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Research article

EFFECT OF TRADITIONAL SUN-DRYING ON PHENOLIC ANTIOXIDANTS OF *AVERRHOA BILIMBI* L.

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ABSTRACT: *Averrhoa bilimbi* L. has various beneficial properties including antidiabetic and antioxidant activity. The effect of traditional sun drying on the stability of fresh *A. bilimbi* fruits was investigated by using different methanol/water extracts, and their total phenolic content (TPC) and total antioxidant capacity (TAC) compared. The TAC was evaluated using established *in vitro* models such as 1,1,diphenyl-2-picryl hydrazyl radical scavenging activity, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) scavenging assay, total reducing power, phosphomolybdenum assay and metal chelating activity. All the extracts of the dried fruit showed lower TPC compared to the fresh bilimbi extracts by 23-88%, TAC of which corresponded accordingly. The investigation revealed that *A. bilimbi* was a good source of antioxidants; however, the drying process of the fruit significantly affected the bioactive compounds.

Keywords: Antioxidants, *Averrhoa bilimbi*, polyphenols, Free Radical Scavenging Activity, Metal Chelating Activity

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are formed by biological redox reactions as a consequence of aerobic life. Their relationship to chronic diseases has led to the general acceptance that increased production of free radicals play an important role in the development of tissue damage and pathological events in living organisms (Halliwell and Gutteridge, 1998). Fruits and vegetables are immensely valued for their nutritional content and their potential health functionality against various degenerative diseases such as cancer, cardiovascular, cataract, diabetes and neurodegenerative diseases like Alzheimer's and Parkinson's (Kaur and Kapoor, 2001) describing them as functional foods, nutriceuticals, and nutraceuticals (Laganayaki and Manian, 2010). Such protection against these diseases has been attributed to the various antioxidants present in them (Ames, et.al., 1993). The major group of phytochemicals that may contribute to antioxidant capacity of fruits includes polyphenols, carotenoids, and vitamins such as vitamin C and E (Loganayaki and Manian, 2010). Plant phenolics which form a large group of natural compounds, ubiquitous in the plant kingdom is known to display a remarkable array of biochemical interactions (Rice-Evans, 2004). These secondary metabolites may act as potent metal chelators and/or free radical scavengers (Hanasaki, et.al., 1994). Thus, these non- enzymatic substances along with the endogenous response system, such as antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) play a significant role in the physiological redox balance together with enzymatic defences (Halliwell and Gutteridge, 1998). *Averrhoa 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 oblong very sour fruits are eaten fresh and also used in production of vinegar, wine, pickles, jams and jellies. Bilimbi has been widely used as traditional medicine to treat cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough. Experimental pharmacological studies have shown that the fruit alleviates hypertension (Goh, et.al, 1995). Aqueous extract of fresh bilimbi have shown to exhibit low antioxidant activites and low nitric oxide inhibition activity (Abas, et.al, 2004).

Fruits and vegetables are consumable in their fresh state and deteriorate within a few days after harvest. The most feasible method usually used by common man to preserve these plant products is the traditional way of sun/shade drying although generally it functions to inactivate the enzyme polyphenol oxidases leading to significant changes in the composition of phytochemicals (Tseng and Zhao, 2012). However certain studies proved that the overall antioxidant properties of certain foods may instead be enhanced due to the formation of Milliard Reaction Products (MRPs), which results from a condensation reaction between amino acids (or proteins) and reducing sugars or lipid oxidation products (Nicoli, et.al., 2005). The 1,1-diphenyl-2-picrylhydrazyl scavenging activities, the reducing power and iron chelating abilities of MRPs have been reported to increase upon irradiation (Chawla, et.al., 2009). Phenolics, which are classified as polyphenols and simple phenols, function by trapping and scavenging free radicals and also regulate nitric oxide, decrease leukocyte immobilization, inhibit cell proliferation and angiogenesis and exhibit phytoestrogenic activity (Pellati, et.al, 2004). The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity has been widely used as a rapid and simple measure of antioxidant activity (Koleva, et.al, 2002). The $ABTS^{\bullet+}$ radical decolourisation assay, which measures the $ABTS^{\bullet+}$ radical scavenging ability of a compound, is especially helpful in determining the antioxidant activity of hydrogen donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavengers of lipid peroxyl radicals) (Loganayaki and Manian, 2010). The spectrophotometric measurement of antioxidant activity of fruit extracts through phosphomolybdenum method is based on reduction of Mo(IV) to Mo(V) by antioxidant compounds resulting in formation of green phosphate/Mo(V) compounds (Prieto, Pineda and Aguilar, 1999). The total reducing power measures the ability of antioxidants to reduce the Fe^{3+}/ferricyanide complex to intensely blue colored ferrous complex (ferri- ferro complex) in acidic medium which may serve as a significant indicator of potential antioxidant activity of the plant extracts (Mier, et.al, 1998). Chelating agents act as effective secondary antioxidants by stabilising the oxidized form of a metal ion by forming σ bonds with the metal leading to a reduced redox potential (Halliwell and Gutteridge, 1990). Iron is an important transition metal which acts as a lipid oxidation pro-oxidant with participation of ferrous ion in direct and indirect initiation of lipid oxidation (Gordon, 1990), capable of triggering Fenton reaction, linked with many diseases, wherein free radicals are generated from peroxides (Amensour, et.al, 2010). While the primary antioxidants scavenge free radicals to inhibit chain initiation and break chain propagation, the secondary antioxidants suppress the formation of radicals and protect against oxidative damage (Loganayaki and Manian). Therefore, the objective of the study is to evaluate and report for the first time the effects of sun- drying on the total phenolic content and antioxidant properties such as DPPH, $ABTS^{\bullet+}$, phosphomolybdenum, total reducing power and ferrous chelating activities of the fruit *Averrhoa bilimbi* under different solvents.

MATERIALS AND METHODS

Plant Material and Drying

Fresh fruits of *Averrhoa bilimbi* were obtained from Mangalore and Udupi, India, during the fruiting season (July- December). Care was taken that the fruits, which were whitish- green in colour and approximately 5- 7.0 cm in size, were not overripe, spoilt or damaged. 5kgs of fruits were cut and sun-dried for a week in September and October at ambient temperature and humidity that ranged between 27- 30ºC, and 48- 52% respectively. The dried fruits were ground in a blender to give 500g of fine powder. The process was conducted twice within the two months.

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), Ethylenediaminetetraacetic acid (EDTA), ferrozine, potassium persulphate, ammonium molybdate and Butylated Hydroxyanisole (BHA) were obtained from Himedia (Mumbai, India).

Ferric chloride, Gallic Acid, Acetone, Methanol, HPLC grade methanol and formic acid 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 chemicals used were of analytical grade.

Sample Extraction

Bilimbi extracts were prepared according to the conditions described by Singh et al (2012) with modification in the temperature and time. 500g of finely blended edible fresh fruit and 50g of dried-fruit powder were subject to continuous agitation in 5000 and 500ml of 0%, 20%, 40%, 60%, 80% and 100% aqueous methanol respectively in a shaking incubator for 3 hours at 40˚C. The extraction efficiency of each solvent:water composition were compared by measuring total antioxidant activity (TAA) of individual extracts. The filtrates were collected using a muslin cloth and concentrated making them solvent free in a rotary vacuum evaporator (Buchi, Germany). The aqueous portion was dried in hot air oven at 40ºC and used to analyse the total antioxidant activity. The extracts were prepared in triplicates.

Total phenolic content (TPC)

Total phenolic content of fruit extracts was determined spectrophotometrically using the Folin-Ciocalteau's reagent (Slinkard and Singleton, 1977). Phenols react with phosphomolibdic acid of FC reagent in alkaline medium and produce blue colored complex, that could be measured at 650nm and expressed as Gallic acid equivalent per 100g of dried/ fresh fruit extract using gallic acid as the standard. The assay was conducted in triplicates in three independent sets of experiment.

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

Free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured according to the method of Blois (1958) with few modifications. Briefly, to 1.0ml of different dilutions of the extracts (50- 200µg/ml) prepared in triplicates, 1.0ml of 200µM DPPH solution was added and the reaction mixture was incubated at room temperature for 20 minutes after vigorous shaking. Indication in the activity of DPPH was observed with a change in the color from purple to yellow. The absorbance at 517nm was measured using a UV-spectrophotometer (Shimadzu UV1800, UV Spectrophotometer, Japan). Butylated hydroxy anisole, gallic acid and rutin were used as the standards. The percentage DPPH radical scavenging activity was calculated using the formula:

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

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.

Ferric Reducing Power

The assay described by Yen and Duh (1993) was used for the determination of reducing power of fruit extracts with slight modifications. To 1.0ml of the extracts and BHA in different concentrations (15-45µg/ml), 0.5ml of phosphate buffer (6.6 pH, 0.2 M) and 0.5 ml of 10% potassium ferricyanide was added and incubated at 50°C for 20 minutes. To terminate the reaction 0.5 ml of 10% trichloroacetic acid was added, further diluted by adding 1.5ml of water after which 0.3ml of 0.1% ferric chloride solution was added producing a green coloured ferri- ferro complex. The procedure was conducted in triplicates before measuring the absorbance at 700nm. The absorbance obtained was plotted against fruit extract concentration wherein increasing absorbance of the reaction indicated the increase in the reducing power.

Trolox Equivalent Antioxidant Activity

The $ABTS^{\bullet+}$ radical scavenging activity was determinced essentially as per the method described by Loganayaki and Manian (2010). 7mM ABTS solution was mixed with 2.45mM potassium persulphate and left in the dark at room temperature for 12-15 hours in order to oxidize ABTS by the action of potassium to produce the $ABTS^{\bullet+}$ radicals.

After consistent absorbance of the $ABTS^{\bullet+}$ free radical solution at 734nm, the solution was diluted till the absorbance measured was 0.7± 0.02. To 1.0ml of the $ABTS^{\bullet+}$ solution, 0.1 ml of the test samples or standard Trolox (5-20mM) were added and incubated at room temperature for 10 minutes in triplicates. The absorbance was measured at 734nm. The $ABTS^{\bullet+}$ solution without sample was used as negative control. The total antioxidant activity of the extracts was expressed as Trolox equivalent antioxidant capacity (TEAC) and compared with BHA, rutin and gallic acid as standards.

Total Antioxidant Activity by phosphomolybdenum method

The total antioxidant activity of fresh and dried- fruit extracts was evaluated in triplicates by the method of Prieto, Pineda and Aguilar (1999) as described in Abdille, Singh, Jayaprakasha and Jena (2005). The absorbance of the mixture was measured spectrophotometrically at 695nm against blank. 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.

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 (1990) was used with slight modification. To different dilutions of the extracts (100- 500µg/ml), 0.05ml of ferrous chloride was added followed by 0.2ml of 5mM ferrozine and incubated at room temperature for 10minutes. The absorbance of the reaction mixtures prepared in triplicates was measured at 532nm. Decrease in absorbance at 562 nm of the red coloured iron- ferrozine complex indicates strong chelating power. Ethylenediaminetetraacetic acid (EDTA) was used as the standard chelator, and BHA, rutin and gallic acid as standards. The percentage chelating activity of the fruit extracts was calculated using the formula

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

where

$A_{control}$ → Absorbance of control

A_{test} → Absorbance of test sample.

The antioxidant activity was expressed in IC_{50} values.

Statistical Analysis

All experiments were conducted in triplicates and repeated in three independent sets of experiments. Data is shown as mean ± standard deviation (SD). Paired sample *t* test was performed for comparison of the antioxidant capacity of the extracts. Correlation analytical data was obtained using the software Origin version 5.0.

RESULTS

The results of Total Phenolic Content, DPPH and ABTS radical scavenging assays, phosphomolybdenuam assay, total reducing power and metal chelating activity gave an interesting observation. Although different solvent systems such as 100% water, 20%, 40%, 60%, and 80% methanol/water, and 100% methanol were used to perform biochemical analysis, invariably, the samples extracted fresh showed higher activity for all antioxidant assays. The drying treatment and various solvent compositions affected the TPC and subsequent antioxidant activities of *Averrhoa bilimbi.* The extraction efficiency of methanol/water system, which involves the criteria to extract maximum polyphenolic compounds in fresh and dried bilimbi and exhibit high antioxidant activities, descended in the following order 60%> 40%> 80%> 100%> 20%> 0%.

Highly significant (551- 11,287mgGAE/100g extract) phenolic content was recorded with freshly used fruits highest being 60% methanol/water extract. The sun dried fruit extracts showed significant (296-1806mgGAE/100g extract) TPC but lower than the fresh fruit extracts ($p< 0.1$, Table. 1) by 23- 88% probably due to the loss of moisture which was approximately 90% during the sun- drying process.
The scavenging effects of all the fresh bilimbi extracts on DPPH radical, particularly 60%, 80% and 40% methanol/water fresh- fruit extracts (0.175, 0.241 and 0.263mg/ml), were higher than the dried bilimbi extracts ($p<0.05$, Fig. 1), which correlated significantly with their TPC ($R^2= 0.915$). The total antioxidant activities, however was much lower than the positive controls gallic acid, rutin and BHA (0.027, 0.031 and 0.028mg/ml).

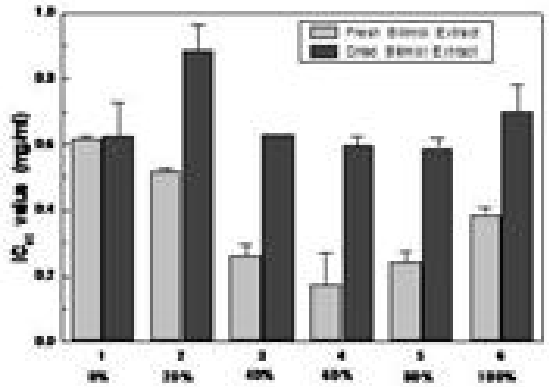

Figure 1. Showing DPPH radical scavenging activity of *Averrhoa bilimbi*. Each value represents mean±SD of three replicates.

Table 1. Showing Total Phenolic Content (mgGAE/ 100g extract), Trolox Equivalent Antioxidant Capacity (mMTrolox Equivalent/ g extract) and Total Antioxidant Activity by phosphomolybdenum method (mM Ascorbic Acid Equivalent/ g extract) of *Averrhoa bilimbi*. Values are means of triplicate determinations ± SD.

Sample	Total Phenolic Content (mgGAE/100g extract)	Trolox Equivalent Antioxidant Capacity (mM/g extract)	Total Antioxidant Activity by phosphomolybdenum method (mM/g extract)
Fresh Bilimbi Fruit Extract			
0%	551.83 ± 56.335	39.10 ± 4.883	77.91 ± 10.84
20%	556.60 ± 13.643	48.77 ± 1.605	82.05 ± 12.06
40%	5384.37 ± 83.969	101.83 ± 0.543	124.17 ± 7.638
60%	11287.5 ± 583.363	149.49 ± 0.770	192.78 ± 16.72
80%	1000.00 ± 106.066	59.23 ± 1.06	102.50 ± 2.5
100%	4183.00 ± 200.818	50.45 ± 0.495	92.50 ± 7.5
Dry Bilimbi Fruit Extract			
0%	296.25 ± 58.336	4.69±0.103	8.31±1.08
20%	410±14.142	3.69±0.375	17.89±2.807
40%	1116.67±18.31	37.79±4.012	61.25±1.768
60%	1806.25±30.943	35.95±0.219	137.05±4.74
80%	653.12±8.839	37.25±1.294	56.25±1.768
100%	503.13±35.355	12.42±0.258	60.0
BHA	-	150.2±2.622	314.9±22.641
Gallic Acid	-	146.3±3.724	304.0±89.52
Rutin	-	148±6.724	508.25±82.80

The TAA by $ABTS^{\cdot+}$ cation radical scavenging activity of dried and fresh bilimbi extracts ranged between 3.69-37.79mmolTEAC/g extract and 39.1-149.49mmolTEAC/g extract respectively (Table.1), the lowest being 20% methanol/water extract of dried fruit. 60% methanol/water extract of fresh fruit exhibited the highest activity (149.49mmolTEAC/g extract) which was on a par with the positive controls rutin (148mmolTEAC/g), BHA(150.2mmolTEAC/g) and Gallic acid (146.3mmolTEAC/g). The TAA significantly correlated with their total phenolics (R^2=0.775, $p<0.05$). Interestingly, the extracts which tested high antioxidant capacity in the DPPH model also showed a high antioxidant capacity in the ABTS model (R^2=0.566). The total antioxidant activity (TAA) of the extracts seems to be sufficient for functioning as potential nutraceuticals when they are ingested along with nutrients.

The TAA of all extracts exhibited significant reduction effects of Mo(IV) to Mo(V) which ranged between 8- 192mMAAE/g extract (Table. 1). 60%, 40% and 80% methanol/water extracts of fresh bilimbi expressed the highest activities at 192, 124 and 102mMAAE/g extract, respectively while 100% water extract of dried bilimbi expressed the lowest TAA (8mMAAE/g extract). The positive controls, however, Rutin, Gallic Acid and BHA showed much higher activity at 508, 304 and 314 mMAAE/g respectively. The TAA of all the fresh bilimbi fruit extracts was higher than that of dried bilimbi fruit extracts ($p<0.05$) correlating well with their total phenolic content (R^2=0.653). At 45µg/ml 60% methanol/water extract of fresh bilimbi fruit exhibited the highest total reducing power (0.403) which was higher than BHA (0.323) (Fig. 2). 100% water extract of dried bilimbi fruit expressed the lowest TRP at 0.079. The TRP of dried fruit extracts was lower than the fresh fruit extracts ($p< 0.05$) attributing to its TPC (R^2=0.767). It also correlated significantly with DPPH and $ABTS^{\cdot+}$ radical scavenging activities (TRP vs DPPH 0.7056 and TRP vs $ABTS^{\cdot+}$ 0.9791).

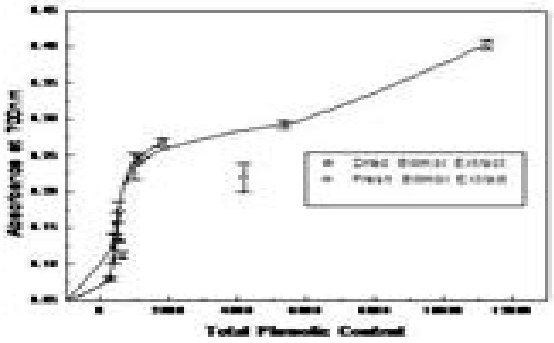

Figure 2. Showing the total reducing power of *Averrhoa bilimbi*. Each value represents mean ± SD of three replicates.

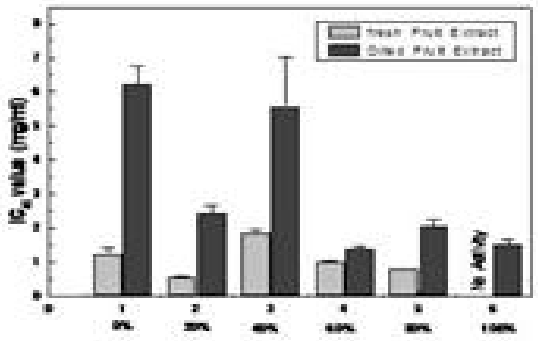

Figure 3. Showing the metal chelating activity of *Averrhoa bilimbi*. Each value represents mean ± SD of three replicates

This may be because the total reducing power relies on the same mechanism wherein it measures the ability of the extract to donate electron to Fe(III). The reducing capacity of bilimbi extracts served as a significant indicator of its potential antioxidant activity, of which the fresh bilimbi extracts, particularly 60% methanol/water extract, were a better potential. In the metal chelating assay, the fresh and dried bilimbi fruit extracts interfered with the formation of ferrous and ferrozine complex, suggesting that they had chelating activity and captured ferrous ion before ferrozine. 20%, 80%, and 60% methanol/water extract of fresh bilimbi registered the highest metal chelating activity (0.565, 0.804 and 1.022mg/ml, Fig. 3). EDTA, a strong metal chelating agent, registered IC_{50} value of 0.059mg/ml, superseding the bilimbi extracts and the positive controls gallic acid, rutin and BHA (0.160, 0.156 and 0.157 mg/ml). 100% methanolic extract of fresh bilimbi, however, did not chelate the ferrous ion. Interestingly, although 20% methanol/water extract of fresh bilimbi had comparatively low TPC and in all the other assays exhibited poor primary antioxidant potential, it was deduced that the extract was richer in secondary antioxidants. 60% and 80% methanol/water extract of fresh fruit possessed potent primary and secondary or preventive antioxidants. 100% water extract of fresh fruit and 100% methanol extract of dried fruit expressed moderate primary and secondary antioxidants. 40% methanol/water extracts of fresh bilimbi and 60% methanol/water extracts of dried bilimbi registered significant primary antioxidants but moderate secondary antioxidants. 100% methanolic extract of fresh bilimbi showed that it was poor in secondary antioxidants.

DISCUSSION

The bilimbi extracts strongly scavenged the free radicals which indicated that the extract had good potential as a source for natural antioxidant to prevent free radical mediated oxidative damage. Of course, there could be few explanations for the loss of phenolics and antioxidant activity due to sun-drying that attributes the deactivation of the polyphenol oxidases by absorbing the water molecule. Perhaps the heating treatment during sun- drying not only deactivates enzymes, but also degrades phytochemicals while some phenolics decompose rapidly in direct sunlight (Mueller-Harvey, 2001). The loss of TPC and TAA of different plant samples after sun- drying process have also been reported[26]. However, contrary to the results obtained in this investigation, numerous studies reported the increase in both TPC and TAA of samples after processing (Tseng and Zhao, 2012; Mejia-Meza, et.al, 2010). In some cases drying process caused little or no change to the content and antioxidant activity of naturally occurring antioxidants like carotenoids and vitamin C[27]. Plant phenolic compounds are present in different binding status depending on plant species. This clearly explains that the drying process may result in high, low or no change in levels of TPC depending on the type of phenolic compounds present in the plant material and their location in the cell (Capecka, Merecczek and Leja, 2005). Solvent used for extraction is another factor to be considered to influence the quantification of antioxidants. Interestingly 60% methanol/water solvent combination was observed to be the best solvent to extract maximum phenolic compounds from both fresh and dried bilimbi fruit as methanol was able to denature polyphenol oxidases. Being an organic and volatile solvent, it is more efficient in plant cell wall degradation, therefore, able to extract a greater amount of endocellular materials than water alone (Lim, Murtijaya, 2006). Researchers mentioned the possibility of breakdown of tannin to simple phenol when high temperature was used during extraction process, which increased the number of compounds with free hydroxyl groups (Oboh, 2006). Extraction of hydrolysable tannins from bilimbi with methanol, methanol/water and water in the elevated temperature (40˚C) is accompanied by different degree of hydrolysis of tannins, resulting in variation of the antioxidant activities. Data in the effects of sun-drying, antioxidant activity and TPC of fruits and vegetables are conflicting due to several factors like different drying conditions, type of extraction solvents, and antioxidant assays used. In conclusion, this indicated that the traditional sun- drying method of fruit preservation had an adverse effect in the TPC and TAA of *A. bilimbi* resulting in all the extracts of the dried fruit possessing lower antioxidant properties than fresh fruits. A more efficient drying method needs to be employed to not only improve the shelf life of *A. bilimbi* but also retain or enhance its TAA.

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