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Research article

EFFECT OF TRADITIONAL SUN DRYING ON INDIGENOUS STAR FRUIT (*AVERRHOA CARAMBOLA*) FROM INDIA

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ABSTRACT

Objective: The objective of the study was to analyse the effect of traditional sun drying on the antioxidant activity of *Averrhoa carambola* L using standard antioxidant assays and analytical techniques.
Materials and Methods: Star fruit was shade dried and its aqueous acetone extract (SD) prepared. The antioxidant activity of aqueous acetone extract of fresh star fruit (SF) was compared with SD using assays like total phenolic content, DPPH free radical scavenging activity, $ABTS^{\cdot+}$ free radical scavenging activity, ferric reducing assay, phosphomolybdenum assay and metal chelating activity. HPLC-ESI-MS and FT-IR was used to identify the phytochemicals in SD and SF.
Results: TPC of SD and SF was 1.0 and 2.7gGAE/100g extract respectively. IC_{50} of SD and SF in DPPH RSA was 100 and 150µg/ml while TEAC of SD and SF were 1.07 and 0.3 respectively. Antioxidant capacity of SD and SF was 392.5 and 113.75mmolesAAE/g extract. IC_{50} of SD was 147 ppm and that of SF, 123 ppm in TRP. The MCA of SD increased with increasing dose while SF showed no significant increase in activity. HPLC-ESI-MS and FT-IR enabled identification of three possible phytochemicals namely protocatechuic acid trimer (**1**) and sinpaic acid teramer (**2**) which contributed significantly to the distinct behaviour of both the extracts towards the different assays.
Conclusion: The antioxidant activities of SF and SD behaved variedly with different assays which was contributed by the bioactive compounds present in both extracts. Clearly, the shade drying process effected the antioxidant potential of star fruit.

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INTRODUCTION

Natural antioxidant defence mechanisms in aerobic organisms, including human beings, protect against oxidative damage, frequent damage removal and possess repair enzymes to eliminate or repair damaged molecules [1,2]. However, these natural defence mechanisms can be inefficient [3]. The importance of fruits and vegetables in our diet is increasingly growing as one of the main sources of antioxidants and reported to be beneficial to age related diseases, cancers, inflammation, heart disease and acceleration of the ageing process [4-8].

These beneficial effects of consumption of fruits and vegetables are attributed to various antioxidants present in them such as phenolics, thiols, ascorbic acid, tocopherols and carotenoids [8,9,10,11]. Scavenging of radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen have been attributed to antioxidants [4, 8, 9,12,13]. Hence, increased consumption of tropical fruits is being recommended by various health advocates for maintaining good health [4, 14, 15].

Star fruit (*Averrhoa carambola* L.*)* is a tree fruit with a distinctive star shape in cross section and commonly known as carambola. The edible fruit of the *Oxalidaceae* family is available locally in various parts of India and predominantly eaten fresh as a vegetable and processed to various products like pickles, jam or jelly. The powdered seed concoction of the fruit is traditionally used for its medicinal properties to treat hemorrhoids, fever, eczema, diarrhoea and asthma [16,17]. Extensive reports on the strong free radical scavenging potential of juice and residue of star fruit cultivated in Singapore and Indonesis have attributed its capacity to the rich procyanidin polymers and β- carotene [18, 19]. The effect of drying methods on its bioactivity however, has not been investigated elaborately. The main objective of the present study was to appraise the effect of traditional sun- drying on the selected fruits available locally in the markets of Mysore, India by assessing its antioxidant activity. Two of the bioactive compounds in fruit extracts using LC-MS and FT-IR were also identified for the first time.

MATERIALS AND METHODS

Plant Material and drying

Fresh Star fruit samples were collected from the local fruit markets in Mysore, India during the months between May and June. Care was taken that the yellowish ripe fruits were not overripe, spoilt or damaged and approximately 10.0cm in size. 5 kgs of chopped fruits of uniform size were subjected to naturalsun drying under shade for 4 days. The ambient temperature during sun drying varied between 28^{o}C to 34^{o}C, and humidity ranged between 48- 52% respectively. The dried fruits were ground in a blender to give 500g of fine powder.

Chemicals and reagents

2, 2′- Azino- bis- (3- ethylbenzothiazoline- 6- sulfonic acid) (ABTS), Rutin (quercetin- 3- rutinoside) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma (Mumbai, India). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 6, Dichlorophenol indophenol (DIP), Ethylenediaminetetraacetic acid (EDTA), ferrozine, potassium persulphate and Butylated Hydroxyanisole (BHA) were obtained from Himedia (Mumbai, India). Ferric chloride, Gallic Acid, Acetone and Methanol were obtained from Merck (India). The other chemicals included L- ascorbic acid (Loba, India), Folin- Ciocalteau reagent, ferrous chloride (Rolex, India), and potassium ferricyanide (Nice, India). All other chemicals used were of analytical grade.

Extraction

The edible portion of fresh star fruits were chopped and used to prepare fresh star fruit extract (SF) while the dried star fruit powder was weighed and extracted to prepare dried star fruit extract (SD). The extraction procedure was followed according to the conditions described by Yap, Ho, Wan Aida [42], Chan, Lee, and Leong [20] with slight modification of the temperature. 200g of the dried fruit powder and 2000g of the fresh fruit were soaked in 500ml and 5000ml, of 60% aqueous acetone for 3 hours under continuous agitation at room temperature (approximately 30°C) respectively. The filtrates were collected using a muslin cloth and subjected to concentration and made solvent free in a rotory vacuum evaporator (Buchi, Germany). The aqueous portion was dried in a hot air- oven at 50ºC. The dried residue of both the dried and fresh fruit extracts was collected and used for the antioxidant assays.

Total phenolic content (TPC)

Total phenolic content of fruit extracts was estimated using the Folin- Ciocalteau's method [21]. Gallic acid was used as the standard reference wherein the total phenolic content (TPC) of the extracts was expressed as mg gallic acid equivalent (GAE)/100g extract.

2, 2- Diphenyl, 1- PicrylHydrazyl free radicalscavenging activity

Fruit extracts were evaluated for free radical scavenging effect on DPPH radical using BHA as standard [22]. To 1.0ml of sample (50- 200ppm), 1.0ml of 200µM methanolic DPPH solution was added and incubated at room temperature for 20 minutes after vigorous shaking. The absorbance was measured at 517nm using a spectrophotometer (Shimadzu UV1800, UV Spectrophotometer, Japan). The percentage DPPH radical scavenging activity was calculated using the following formula:

$$\% \text{ RadicalScavengingActivity} = (A_{control} - A_{test} / \text{Acontrol}) \times 100$$

where

$A_{control}$ → Absorbance of control

A_{test} → Absorbance of test sample.

The total antioxidant activity was expressed in IC_{50} values which is the concentration of the extract necessary to decrease the initial concentration of DPPH by 50% under the experimental conditions. The assay was conducted in three independent experiments.

Reducing Power

Reducing power of SD and SF was evaluated using the protocol described by Yen and Duh [23]. The reaction mixture of this assay comprised of the extracts and BHA in different concentrations (15-270ppm), 0.5ml of phosphate buffer (6.6 pH, 0.2 M) and 0.5 ml of 10% potassium ferricyanide. It was incubated at 50°C for 20 minutes and the reaction was ceased by adding 0.5 ml of 10% trichloroacetic acid. 1.5 ml of water and 0.3ml of 0.1% ferric chloride solution were added producing a green coloured ferri- ferro complex. The absorbance was measured at 700nm wherein increasing absorbance of the reaction mixture indicated the increase in the reducing power. The experiment was conducted in triplicates.

2, 2´- Azino- bis- (3- ethylbenzothiazoline- 6- sulfonic acid) ($ABTS^{\cdot+}$) free radical scavenging assay

The $ABTS^{\cdot+}$ radical scavenging activity was estimated as per the method described by Loganayaki and Manian [24]. 7mM ABTS solution was mixed with 2.45mM potassium persulphate and left in the dark at room temperature for 12- 15 hours. This was carried out in order to oxidize ABTS by the action of potassium to produce the $ABTS^{\cdot+}$ radicals. After consistent absorbance of the $ABTS^{\cdot+}$ free radical solution at 734nm, the solution was diluted till the absorbance measured was 0.7± 0.02. To 1.0ml of the $ABTS^{\cdot+}$ solution, 0.1 ml of the test samples or standard Trolox (100-400ppm) were added and incubated at room temperature for 10 minutes. The $ABTS^{\cdot+}$ solution without sample was used as negative control. The absorbance was measured at 734nm using water as blank. The percentage radical scavenging activity of the samples (fruit extracts and Trolox) was calculated by the following formula:

$$\% \text{ RadicalScavenging Activity} = (A_{control} - A_{test} / A_{control}) \times 100$$

where

$A_{control}$ → Absorbance of control

A_{test} → Absorbance of test sample.

The percentage $ABTS^{\cdot+}$ radical scavenging activity of the extracts was plotted as a function of the concentration of the sample and standard Trolox. The Trolox equivalent antioxidant capacity (TEAC) was measured by dividingthe gradient of the sample plot with the gradient of the Trolox plot (Hazra, Biswas and Mandal, 2008). The experiment was conducted in triplicates.

Total Antioxidant activity by phosphomolybdenum method

The total antioxidant capacity of fresh and dry fruit extract was evaluated by the method of Prieto et al. [25] as described in Abdille et al. [26]. The absorbance of the mixture was measured at 695nm against blank using a spectrophotometer. The water soluble antioxidant capacity was expressed as equivalents of ascorbic acid (mmole/g of extract) and compared with BHA, rutin and gallic acid as standards. The assay was conducted in three independent experiments.

Ferrous ion chelating ability

The chelating ability of the extract measures how effective the compounds in it can compete with ferrozine for ferrous ion. The method described by Decker and Welch [27] was used with slight modification. To the various concentrations of the extracts (100ppm- 500ppm), 0.05ml of ferrous chloride was added followed by 0.2ml of 5mM ferrozine. The reaction mixture was incubated at room temperature for 10 minutes.

The absorbance of the reaction mixtures was measured at 532nm. Ethylenediaminetetraacetic acid (EDTA) was used as the standard chelator and the ferro- ferrozine complex solution was used as control. A graph of absorbance versus fruit extract concentration was plotted to observe the metal chelating ability. The assay was conducted in three different experiments.

HPLC- ESI- MS analyses

The Thermo LCQ Deca XP MAX ion trap mass spectrometer (USA) with software Xcalibur equipped with Thermo Finnigan Surveyor HPLC was used to separate and identify the probable bioactive compounds present in the fresh and dried star fruit extracts. The heated capillary and spray voltage were maintained at a temperature of 275 ºC and 4.5kV. Nitrogen is operated at 40psi for sheath gas flow rate and 26psi for auxillary/sheath gas flow rate. The full scan mass spectra from *m/z* 50-2000 were acquired in positive ion mode with a scan speed of 1s per scan. Themass spectrometry was performed using helium as collision gas, operated at 0.1mtorr.

Chromatographic separations were done using a BDS HYPERSIL C-18 column (250 X 4.6mm, 5µm particle size) equipped with PDA/ UV detector with 280nm as the detecting wavelength in room temperature (27º C) under the following conditions: 200µl min^{-1}; solvent A, methanol; solvent B, 0.1% formic acid in water starting from 0-20minutes (40- 52% A), 20- 40min (52- 80% A) and 40-60min (80% A). Methanol was considered in the mobile phase as it is an H- bonding organic modifier which forms various degrees of H- bond with the phenolic compounds in various structures and stereo configuration resulting in longer retention time and resolution, whereas formic acid was added to the mobile phase to enhance the ionization of interested compound.

Fourrier Transform Infrared Spectroscopy

IR spectra was recorded in KBr pellet on a Jasco FT/IR- 5300 spectrometer in spectral range 400-4000cm^{-1}.

Statistical Analysis

All experiments were conducted in triplicates and repeated in three independent sets of experiments. Data is shown as mean ± standard deviation (SD). Correlation analytical data was obtained using the software Origin version 5.0.

RESULTS

Drying yields

Fresh ripe fruits on sun drying for 4 days gave a 10-12% yield of dry fruits indicating high moisture content (88-90%) of fresh fruits.

Total Phenolic Content

Highly significant TPC was recorded with both freshly used fruits and dried fruit extracts.TPC of SF was found to be 2.3 ± 0.07 g GAE /100g extract and of SD 1.0 ± 0.07 gGAE/100g extract ($p<0.05$, Table 1).

DPPH assay and reducing power

The bleaching of purple coloured DPPH solution expressed as percent radical scavenging activity increased with increasing dose of SF and SD. The IC_{50} value of SD was 147 ppm while that of SF was 123 ppm ($p< 0.05$.Table 1). The radical scavenging activities, however was much lower than the positive controls gallic acid, rutin and BHA (27, 31 and 28ppm). The ferric reducing activity was dose dependent and was observed to be more efficient by SF which exhibited an absorbance of 1.658 as compared to 0.633 of SD at 300ppm ($p< 0.05$, Fig 2). BHA, however, expressed much higher ferric reducing power at absorbance of 2.5.

Antioxidant activity by $ABTS^{\bullet+}$ and phosphomolybdenum methods

The Trolox Equivalent Antioxidant Capacity (TEAC) of SD and SF was 1.05± 0.005 and 0.37± 0.023 respectively ($p< 0.05$, Table 1) while the TAA of both extracts in the form of significant reduction effects of Mo(VI) to Mo(V) were 113 and 392 mMAAE/g extract (Table. 1) For SF and SD respectively. The antioxidant activity of SD was on par with positive controls gallic acid and BHA at 304 and 314mMAAE/g extract, respectively, while the positive control Rutin showed much higher activity at 508mMAAE/g.

Ferrous ion chelating ability

Using the assay, both SD and SF extracts were studied for their ferrous ion chelating ability at concentrations of 100ppm, 200ppm and 400ppm and results are summarised in Fig 2 ($p< 0.05$). The metal chelating activity of SD increased with increasing dosage while no significant chelating activity of SF was observed despite increase in dose.

HPLC- ESI- MS

Two metabolite peaks were identified and labelled as compound 1 and 2 as they were observed in both the fresh and dried extracts. Compound 1eluted at 10.58 (Fig. 3(a)) and11.15 minutes (Fig. 4(a)) for SD and SF respectively showing a molecular $[M+H]^+$ ion at *m/z* 472 (Fig. 3(b), Table 2). Collision- induced dissociation of molecular ions and daughter ions were characteristic of protocatechuic acid trimer giving daughter ions *m/z* 315, 157 and 111. [41] (Figure 3(b), 4(b) and Table 2). Compound 2 of SF and SD eluted at 13.90 (Fig. 3A) and 13.74 minutes (Fig. 4(a)). The $[M+H]^+$ molecular ion of which was *m/z* 905 and daughter ions (Fig-3(c), 4(c), Table. 2) were *m/z* 679, 453 and 226 due to loss of monomeric units of sinapic acid indicating the prevalence of sinapic acid tetramer. The monomeric unit of sinapic acid was confirmed through its characteristic fragmentation at *m/z* 212 and 195 [28].

FT-IR

The vibration spectral analysis was carried out by comparing the experimental spectra obtained with that reported in literature. The study showed characteristic bands of protocatechuic acid and sinapic acid [20, 29]. Characteristic strong bands at 1674 due to C=O stretch of α, β- unsaturated acid, 1529 and 943 cm^{-1} of protocatechuic acid were observed. The IR carbonyl stretch (C=O) of carboxylic acid of sinapic acid showed up in the region of 1650- 1700 cm^{-1}, whereas phenols reported to absorb strongly in the region of 3400- 3700 cm^{-1} were observed. Vibration bands at 3311 cm^{-1} characteristic to sinapic acid were observed. The C-H stretching band of methyl part of OCH_3 group of sinapic acid was observed at 2925cm^{-1} which could be assigned to the non- symmetric C-H stretching vibration.

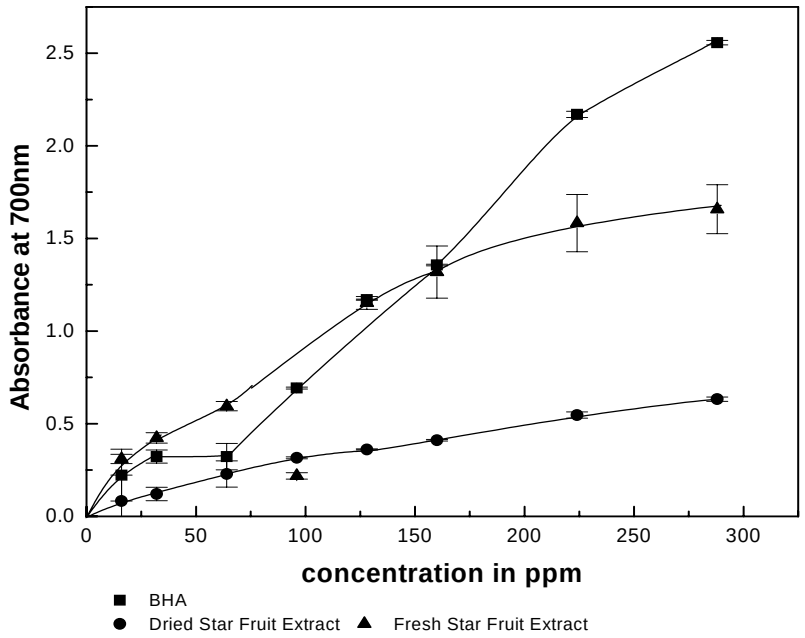


Figure-1. Total reducing power of SD and SFusing BHA as standard.

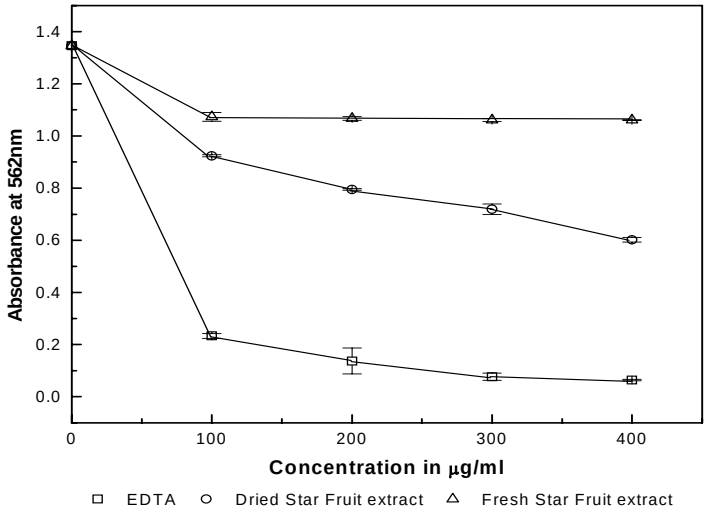
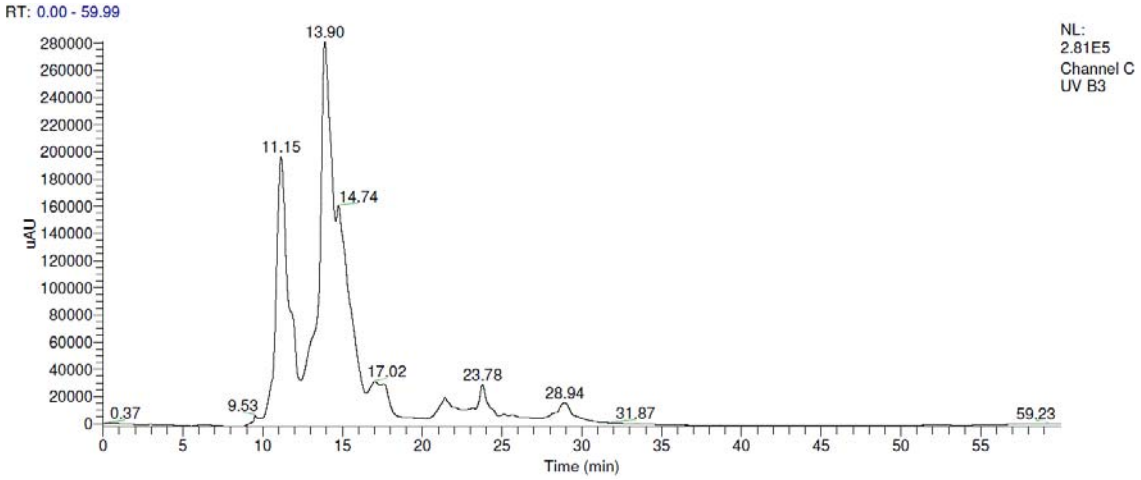
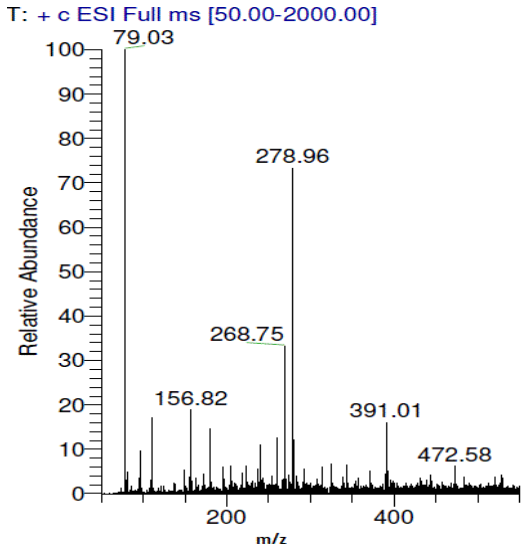


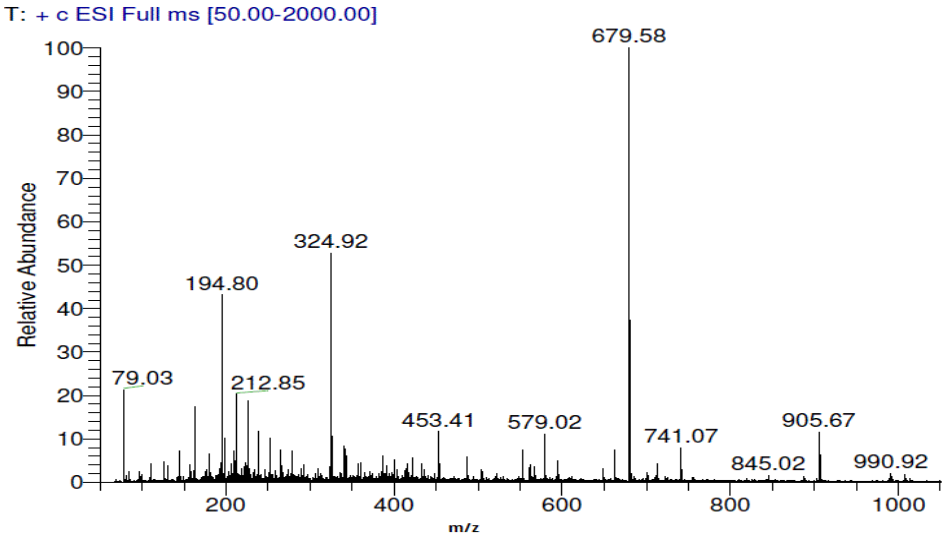
Figure 2. Ferrous ion chelating ability of SD and SF with EDTA as standard.



(a)

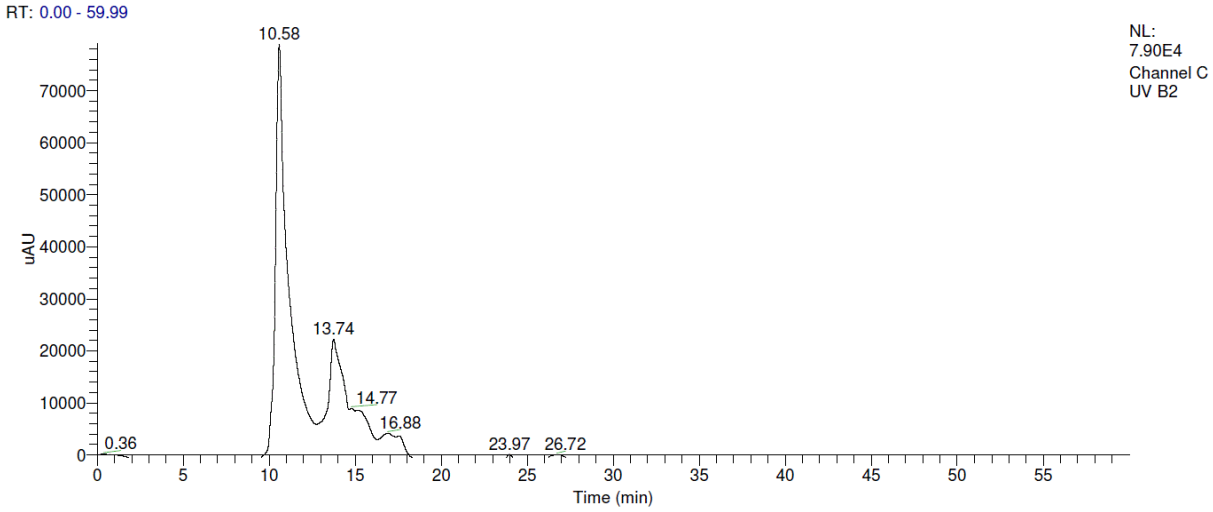


(b)

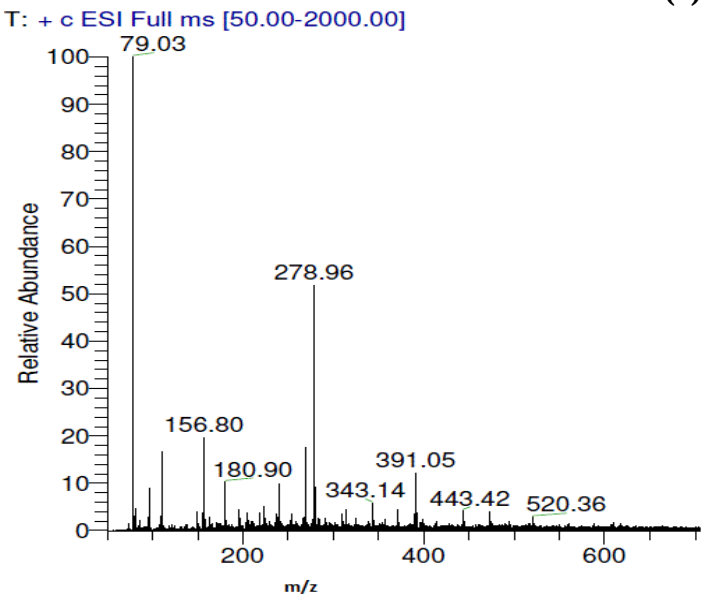


(c)

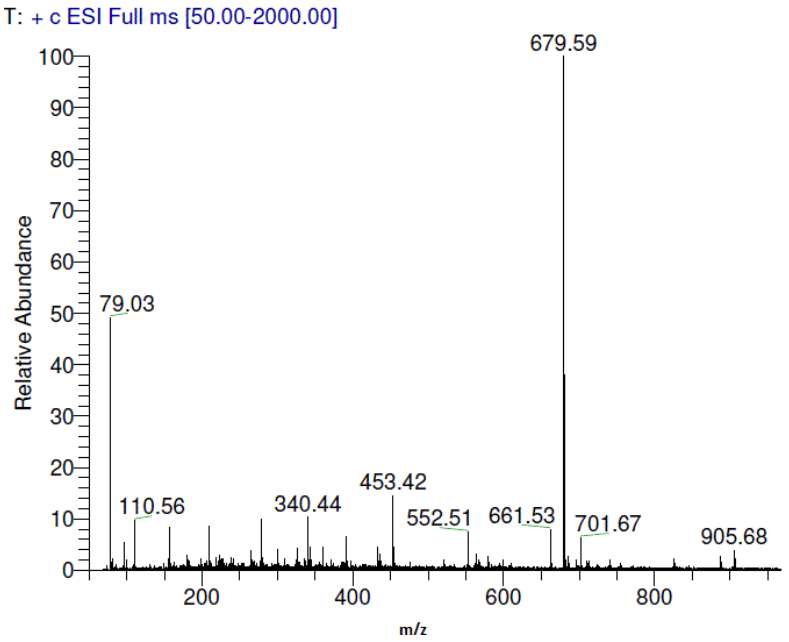
Figure-3. (a) HPLC chromatogram of the SD and ESI- MS of (b) Compound 1 and (c) Compound 2



(a)

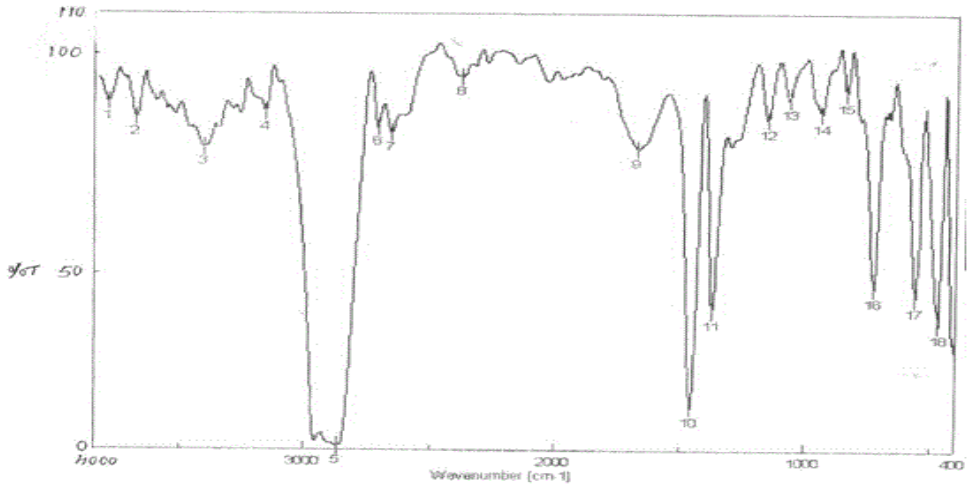


(b)

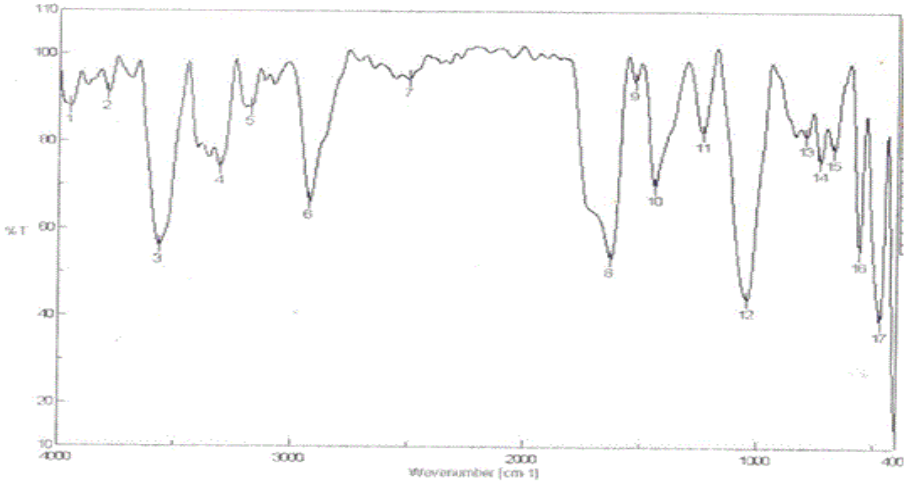


(c)

Figure-4. (a) HPLC chromatogram of the SD and ESI-MS of (b) Compound 1 and (c) Compound 2



(a)



(b)

Figure-5 (a) Infrared Spectra of SD and (b) Infrared Spectra of SF

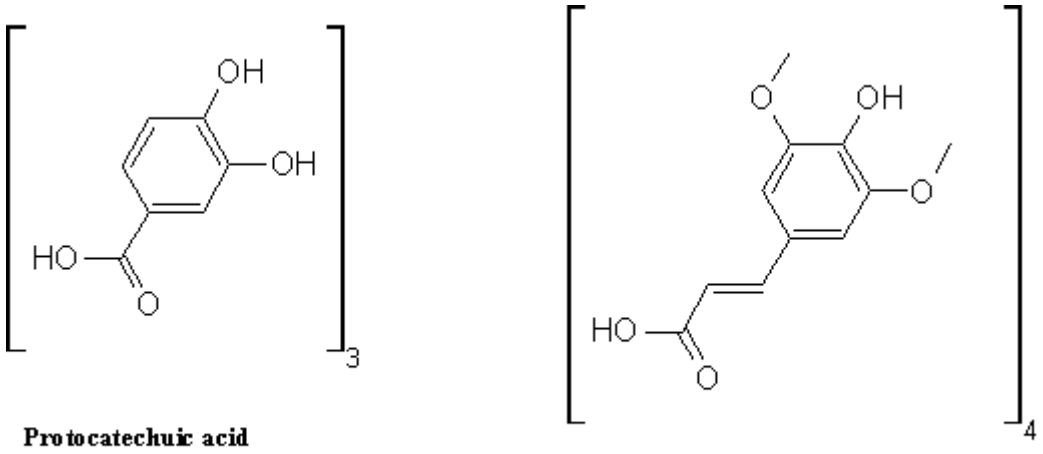


Figure-6. Structure of (a) protocatechuic acid, (b) Sinapic acid

Table 1. Table showing the Total Phenolic Content, IC_{50} of DPPH Radical Scavenging Activity, Trolox Equivalent Antioxidant Capacity and Antioxidant Capacity using Phosphomolybdenum method of extract of fresh and dried star fruit.

Extract	TPC (GAE g/100g extract)	IC_{50} (DPPH RSA) (μg/ml)	TEAC	Antioxidant Capacity (m moles AA equivalents/g extract)
SF	2.3 ± 0.7	100 ± 6.2	0.37± 0.023	113.75±12.37
SD	1.0 ± 0.7	150 ± 3.4	1.05± 0.005	392.5±3.535

Table 2. LC-MS/MS spectral information of the 2 compounds identified in SD and SF

Compound Names	RT (min)		$[M+H]^+$ (Frag. $MS^2 m/z$)
	SD	SF	
Protocatechuic acid trimer	10.58	11.15	472 (514, 156, 110)
Sinapic acid tetramer	13.90	13.74	905 (679, 453, 226, 212, 195)

DISCUSSION

Fruit extracts rich in phenolic compounds is a major area of health and medical- related research due to their potent antioxidant properties marking effects in prevention of various oxidative stress associated diseases such as cancer, cardiovascular diseases, inflammation, neurodegenerative disease etc and their general involvement in defence against aggression by pathogens, parasite and predator. Thus the total phenolic content of the fruit extracts serves as an effective indicator of the overall antioxidant potential of the fruit. Successful extraction of these compounds depends on various factors like the type of solvent, extraction time and temperature, sample-to-solvent ratio and physical characteristics of the sample [30]. In particular, aqueous acetone is generally found to be more efficient in the extraction of higher molecular weight polyphenols like flavanol polymers [31]. Acetone was the preferred choice because of its polar nature, volatility, miscibility with polar and non- polar solvents and relatively low toxicity [32]. This constituent along with the rest of the extraction criteria was efficient in obtaining distinct yield of phenolics from the fresh star fruit. The TPC however dropped by 57% in the sun- dried star fruit, an observation supported by the LC-MS chromatogram wherein several unidentified metabolite peaks were not observed in SD as in SF.

It could be due to the decomposition of phenolics exposed to direct sunlight [33, 34] or the extraction method for SD was less efficient than that for SF. A more efficient condition may be developed to extract maximum phenolics from the dried fruit. The Folin-Ciocalteau antioxidant capacity assay is also considered as an assay to analyse the antioxidant capacity because of its basic mechanism of oxidation/ reduction reaction [30] which depends on the electron transfer in alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complex to form the characteristic blue complexes ($[PMoW_{11}O_{40}]^{4-}$) [35,36]. The DPPH assay is based on the reduction of the purple DPPH radical, which is a stable nitrogen radical, to yellow coloured diphenyl picrylhydrazine in the presence of hydrogen donor. The higher DPPH radical scavenging activity of a compound, the lower is its IC_{50} value. SD showed approximately 33% lesser DPPH Scavenging activity than SF. Sinapic acid, among the hydroxyl cinnamic acids is considered as a strong $DPPH^{\cdot}$ scavenger right after caffeic acid [40]. This compound was predominantly present in SF and SD suggesting their radical scavenging potential. A similar result was observed in ferric reducing assay, which measures ferric- to ferrous reduction capacity through electron transfer of water soluble antioxidants in acidic pH. SF expressed three fold more reducing power than SD. In contrast to the previous assays, SD expressed at least three folds higher antioxidant activity compared to SF in the $ABTS^{\cdot+}$ radical scavenging activity and phosphomolybdenum reducing activity. The electron transfer ability in $ABTS^{\cdot+}$ radical scavenging activity and phosphomolybdenum reducing activity can be attributed to compound 1, protocatechuic acid trimer, which was the major compound in SD. The metal chelating activity is a model to assess the ability of an antioxidant to inhibit Cu^{2+}- catalyst LDL oxidation- a critical point considered in the development of atherosclerosis in the human body- through metal chelation [37]. It was observed that sinapic acid significantly reduced the Cu^{2+}- catalysed LDL oxidation representing a strong metal chelating activity of the hydroxyl cinnamate [38]. Protocatechuic acid was reported to show two to three fold more effective antioxidant activity compared to standard antioxidant, Trolox using various scavenging models like DPPH, ABTS, Ferric reducing power, Ferrous chelating ability among other assays [39]. The report suggested their contribution to SD expressing better metal chelating activity than SF. HPLC coupled with mass spectrometry is being widely used to identify phenolic compounds, and ESI mass spectrometric liquid interface is thought to provide advantages in terms of sensitivity and capability to analyze large, thermally labile and highly polar compounds [7]. Mass spectral analysis of both the extracts highlighted the prominence of the two compounds while exhibiting loss of compounds in SD. This explained the distinct behaviours of SF and SD in different *in vitro* radical scavenging assay models conducted in this study. It is reported that the number of free hydroxyl groups in the molecule enhances the reduction activity owing to steric hindrance [30]. The mass spectral analysis elucidated the structures of probable compounds in SF and SD while IR analysis identified their characteristic functional groups and conformation. An NMR investigation, however, can further confirm their structural conformation.

CONCLUSION

The traditional method of sun drying of star fruit (*Averroha carambola* L.) under shade appears to affect the composition of antioxidant constituents of fruit. It may be aiding the enzymatic degradation of phenolics resulting in lower TPC in shade dried fruit extract or a more eficient extraction method to extrct the phenolic in SD has to be formulated. The distinct behaviour of SD and SF towards various radical scavenging assays (DPPH, reducing power, $ABTS^{\cdot+}$ and phosphomolybdenum assays) suggested the antioxidant components present in both extracts to be different. This was supported by the LC-MS and IR analysis. Antioxidant compounds which scavenge different free radicals in different systems could be important for food and other pharmaceutical applications. Further studies of their isolation and characterisation are underway.

ACKNOWLEDGEMENT

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The authors declare that there is no conflict of interest.

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