenolic and flavonoid contents of the two extracts were found to be significantly different. A positive correlation was observed between the antioxidant activities and the phenolic content of the extracts.

Keywords: *Averrhoa carambola,* Antioxidant Assays, Total Phenolic Content, Total Flavonoid Content, Correlation

Introduction

Plants form the basis of traditional system of medicine that has been in existence for thousands of years across civilizations. Plants continue to be a source of therapeutic molecules and a source of novel bioactive compounds (1). Apart from contributing primary macromolecules like carbohydrates, proteins, fats, vitamins and minerals to the diet, plants also provide secondary metabolites like alkaloids, phenolics, flavonoids that are known to impart different functional activities to food and thus significantly affect human health (2). Metabolic activities in a cell generate various reactive species that interact with the different cellular components and cause the denaturation of lipids, proteins and nucleic acids; consequently leading to oxidative stress (3–5). In order to mitigate the effects of oxidative stress, cells possess an array of endogenous antioxidant mechanisms (6). However, at times the oxidative stress may far exceed the ability of the endogenous system to balance it. Under such circumstances, dietary antioxidants play a pivotal role in maintaining cellular homeostasis.

Averrhoa carambola, commonly known as carambola or star fruit, is a tropical, evergreen, small tree or shrub native to Southeast Asia and Indian Subcontinent. The fruit is reported to be a laxative, antidysenteric, antiphlogistic, febrifuge, antiinflammatory, antispasmodic and is used in hepatic disorders and piles (7). Analysis of polyphenolic antioxidants in star fruit was first reported in 2004 by Shui and Leong (8). Subsequent works have reported *A. carambola* to be rich in phenolics and flavonoids and a potent source of natural antioxidants by several workers (9–14) total phenolic content, total flavonoid, antioxidant capacity, and antioxidant

vitamins of bilimbi (*Averrhoa carambola)*

In the present work, we evaluated the effect of the polarity of the solvent on the extraction of phenolics and flavonoids from the ripe fruits of *A. carambola* and on the antioxidant activity of the extracts. Further, we also elucidated the mechanisms of antioxidant activity and explored its correlation with the contents of phenolics and flavonoids in the extracts. Such information would be of significance while extracting and characterizing the antioxidant constituents of *A. carambola* fruits in future.

Materials and Methods

Chemicals

All solvents and reagents used for extraction and assays were of analytical grade and were obtained from Merck (Mumbai, India) and HiMedia (Mumbai, India). Deionized water used in the study (Sartorius Stedim Arium Water Purification System, Germany).

Plant sample

Ripe fruits of *A. carambola* L. were collected from their natural habitats from the wild. The plant was identified and authenticated by Prof. L.R. Saikia, Department of Life Sciences, Dibrugarh University and voucher specimens were deposited in the department museum.

Preparation of extracts

Fruits were washed and air dried and grinded using a mechanical grinder. The powdered material was soaked in petroleum ether (1:10 w/v) for 48 hours at 25º ± 2 °C. The extract was filtered and concentrated in a rotatory evaporator (IKA RV-10, Germany). The residue was dried and the process was repeated with ethyl acetate. The extracts were evaporated to dryness in pre-weighed beakers and stored aseptically at 4 °C until further use.

Determination of total phenolic content

Folin-Ciocalteu method was used for determining the total phenolic content (15). A 0.5 mL aliquot of the diluted sample (0.50 mg/mL) was mixed with 2.5 mL of 10% Folin-Ciocalteu's reagent and incubated for two minutes followed by addition of 2 mL of 7.5% Na_2CO_3. The solution was then incubated for one hour at room temperature and absorbance was recorded at 765 nm. Gallic acid was used as standard for preparing the calibration curve and the content was expressed as Gallic Acid equivalent (mg of GAE/g of extract).

Determination of total flavonoid content

The total flavonoid content was determined by following the Dowd method (16). Five mL of aluminum trichloride ($AlCl_3$) (2 % w/v) was mixed with an equal volume of the sample solution (0.20 mg/mL in methanol). Absorption was recorded at 415 nm after incubating for 10 minutes. The total flavonoid content was estimated using the standard curve of rutin and was expressed as Rutin Equivalent (mg of RE/ g of extract).

DPPH free radical scavenging activity

The free radical scavenging activity of *A. carambola* L. extracts was determined by DPPH radical scavenging activity (17). DPPH (0.1mM) was mixed with an equal volume of different concentrations of the extracts. A control was prepared with DPPH and methanol (1:1 v/v). After thirty minutes of incubation at room temperature in dark, the absorbance of the control and the tests was measured at 517 nm and percent scavenging of DPPH radical was calculated as follows:

DPPH radical scavenging activity (%) = $[(A_{control} - A_{sample})/ (A_{control})] \times 100$

Where, $A_{control}$ is the absorbance of the control; A_{sample} is the absorbance of the sample/ standard. Ascorbic acid was used as the standard during the experiment. The concentration of an extract required to scavenge 50 % of DPPH free radicals was defined as the IC_{50} value and was expressed in µg/mL of the extract.

ABTS free radical scavenging activity

The ABTS radical scavenging activity of the extracts was determined by the method of Alam et al. (2013) (18). The $ABTS.^{+}$ solution was prepared freshly and diluted appropriately to obtain an absorbance of 0.700 at 734 nm. Different concentrations of plant extracts were mixed with equal volume of the ABTS solution and incubated for 10 minutes. Absorbance was recorded at 734 nm against blank and the ABTS radical scavenging activity of the samples was expressed as percentage:

ABTS radical scavenging activity (%) = $[(A_{control} - A_{sample})/(A_{control})] \times 100$

Where, Acontrol is the absorbance of control; Asample is the absorbance of sample/ standard. Ascorbic acid was used as the standard. The concentration of the plant extract required for achieving 50% inhibition was determined statistically and reported as IC_{50}.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was estimated by the Griess reagent assay (19). Different concentrations of the sample/ standard (0.5 mL) were mixed with 2 mL of 10 mM sodium nitroprusside and 0.5 mL phosphate buffered saline (PBS). The mixture was incubated for 150 min at 25 °C after which, 0.5 mL of the reaction mixture was transferred to another tube and 1 mL sulphanilic acid reagent (0.33% sulphanilic acid in 20% glacial acetic acid) was added to it. After incubating for 5 minutes, Naphthyl ethylene diamine dihydrochloride (0.1%, 1 mL) was added to the reaction mixture and incubated again for 30 minutes. Absorbance was recorded at 540 nm against blank and percentage of scavenging was calculated as follows:

Nitric Oxide Scavenging Activity (%) = $[(A_{control} - A_{sample})/(A_{control})] \times 100$

Where, $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of sample/ standard. Ascorbic acid was used as the standard. Results were expressed as IC_{50} values.

Iron chelating activity

Iron chelating activity was determined by the method described previously (20). A 150 µL aliquot of different concentrations (0.05 mg/ mL- 6.25 mg/mL) of plant extracts was mixed with 1000µL of deionized water and 150µL of FeCl2 solution (0.1mg/mL). After 30 minutes, 200µL of ferrozine solution (40mM) was added. The resulting mixture was incubated for 10 minutes at room temperature and absorbance was recorded at 562 nm. Iron chelating activity was calculated using the following expression:

Inhibition of ferrozine–Fe^{2+} complex formation (%) = $[(A_{control} - A_{sample})/(A_{control})] \times 100$

Where, $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the test reaction. EDTA was used as the standard. Results were expressed as IC_{50} values.

Ferric reducing antioxidant power (FRAP)

The ability of the extracts to reduce Fe^{3+} was assayed by the method described by Benzie and Strain (1996) (21). A 500µL aliquot of sample (40 µg/mL) was mixed with deionized water (500µL) and 1% potassium ferricyanide (50µL). After 20 minutes of incubation at 50°C, 500µL of 10% trichloroacetic acid (w/v) and ferric chloride solution (0.1%, w/v) were added. Change in absorbance of the reaction mixture was monitored at 700 nm. FRAP was calculated with reference to the positive control (BHT; 1000µg/mL).

Total antioxidant capacity

Total antioxidant capacity was determined by the phosphomolybdenum assay (22). The reagent solution was prepared by mixing equal volumes of 0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium

phosphate. An aliquot of 0.3 mL of the sample (500 µg/mL) was added to 2.7 mL reagent solution and incubated at 95°C for 90 minutes. Absorbance was recorded at 765 nm against blank. Ascorbic acid was used as standard in the assay.

Statistical analysis

Replicates were maintained during all experiments. Results were expressed as mean ± sd of triplicates. The significance of differences was tested at α= 0.05. IC_{50} values were determined by curve fitting using SigmaPlot 10.0 (Systat Software, San Jose, CA).

Results and Discussion

The total phenolic and total flavonoid contents of petroleum ether extract of A. carambola (AcPe) and ethyl acetate extract of A. carambola (AcEa) are recorded in Table 1. The total phenolic content (TPC) of AcEa (0.22 ± 0.009 µgGAE/ mg extract) was significantly higher than that of AcPe (0.19 ± 0.008 µg GAE/ mg extract) while the latter recorded significantly higher total flavonoid content (TFC) (0.28 ± 0.011 µg RE/ mg extract) than AcEa (0.25 ± 0.010 µg RE/ mg extract) (Figure 1).

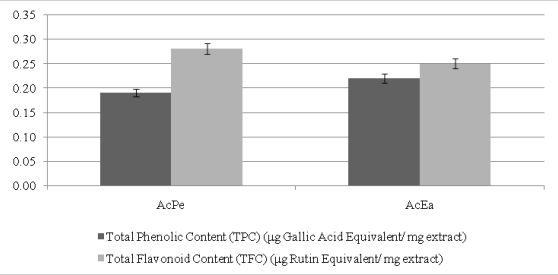

Figure 1. Total Phenolic Content and Total Flavonoid Content of *A. carambola* extracts (AcPe: *A. carambola* Petroleum Ether extract; AcEa: *A. carambola* Ethyl Acetate extract)

The antioxidant activities of the two extracts are recorded in Table 2. AcEa exhibited significantly higher scavenging of DPPH, ABTS and NO free radicals and iron chelation as compared to AcPe. AcEa also recorded significantly higher BHT and ascorbic acid equivalents in the FRAP and TAC assays respectively. The results of the various antioxidant assays were observed to be correlated with the total phenolic and flavonoid contents of the two extracts (Table 3). All the various antioxidant activities exhibited positive correlation with TPC to different degrees of significance. However, all of them showed negative correlation with TFC. Aerobic organisms are constantly exposed to free radicals and other reactive oxygen species (ROS), which are generated as a natural byproduct of the normal essential metabolism of the cell or from other endogenous and exogenous sources. These

Table 1: Total Phenolic Content and Total Flavo-noid Content of *A. carambola* extracts

	Total Phenolic Content (TPC)	Total Flavonoid Content (TFC)
	(µg Gallic Acid Equivalent/ mg extract)	(µg Rutin Equivalent/ mg extract)
AcPe	0.19 ± 0.008	0.28 ± 0.011
AcEa	0.22 ± 0.009	0.25 ± 0.010

are harmful in excess but some level of these is essential for important cellular functions like cell signaling and apoptosis (23). In a cell, the levels of free radicals and ROS are balanced by small molecular weight peptides and cofactors and enzymes like superoxide dismutase and catalase that act as endogenous antioxidants. Their effect are further supplemented by dietary antioxidants like vitamins C and E (24). Since oxidative stress has been shown to contribute to conditions like atherosclerosis, inflammation, certain cancers and ageing (25), antioxidants are believed to counter these and contribute positively to good health. However, extensive use of synthetic antioxidants is speculated to be responsible for liver damage and carcinogenesis (26), which has led to the search for antioxidants from natural sources. Several workers have reported and reviewed the significance of plants as rich sources of natural antioxidants

Table 2: Antioxidant Activities of *A. carambola* extracts

	IC_{50} (µg/mL)				FRAP	TAC
	DPPH	ABTS	NO	Iron Chelating	(µg BHT equivalent)	(µg Ascorbic acid equivalent)
AcPe	4.19 ± 0.17	4.06 ± 0.16	8.70 ± 0.35	130.16 ± 5.21	0.54 ± 0.02	103.56±4.14
AcEa	0.011 ± 0.001	0.94 ± 0.04	0.98 ± 0.04	317.90 ± 12.72	0.96 ± 0.04	206.00±8.24
Standard	4.20 ± 0.01*	0.40 ±0.01*	87.15 ± 4.36*	4.78 ± 0.19#	&	*

* = Ascorbic Acid; # = EDTA; &= BHT

and have characterized several plants (27–29). Plant based natural antioxidants are a complex heterogeneous mixture of phytochemicals that exert their effects through various modes of action. Depending on their chemical structure, these can act as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors and metal ion chelating agents. Hence, in the current study, we used an array of assays to assess the antioxidant potential of the extracts of A. carambola fruits. We observed that the ripe fruit of A. carambola is a source of both non-polar and mildly polar antioxidants. This is evident from the responses of petroleum ether and ethyl acetate extracts during the various antioxidant assays used in the study. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical with a deep violet color which has absorption maxima at 517 nm. It is known that when a solution of DPPH is mixed with that of an antioxidant (AH) which can donate a hydrogen atom, DPPH free radical is reduced resulting in the loss of the violet color. In order to evaluate the antioxidant potential by the free radical scavenging assay, the reduction in absorption of DPPH solution is monitored at 517 nm (20). Similarly, the ABTS assay is based on interaction between the antioxidant and ABTS radical cations (ABTS•+) which has a characteristic absorption maxima at 645, 734 and 815 nm (30). The assay measures the ability of a hydrogen-donating antioxidant to scavenge the ABTS•+ and is indicated by the reduction of the blue-green color of ABTS•+ to colorless during the reaction (31). A. carambola fruit extracts were able to scavenge both DPPH and ABTS free radicals, thus suggesting their ability to neutralize these free radicals by donating hydrogen atoms.

Our findings are in agreement with other works that have reported plant extracts as potent free radical scavengers (32–35). Nitric oxide (NO) is a signaling molecule which is derived by both enzymatic and non-enzymatic pathways in the human body (36). It is involved in various biological functions and either over- or under- production of NO can have implications on health; which can be modulated through dietary factors (36). During the assay, NO was generated, in vitro, from aqueous sodium nitroprusside, which on interaction with oxygen produced nitrite ions that could be estimated using Griess reagent (19). Presence

Table 3: Correlation of Antioxidant Activities with Total Phenolic Content and Total Flavonoid Content of A. carambola extracts

Antioxidant activity	Correlation Coefficient (R^2)	
	TPC	TFC
DPPH	0.869*	-0.898*
ABTS	0.881*	-0.892*
NO	0.888*	-0.887*
Iron Chelating	0.937**	-0.827*
FRAP	0.950**	-0.805 NS
TAC	0.944**	-0.815*

TPC: total phenolic content; TFC: total flavonoid content

* = difference significant at $p < 0.05$, ** = difference significant at $p < 0.01$ and *** = difference significant at $p < 0.001$

of A. carambola extract in the reaction mixture scavenged NO by competing with oxygen, thus leading to reduced production of nitrite ions. Our findings are in agreement with those for the antioxidant activities of A. carambola and A. bilimbi (8,12,37–40). Metal ions are essential for several fundamental biological processes. Trace amounts of metal ions are required for enzyme-catalysed reactions and other metabolic processes (41). However, in excess, these may accumulate and prove toxic to cells (42). Redox-active metals like Fe2+ and Fe3+ can initiate Fenton reaction which leads to the production of ROS that ultimately contribute to oxidative stress (42). Ferrozine chelates Fe2+ with the formation of a red-coloured complex. This reaction is restricted in the presence of other chelating agents, like plant extracts, and results in a decrease of the red colour due to the ferrozine-Fe2+ complexes. Measurement of the reduction in colour is utilised to determine the iron chelating ability of the samples (43). Our results demonstrated that A. carambola fruit extracts exhibited significant iron chelating activity which showed correlation with total phenolic content. Previous studies have also reported similar observations for plant based antioxidants (42,44,45). The FRAP assay measures the capacity of antioxidants to reduce ferric iron. The assay involves the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4- diene chloride (TPTZ) to the ferrous form at low pH. This reaction is monitored at 593 nm to measure the reduction in absorption (18,21). A. carambola fruit exhibited ability to reduce ferric-tripyridyltriazine complex (Fe3+-TPTZ) which showed positive correlation with phenolic content. Our results are in agreement with previous studies which have demonstrated that plant polyphenols are powerful antioxidants (42). The total antioxidant capacity assay or the phosphomolybdate antioxidant power is a spectroscopic method for the quantitative determination of the overall antioxidant capacity of the samples. In the presence of an antioxidant, Mo (VI) is reduced to Mo (V) with the subsequent formation of a green phosphate Mo (V) complex with absorption maxima at 700 nm (18). The extracts of the fruit of A. carambola exhibited significant levels of total antioxidant capacity which showed strong correlation with phenolic content. During our investigation it was observed that the total flavonoid content was always higher than the total phenolic content of the extracts. However, there was a strong positive correlation between the antioxidant activities and the total phenolic content of A. carambola fruit. Thus, it may be suggested that the principal antioxidant component may be phenolic in chemical nature. Our findings are in agreement with several other studies that attributed the antioxidant properties of plant extracts to their high total phenolic content (20). Previous works on A. carambola have reported the antimicrobial activity, phytochemical constituents, polyphenolic content, polyphenolic antioxidants and DNA protective activity of fruits (8,11–13,39,46–48). Secondary metabolites from the bark of A. carambola have been reported for alpha-glucosidae, tyrosinase, elastase and antioxidant potential (49). However, detailed analysis of antioxidant activity of the ripe fruit of A. carambola and plausible modes of action was not available in literature. Our investigation, to the best of our knowledge, appears to be the first to investigate the effect of the kind solvent on the extraction of antioxidants from A. carambola fruits and to explore the correlation of antioxidant activity with phenolic and flavonoid contents. The results show that the ripe fruits of A. carambola effectively counteracted oxidative species through multiple mechanisms.

Conclusions

The present study reported the phenolic and flavonoid contents of *A. carambola* fruit and deciphered the different modes of action through which the fruit extracts exerted antioxidant activity. Further, the dependence of antioxidant activity on the phenolic and flavonoid contents was also contemplated. In future studies, experiments utilizing *in vivo* models may provide deeper insight to the significance and

mechanism of antioxidant activity in biological systems. To the best of our knowledge, our study is the first to report the antioxidant activity of *A. carambola* ripe fruit with plausible modes of action and suggest its association with its phenolic content.

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