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FULL PAPER

Antioxidant and antimicrobial activity of sapodilla (*Manilkara zapota* L.) fresh, juice and bar

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Abstract

This study evaluates the antioxidant and antimicrobial activity of sapodilla fresh, juice and bar using three extraction solvents with different polarities (70% acetone, 80% ethanol and water). For evaluating antioxidant capacity, Ferric Reducing Antioxidant Power (FRAP) and 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) assays were used. The total flavonoid content (TFC) and condensed tannin of the sapodilla juice and bar extracts were determined as well. Antimicrobial activity was measured using *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*. Both sapodilla bar and juice produced more inhibition of the DPPH assay with 70% acetone and 80% ethanol than with the water extraction. The reducing ability of fresh sapodilla was on the order of 70% acetone > 80% ethanol > water. The 70% acetone was the best solvent for yielding condensed tannins. For total flavonoid content, fresh sapodilla and the sapodilla bar and juice showed the highest flavonoid content in 80% ethanol. The results showed that the 70% acetone extract for sapodilla bar and juice exhibited stronger antibacterial activity against gram-positive bacteria. Despite some antioxidants being lost in processed food, these still retained important sources of bioactive compounds. Antimicrobial activity done showed different selectivity for sapodilla juice and bar.

1. Introduction

Sapodilla is an excellent source of antioxidants with over 3000 mg/L ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh sample (Shui *et al*., 2002). Polyphenols found in immature sapota fruit have been identified as 3'-caffeoylquinic (Lakshminarayana, 1969). Unripe fruits of *Manilkara zapota* L. contain a high amount of tannin, which may be responsible for the fruit's high antioxidant capacity, although the amount of tannin decreases as it ripens (Lakshminarayana, 1969).

Pathogens, such as *Salmonella* spp. are a primary cause of food poisoning, and this problem occurs in several parts of the world. Some bacterial strains may cause intestinal infection, which may lead to death (Munazir, 2012). Infectious diseases are a critical problem that is primarily caused by bacteria and fungi from various polluted sources, which may then contaminate food. There is a sudden increase in foodborne illness throughout the world due to the consumption of contaminated food which consists of pathogenic bacteria (Rodriguez *et al*., 2010). Hence, the effort of finding potential antimicrobial agents is of paramount priority. However, studies also indicated that even new families of antimicrobial agents will have a short life expectancy (Coates *et al.,* 2002). Due to this reason, researchers nowadays are shifting their attention towards the use of natural sources as antimicrobial agents that act against the pathogenic bacteria.

Processing *M. zapota* L. into fruit products also offers a convenient way to market the fruit, especially because such food products based on sapodilla fruit are not popular in the local market. Fruits processed into value-added products (fruit bars and juice), are not only nutritious but can also be accepted by consumers. Therefore, developing fruit bars and juice from *M. zapota* L. will increase its commercial value and reduce waste in the production of fruit during any given season. In accordance with these facts, this study was conducted with the aim of identifying the antioxidant activities in *M. zapota* L. juice and bar which extracted using different type of solvents as well as investigating the antimicrobial activities of *M. zapota* L. against several microorganisms to explore the ability of this fruit as a potential source of natural antimicrobial agent.

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2. Materials and methods

2.1 Sample collection

Fresh fruits were harvested from Kuala Kurau, Perak Darul Ridzuan (5.0164°N, 100.4318°E). The herbarium number of *M. zapota* L. is 11475.

2.2 Sample preparation of sapodilla bar

Sapodilla fruits were weighted, cleaned with tap water, peeled and all the seeds were removed. Then, the fruits were homogenized in a blender (Panasonic MX-7995, Malaysia). Approximately 72.7% of sapodilla pulps were boiled, and then sugar (30%), pectin (1%), milk powder (6%), citric acid (0.6%) and a pinch of salt were added. Then, the homogenized pulp was poured approximately 5-8 mm thick on the tray that was coated with butter and dried at 60±2˚C for 8 hrs in a convection air drier. Finally, the fruit bar was freeze-dried ((LD53, Kingston, New York).

2.3 Sapodilla juice

Both the glass bottle and cap were sterilized in boiling water for 5 mins and then drained and dripped dry. The fruits were cleaned, cut into small pieces and blended in a blender (Panasonic MX-7995, Malaysia). Fruit juice was extracted using a muslin cloth, and the weight of the extracted juice was recorded. An amount of water was weighed to be the same as the weight of extracted juice (1:1 ratio) and added to the extracted juice. The initial soluble solid content in degrees Brix (B1) was measured using a handheld refractometer, and the total weight of the diluted juice (W1) in grams was recorded. The initial titratable acidity of the diluted juice was determined. Each sample was titrated with 0.1 N of sodium hydroxide (NaOH) to an endpoint pH of 8.2. The mixture was warmed to dissolve all the sugars, and the juice was pasteurized at 85˚C for 3 mins. Then, the caps were closed and filled while hot. Lastly, the juice was blast frozen (-18˚C) prior to freeze-drying and grinding into powder.

2.4 Sample extraction

Sample extraction was carried out as described by Ikram *et al.* (2009) with slight modifications (ratio of sample to solvent). Approximately 1 g of sample was mixed with 100 mL of 70% acetone (v/v). For the sapodilla bar, the sample was homogenized first using a homogenizer. For sapodilla juice, the sample was ground into powder. Then, the homogenized sapodilla bar and powdered juice samples were placed in a conical flask (wrapped with aluminium foil) and agitated at 150 rpm (27˚C) overnight with the aid of an orbital shaker (Lab Companion, Model SI600R). Then, the mixture was centrifuged at 2500 rpm for 20 mins to obtain a clear solution.

2.5 Antioxidant analysis

2.5.1 DPPH free radical scavenging activity

Based on the method described by Sanchez-Moreno *et al.* (1998), the capacity of the extract to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured. Briefly, 2 mL of the sample extract was added to the 2 mL of a methanolic solution of DPPH radical (0.1 mM). The mixture was vortexed for 1 min and maintained in the dark for 30 mins. The absorbance of the mixture was read at a wavelength of 516 nm against a blank of the methanolic solution of DPPH, which was mixed with distilled water using a UV-vis spectrophotometer, Shimadzu, Japan.

2.5.2 Ferric reducing antioxidant property (FRAP)

Ferric reducing antioxidant power (FRAP) reagent was prepared as described by (Benzie and Strain, 1996). A blank sample was prepared by replacing the amount of extract with distilled water. After that, the absorbance was read at 593 nm using a UV-vis spectrophotometer, Shimadzu, Japan. Ferrous sulphate solution was used as a standard.

2.6 Determination of total flavonoids content

The total flavonoid content (TFC) was determined using the aluminium trichloride method as described by Wu and Ng (2008). Briefly, 0.5 mL of sample extract was mixed with 2.5 mL of distilled water in a test tube followed by the addition of 0.5 mL of 5% (w/v) sodium nitrite solution and maintained for 5 mins. Then, 0.3 mL of 10% (w/v) aluminium chloride was added, and the mixture was incubated for another 6 minutes. After that, 1 mL of 1M sodium hydroxide (10%, w/v) was added and immediately make up the solution up to 0.55 mL with distilled water, vortexed and the absorption of the solution was measured at 510 nm wavelength by using UV-vis spectrophotometer, Shimadzu, Japan against a reagent blank. A blank sample was prepared by replacing the amount of extract with distilled water. Total flavonoid content was expressed as mg quercetin equivalent (QE)/g of sample.

2.7 Determination of condensed tannin

The vanillin-HCl method described by Broadhurst and Jones (1978) was used to measure tannin. Approximately 0.5 mL of the sample extract was mixed with the 3 mL of vanillin reagent (4%, w/v, vanillin in methanol). Then, 1.5 mL of concentrated hydrochloric acid was added, and the mixture was vortexed. The mixture was maintained in the dark for 15 mins and 27˚C, and the absorbance of the mixture was measured at

500 nm using a UV-vis spectrophotometer, Shimadzu, Japan. A blank sample was prepared using the same procedure without the extract and was used to prepare the calibration curve. Tannins content was expressed as mg catechin equivalent (CE)/g of sample.

2.8 Antimicrobial analysis

2.8.1 Preparation of culture inoculated for disc diffusion assay

The disc diffusion method described by the National Committee for Clinical Laboratory Standard (NCCLS) (2015) was used for determination of antimicrobial activity of fresh sapodilla and the sapodilla bar and juice. Each bacterial strain in nutrient agar was transferred into the nutrient broth and incubated approximately 4 to 6 hours before use. After that, the cultures were swabbed on to the Mueller-Hinton agar using a sterilized cotton swab. After that, a petri dish was divided into 5 parts containing 3 types of extraction solvents and positive and negative controls. Sterile blank paper disks were impregnated with 50 µL of the sample extracts, dried and placed on the inoculated agar plates. Gentamycin was used as the positive control (Owolabi *et al*., 2007) while methanol as the negative control in accordance with Sati and Joshi (2011). Plates were incubated at 37˚C for 18 to 24 h. Antimicrobial activity was determined by measuring the diameter of the clear zones of inhibition.

2.9 Statistical analysis

Data were analysed by using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range method was used to compare any significant differences between solvents. Values were expressed as means ± standard deviations. Differences were considered significant at $p<0.05$. All the analysis were carried out in triplicates.

3. Results and discussion

3.1 Antioxidant analysis

3.1.1 DPPH radical scavenging activity

Table 1 shows the percentage of DPPH inhibition in sapodilla bar and juice. From the table, the water extract appeared to be lowest for antioxidants in the sapodilla for both products (bar and juice). According to previous studies by Woo *et al.* (2013), the percentage of DPPH inhibition for sapodilla fruit in 40% ethanol was 63.20±0.06%. Since the phytochemical constituents, which are responsible for scavenging free radicals, were higher in the 70% acetone and 80% ethanol compared with the water extraction, it can be concluded that higher DPPH radical scavenging activity should be found in combination of water and organic solvents that maximizes the interaction of DPPH radicals with antioxidants present in the sample (Chew *et al*., 2012). Since amount of total phenolic content could contribute to antioxidant activity in some fruits, prolonged exposure of sapodilla fruit bars to temperature above 60˚C during drying in a convection oven might have released some of the bound phenolic compounds from the food matrices, resulting in the increased amount of extracted phenols in fruit bars (Dewanto *et al*., 2002). This significant increase in the total phenolic content might have resulted from the addition of other ingredients and the various reactions that occurred during drying (Rabeta *et al*., 2016).

Table 1. Percentage of DPPH inhibition in sapodilla bar and juice

Sample	70% acetone	70% ethanol	water
Sapodilla Juice	36.75±0.89[a]	36.75±1.06[a]	14.06±1.40[b]
Sapodilla Bar	64.03±2.12[a]	60.08±5.97[a]	40.36±0.81[b]

Values are means (n=3)±SD. Values with different superscript are significantly different at $p<0.05$

3.1.2 Ferric reducing antioxidant potential (FRAP)

Based on the result (Table 2), it can be seen that all the samples for fresh sapodilla juice and bar were significant ($p<0.05$) in each extract. The reducing ability of fresh sapodilla was on the order of 70% acetone > 80% ethanol > water. The 70% acetone extract had the highest reducing power with 9157.00 µM Fe(II)/g. The reducing power in 80% ethanol and water extraction were 8725.33 µM Fe(II)/g and 6367 µM Fe(II)/g, respectively. Addai *et al.* (2013) reported that the reducing power in the extraction of papaya in 70% acetone was 7265.17 µM TE/g, was 7644.33 µM TE/g in 70% ethanol and was 6391.79 µM TE/g in water extraction. On the other hand, the 80% ethanol exhibited a high amount of reducing power with 5880.33 µM Fe (II)/g in sapodilla bar. The absorbance value measured in the 70% acetone was 2762.33 µM Fe(II)/g and 2570.33 µM Fe(II)/g in 80% ethanol, respectively. The water extract showed the lowest amount of reducing power at 1523.33 µM Fe(II)/g.

Table 2. FRAP assay in sapodilla bar and juice

Sample	70% acetone	70% ethanol	water
Fresh Sapodilla	9157.00± 128.33[a]	8725.33± 230.76[b]	6367.00± 79.73[c]
Sapodilla Bar	5570.67± 90.84[b]	5880.33± 170.54[a]	3915.33± 150.89[c]
Sapodilla Juice	2762.33± 115.85[a]	2570.33± 154.08[b]	1523.33± 177.68[c]

FRAP assay was expressed as µM Fe(II)/g. Values are means (n=3) ± SD. Values with different superscript are significantly different at $p<0.05$

3.2 Total flavonoid content

The results of total flavonoid (Table 3) present in the

sample, showed significant difference (p<0.05) in the total flavonoid of all sample extracts. The highest content of total flavonoids in fresh sapodilla was obtained from 80% ethanol extract (2413.33±13.5 µg QE/g). After 80% ethanol, 70% acetone had the highest content of flavonoids (1735.33±223.39, µg QE/g) and water produced the lowest extraction of flavonoids (1275.67±38.94 µg QE/g) in fresh sample. A previous study by Jayakumar and Kanthimathi (2011) demonstrated that 95% ethanol extract of sapodilla fruit yielded 390 µg QE/g. According to Addai *et al.* (2013), the flavonoid content of papaya in 70% acetone was 302.4±0.67 µg QE/g and was 310.6±0.54 µg QE/g in 80% ethanol and 210.4±0.63 µg QE/g in water extract. A similar trend was observed in the sapodilla bar, which had the highest flavonoid content in 80% ethanol (1791.0±61.83 µg QE/g) followed by 1637.33±20.03 µg QE/g in 70% acetone and water (1170.33±2.51 µg QE/g). Among different solvents utilized for the extraction of flavonoids, 80% ethanol was shown to be the best solvent, and water was observed to be a poor solvent. Acetone was found to be a moderate solvent in the extraction of flavonoids. The interaction of ethanol with flavonoid can be described through non-covalent interactions and provides rapid diffusion into the solution (Castro and Tena, 1996).

Table 3. Total flavonoid content in sapodilla bar and juice

Sample	70% acetone	70% ethanol	water
Fresh Sapodilla	1735.33± 223.39^{b}	2413.33± 13.5^{a}	1275.67± 38.94^{c}
Sapodilla Bar	1637.33± 20.03^{b}	1791.00± 61.83^{a}	1170.33± 2.51^{c}
Sapodilla Juice	949.00±1 7.08^{b}	1079.00± 93.66^{a}	945.33±1 2.85^{b}

Total flavonoid content was expressed as µg QE/g. All data are the mean ± SD of three replicates. Values are followed by identical letters are not statistically different from each other p<0.05

3.3 Total condensed tannin

Tannins not only function as primary antioxidants but secondary plant metabolites and one of the major groups of antioxidant polyphenols that found in food (Kumari *et al.,* 2012). Condensed tannin content of the three samples was determined in this study (Table 4). The highest content of condensed tannins obtained in the 70% acetone among all of the samples. The 70% acetone significantly extract more condensed tannins compared to 80% ethanol and water extraction. Fresh sapodilla extract with 70% acetone (2994.67±21.38 µg CE/g) exhibited the highest condensed tannin, whereas 80% ethanol (2694.00±73.02 µg CE/g) and water extracts produced the lowest amount of condensed tannins (1738.00±74.80 µg CE/g). Similar to the sapodilla bar, the 70% acetone extraction had a proportion of condensed tannin of 1664.00±112.59 µg CE/g, followed by 70% ethanol (1390.00±13.11 µg CE/g) and water (868.67±42.73 µg CE/g). For sapodilla juice, the amount of condensed tannins in 70% acetone was 1107.33±84.12 µg CE/g, whereas water exhibited the lowest extraction of condensed tannins (472.00±32.19 µg CE/g). Previous studies by Melo *et al*. (2006) showed that the condensed tannin of banana was 2526 µg CE/g of fresh weight in an acetone extract. According to Macheix and Fleurite (1990), condensed tannins are not very soluble, and the extraction requires aqueous acetone to break the natural macromolecule linkages of the phenols. The better extraction power of the mixture of alcohol with water indicates that the mixing of a non-polar solvent with water may increase the polarity index of the solvents (Addai *et al*., 2013).

Table 4. Total condensed tannin in sapodilla bar and juice

Sample	70% acetone	70% ethanol	water
Fresh Sapodilla	2994.67± 21.38^{a}	2694.00± 73.02^{b}	1738.00± 74.80^{c}
Sapodilla Bar	1664.00± 112.59^{a}	1390.00± 13.11^{b}	868.67±4 2.73^{c}
Sapodilla Juice	1107.33± 84.12^{a}	776.00±4. 16^{b}	472.00±3 2.19^{c}

Total condensed tannins were expressed as µg CE/g. Value are means (n=3) ± SD. Values with different superscript are significantly different at p<0.05

3.4 Antimicrobial activity

The results of the antibacterial activity of the acetone, ethanol and water extracts of all three samples are given in Table 5. The antimicrobial activity of the tested extracts showed different selectivity for each microorganism. The results showed that the 70% acetone extract for sapodilla bar and juice exhibited stronger antibacterial activity against gram-positive bacteria, whereas their activities towards the gram-negative bacteria was found to be weak. The gram-positive bacteria seemed to be more easily inhibited than gram-negative bacteria. For sapodilla bar, the highest zone of inhibition was 12.7 mm against *B. subtilis* (extracted with 70% acetone).

Sapodilla juice with 70% acetone exhibited greater antimicrobial effect towards *B. subtilis* at 9.3 mm as compared to other extraction solvent. Both samples showed weak inhibition against *Salmonella* spp. at 5.0 mm for sapodilla bar and 3.0 mm for sapodilla juice (extracted with 70% acetone). *Salmonella* spp. was found to be most susceptible to the fresh sapodilla extract with an inhibition zone 2.0 mm. The 80% ethanol extracts of the sapodilla bar showed weak inhibition against *E. coli* (1.0 mm), and a larger inhibition zone was

Table 5. Antimicrobial activity of fresh sapodilla, sapodilla bar and juice

Microorganisms	Zone of Inhibition (mm)									
	Fresh Sapodilla			Sapodila Bar			Sapodila Juice			Antimicrobial Disc
	Acetone	Ethanol	Water	Acetone	Ethanol	Water	Acetone	Ethanol	Water	Gentamycin
E. coli	5.0	-	-	7.3	1.0	-	7.0	3.0	-	16.0
Salmonella spp.	2.0	-	-	5.0	2.0	-	3.0	2.0	4.0	19.0
B. subtilis	7.3	5.0	3.3	12.7	8.3	7.3	9.3	9.0	6.5	22.0
S. aureus	8.0	8.0	5.0	9.0	10.0	7.0	10.0	8.7	7.0	20.0

- no inhibition zone

found against *S. aureus* (10.0 mm). In 80% ethanol extract, fresh sapodilla was inactive against *E. coli* and *Salmonella* spp. but showed a minor inhibition effect against *B. subtilis* (5.0 mm) and *S. aureus* (8.0 mm) while the sapodilla juice extract was quite effective against *B. subtilis* (9.0 mm) and *S. aureus* (8.7 mm).

It is worth noting that all the extracts showed greater antibacterial activity against gram-positive bacteria than gram-negative bacteria. All the extracts showed activity against *B. subtilis* and *S. aureus,* which are generally more sensitive to the sample extracts. The reason for this difference in sensitivity towards the plant extracts might be due to the difference in the morphological constitution of these microorganisms. According to Chaghaby (2014), gram-negative bacteria have an outer phospholipidic membrane consisting of lipoprotein and lipopolysaccharide that makes the cell wall impermeable to plant extracts. This membrane renders the gram-negative bacteria generally less susceptible to plant extracts than the gram-positive bacteria (Chan *et al*., 2012). On the other hand, gram-positive bacteria are more susceptible and only have an outer peptidoglycan layer that is not an effective permeability barrier (Chanda *et al.*, 2010). The antibacterial activity of the extracts might be due to the presence of bioactive compounds such as tannins, flavonoids and phenolic acid (Ouattara *et al*., 2011). Fresh sapodilla extracts have the lowest inhibition against both gram-positive and gram-negative bacteria. No results have been published on the antimicrobial activity of *M. zapota* L., but studies from (Patel and Rao, 2012), who screened the antibacterial activity of *Manilkara hexandra,* showed that acetone extracts exhibited the lowest antibacterial activity in ripe fruit. Based on the results, the *B. subtilis* inhibition zone is only 2.0 mm, whereas *E. coli* exhibited a 3.0 mm inhibition zone. Previous study found that the formulated juice had better microbial stability (Lim *et al.,* 2018).

4. Conclusion

Although some antioxidant losses could have occurred, the present results suggest that fruit bar and juice may still represent important sources of bioactive compounds in the diet with noticeable antioxidant capacity. Sapodilla bar and juice have higher antimicrobial activities compared to fresh sapodilla. However, the results of flavonoid content and condensed tannins in the sapodilla bar and juice in this study were lower than fresh sapodilla. The results should indicate a higher inhibition zone in fresh sapodilla. This result might be due to the effect of the addition of sugar to sapodilla bar and juice, which acts as a preservative. Greater inhibition in the samples might be due to the presence of the solvent used. The overall study has demonstrated the potentiality of sapodilla fruit bar and juice as cheap resources of natural microbial agents against pathogenic microorganisms which may overcome the current problem of expensive medical cost in the societies.

Conflict of Interest

We declare that we have no conflict of interest.

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