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Comparative Evaluation of Antioxidant Properties of Methanolic Extract of Red and Green Custard Apple Fruits

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Authors' contributions

This work was carried out in collaboration between all authors. Author MS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FAMB, ASA, SHMS, RA, NIAR and MSA managed the analyses of the study. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Annona squamosa (custard apple) or also called Sugar Apple. It is native to the tropical Americas and West Indies, but the exact origin is unknown. It is now the most widely cultivated of all the species of Annona, being grown for its fruit throughout the tropics and warmer subtropics, such as Indonesia, Thailand, and Taiwan; it was introduced to southern Asia before 1590. There are two varieties of custard apple which were available in the local Malaysian market. In the present study we aimed to carry out comparative antioxidant activity of methanolic extract of both custard apple fruits by using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method. The results showed that the methanolic extract of red custard apple showed better antioxidant activity than methanolic extract of

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green custard apple. However, the standard ascorbic acid showed better antioxidant activity than both the extracts. In conclusion, the red custard apple is good candidate for further investigation.

Keywords: Annona squamosa; custard apple; antioxidant.

1. INTRODUCTION

Annona squamosa (custard apple) is also called Sugar Apple. It is native to the tropical Americas and West Indies, but the exact origin is unknown. It is now the most widely cultivated of all the species of Annona, being grown for its fruit throughout the tropics and warmer subtropics, such as Indonesia, Thailand, and Taiwan; it was introduced to southern Asia before 1590 [1]. It is naturalized as far north as southern Florida in the United States and as south as Bahia in Brazil, and is an invasive species in some area.

There are two varieties of *A. squamosa* Linn, a green one from Brazilian and red one from Southeast Asia. It is used for the treatment of dysentery, cardiac problem, fainting, worm infections, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors and ulcers [2-4]. The white creamy fruit pulp is soft and edible and is employed in preparing cool drinks and flavouring ice puddings.

Well-known antioxidants include enzymes and other substances, such as vitamin C, vitamin E, and β-carotene, which are capable of counteract the damaging effects of oxidation. Antioxidants are also commonly added to food products such as vegetable oils and prepared foods to prevent or delay their deterioration from the action of air. Antioxidants may possibly reduce the risks of cancer. Antioxidants clearly slow the progression of age-related macular degeneration [5].

Free radicals have been implicated in the pathology of many diseases, including cancer, atherosclerosis, diabetes and neurodegenerative disorders, in addition to aging. Natural antioxidants represent promising tools to protect against the damage to the cellular organelles caused by these free radicals [6].

The antioxidant activity of *A. squamosa* was well known [2], but there is no studies reported so far that show comparative antioxidants properties of methanolic extract of red and green custard apples. Therefore, we decided to carry out detail antioxidants investigation on both varieties of custard apple.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of *Annona squamosa*

The fruits of red and green custard apple (Fig. 1) were collected from the local market Ipoh, Perak, Malaysia and identified.

2.2 Extraction of Green and Red Custard Apple

The collected fruits were washed thoroughly in distilled water to remove contaminants; the peels and seeds were removed, then it was cut into small pieces and subjected to extraction by maceration in pure methanol (100%) at room temperature with occasional shaking for seven days (Table 1). The macerates were filtered and the filtrate was dried at low temperature (40-50℃) under vacuum. The extracts were stored in air-tight containers in a refrigerator at 4℃ until further use.

Fig. 1. Red and green custard apple (*Annona squamosa* Linn*)*

2.3 Qualitative Phytochemical Screening

The methanolic extracts obtained as above were tested for the following qualitative chemical tests for the identification of various phytoconstituents [7-8].

Table 1. Yields and nature of methanolic extract of green and red custard apple fruits

Plant source	Quantity used for methanol extraction		Nature of the extracts	Yield (%)
	Powder (g)	Solvent (ml)		
Green custard apple	100	250	Yellowish white semi solid	6.23
Red custard apple	100	250	Yellowish white semi solid	8.42

2.3.1 Tests for alkaloids

2.3.1.1 Dragendorff's test

To the extract, 1 ml of Dragendorff's reagent was added. An orange red precipitate indicates the presence of alkaloid.

1. Wagner's test: To the extract, Wagner's reagent was added. Reddish brown precipitate indicates the presence of alkaloid.
2. Mayer's test: To the extract, 1 or 2 ml of Mayer's reagent was added. A dull white precipitate indicates the presence of alkaloid.
3. Hager's test: To the extract, 3 ml of Hager's reagent was added. Yellow precipitate indicates the presence of alkaloid.

2.3.2 Tests for carbohydrates

1. Molisch test: To the extract, 1 ml of α-naphthol solution was added and concentrated sulfuric acid was added along the sides of test tube. Purple or reddish violet color at the junction between the two liquids indicates the presence of carbohydrates.
2. Fehling's test: To the extract, equal quantities of Fehling's solution A and B was added. Upon heating gently, a brick red precipitate indicates the presence of carbohydrates.
3. Benedict's test: To 5 ml of Benedict's reagent, 8 drops of solution under test was added, mixed and the mixture was boiled vigorously for two minutes and cooled. A red precipitate indicates the presence of carbohydrates.

2.3.3 Tests for proteins

1. Biuret test: To the extract, 1 ml of 40% sodium hydroxide and 2 drops of 1% copper sulfate solutions were added. A violet color indicates the presence of proteins.
2. Xanthoproteic test: To the extract, 1 ml of concentrated nitric acid was added, a white precipitate formed, it was boiled and cooled. Then, 20% of sodium hydroxide or ammonia was added. Orange color indicates the presence of aromatic amino acids.
3. Lead acetate test: To the extract, 1 ml of lead acetate solution was added. A white precipitate indicates the presence of proteins.

2.3.4 Test for amino acids

Ninhydrin test: Two drops of freshly prepared 0.2% ninhydrin reagent was added to the extract and heated. Development of blue color indicates the presence of proteins, peptides or amino acids.

2.3.5 Tests for steroids and sterols

1. Libermann Burchard test: The extract was dissolved in 2 ml of chloroform in a dry test tube. Ten drops of acetic anhydride and 2 drops of concentrated sulfuric acid were added. The solution becomes red, then blue and finally bluish green in color indicating the presence of steroids.
2. Salkowaski test: The extract was dissolved in chloroform and an equal volume of concentrated sulfuric acid was added. Bluish red to cherry red color is observed in chloroform layer, whereas the acid layer assumes marked green fluorescence indicating the presence of steroids.

2.3.6 Tests for glycosides

1. Legal test: The extract was dissolved in pyridine and sodium nitroprusside solution added to it and made alkaline. Pink red or red color indicates the presence of glycosides.

2. Baljet test: To the extract, sodium picrate solution was added. Yellow to orange color indicates the presence of glycosides.
3. Borntrager's test: Few ml of dilute sulfuric acid was added to the test solution. Boil and filter the solution. The filtrate was extracted with ether or chloroform. The organic layer was separated and treated with ammonia. Pink, red or violet color indicates the presence of glycosides.
4. Keller Killiani test: Sample was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulfuric acid. At the junction, reddish brown color was formed, which gradually becomes blue indicating the presence of glycosides.

2.3.7 Test for flavonoids

Shinoda test: To the extract, magnesium turnings were added, followed by the addition of concentrated hydrochloric acid. A red color indicates the presence of glycosides.

2.3.8 Tests for tannins

1. To the extract, ferric chloride was added. Dark blue or greenish black color indicates the presence of tannins.
2. To the extract, potassium dichromate solution was added. A precipitate indicates the presence of tannins.

2.3.9 Test for triterpenoids

In the test tube, 2 or 3 granules of tin was added and dissolved in 2 ml of thionyl chloride solution. Then, test solution was added. Production of pink color indicates the presence of triterpenoids.

2.3.10 Tests for fixed oils

1. Spot test: A small quantity of extract was pressed between two filter papers. Oil stains on paper indicate the presence of fixed oils.
2. Saponification test: To the extract, few drops of 0.5 N alcoholic potassium hydroxide were added along with a drop of phenolphthalein. The mixture was heated on a water bath for 1–2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils.

2.4 *In-vitro* Antioxidant Activity Using 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) Method

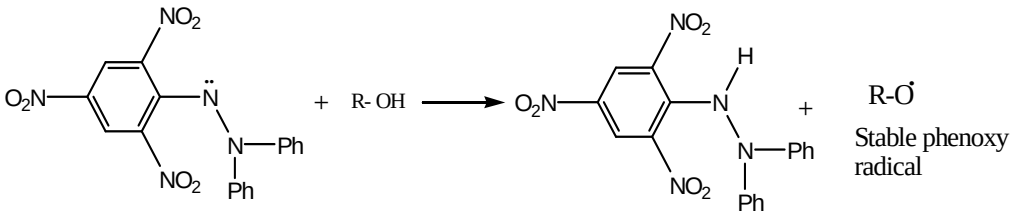
The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with hydrogen donor's changes to yellow in colour (see scheme 1). It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490 nm.

2.4.1 Preparation of the extracts

Accurately weigh 21 mg of each of the extracts and dissolve in 1 ml of freshly distilled DMSO separately to obtain solutions of 21 mg/ml concentration. These solutions were serially diluted separately to obtain the lower concentrations.

2.4.2 Preparation of standard solution

Accurately weigh 10 mg of ascorbic acid and dissolve in 0.95 ml of freshly distilled DMSO to get 10.5 mg/ml concentration. These solutions were serially diluted with DMSO to get the lower concentrations.



Purple DPPH
(2,2 diphenyl-1-picryl hydrazyl)

Yellow

Scheme 1. Reaction of DPPH with hydrogen donors

2.4.3 Procedure

To 200 µl of DPPH solution, 10 µl of each of the extract or standard solution was added separately in wells of the microtitre plate. The plates were incubated at 37℃ for 30 min and the absorbance of each solution was measured at 490 nm (Hwang et al. 2001), using UV-VIS spectrophotometer. The concentration which is showed 50% inhibition (IC_{50}) will be calculated.

3. RESULTS AND DISCUSSION

The percentage yield of methanolic extract of green and red custard apple fruits were found to be 6.23% and 8.42%, respectively (Table 1). Based on the phytochemical screening carried out, both the extracts showed the presence of alkaloids, carbohydrates, protein, amino acids, steroids, glycosides, flavonoids, fixed oils and absence of tannins and triterpenoids (Table 2).

In earlier studies reported that the *Annona squamosa* leaves extract and its constituents exhibit significant free radical activity [8]. In the antioxidant activity, the green custard apple at 1000, 500, 250 and 125 µg/ml showed 39.61%, 22.86%, 12.38% and 4.38% of inhibition, respectively. The red custard apple at 1000, 500, 250 and 125 µg/ml showed 70.67%, 39.62%, 36.76% and 10.86% inhibition, respectively. The IC_{50} value of green and red custard apple were >1000 and 650.00 µg/ml respectively (Table 3). This showed that the red custard apple showed better antioxidant activity than the green custard apple. However, the standard ascorbic acid showed better antioxidant activity than both the extracts. This may be due to the presence of active constituents which are present in custard apple. The high amount of phenolic and flavonoids in red custard apple may be responsible for the antioxidant activity. This results also well correlates with the previous report [9].

Table 2. Preliminary phytochemical screening of methanolic extracts of Green and red custard apple fruits

S. no	Chemical test	Methanolic extract of green custard apple fruits	Methanolic extract of red custard apple fruits
1	Alkaloids	+	+
2	Carbohydrates	+	+
3	Proteins	+	+
4	Amino Acids	+	+
5	Steroids and Sterols	+	+
6	Glycosides	+	+
7	Flavonoids	+	+
8	Tannins	-	-
9	Triterpenoids	-	-
10	Fixed oils	+	+

+ Present; - Absent

Table 3. DPPH radical scavenging activity of methanolic extracts of green and red custard apple fruits

Concentration (µg/ml)	% of inhibition		
	Methanolic extract of green custard apple fruits	Methanolic extract of red custard apple fruits	Standard ascorbic acid
1000	39.61	70.67	
500	22.86	39.62	
250	12.38	36.76	
125	4.38	10.86	
IC_{50}	>1000	650.00	58.24

Values are mean n=2

4. CONCLUSION

The active constituents in good amount were may be responsible for antioxidant abilities of the red custard apple. In conclusion, the red custard apple is the good candidate for further investigation of antioxidant properties. Further studies also required to isolate its active constituents and to determine the mechanism of action of the antioxidant effect.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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