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RESEARCH ARTICLE

In vitro Antioxidant Activities of Methanol and Aqueous Extract of *Annona squamosa* (L.) Fruit Pulp

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Abstract
The present study evaluated the antioxidant activity of the fruit of *Annona squamosa* by means of *in vitro* studies involving two different solvent extracts: methanol and aqueous. The antioxidant properties of the extract were determined by scavenging 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), lipid peroxidation (LPO), nitric oxide (NO), superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), reducing power and total antioxidant. The results showed that, compared to aqueous extract, a methanolic fruit extract of *A squamosa* has a higher percentage of inhibition of DPPH radical scavenging activity (97.99%), LPO (94.15%), NO scavenging activity (70.96%), O_2^- scavenging activity and $OH^\bullet$ scavenging activity (78.68% and 85.25%, respectively), total antioxidant activity (206 μg α-tocopherol/g) and reducing power (56.0 μg of ascorbic acid/g). The results obtained in the *in vitro* models clearly suggest that methanol extract has higher antioxidant activity than the aqueous extract due to a higher presence of phenolic and flavonodal constituents in the methanol extract.

1. Introduction

Reactive oxygen species, such as superoxide anion (O_2^-) radicals, hydroxyl radicals ($OH^\bullet$) and hydrogen peroxide (H_2O_2), can cause oxidative damage to macromolecules, including DNA, proteins, lipids, and small cellular molecules [1]. Free radicals have been implicated in the pathology of many diseases, including cancer, atherosclerosis, diabetes, and neurodegenerative disorders, in addition to aging [2]. Natural antioxidants represent promising tools to protect

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against the damage to the cellular organelles caused by these free radicals [3].

Herbs, shrubs, and trees are nature's gift to living beings. Many synthetic antioxidant components have been shown to possess toxic and/or mutagenic effects, and this finding has promoted research into the properties of naturally occurring antioxidants [4]. Vegetables and fruits contain several antioxidants, nutrients, vitamins, carotenoids, and several classes of phytochemicals that contribute to their total antioxidant capacity [5,6].

Antioxidants are compounds that inhibit or delay oxidation of other molecules by inhibiting both initiation and propagation of oxidizing chain reactions [7]. They protect organisms against radicals and are vital in neutralizing the destruction caused by radicals [8]. Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxycinnamic derivatives, catechins, etc.) of plant origin have been reported to act as scavengers and inhibitors of lipid peroxidation [9].

A squamosa Linn (Annonaceae), which is popularly known as custard apple, has been cultivated all over India. It is traditionally used for the treatment of dysentery, cardiac problems, fainting, worm infections, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors, and ulcers and is also an abortifacient [10,11]. The white creamy fruit pulp is soft and edible and is employed in preparing cool drinks and flavoring ice puddings. The unripe fruit, when dried and powdered, has been used to treat ulcers, diarrhea, dysentery, and atonic dyspepsia. It is also mixed with gram flour and used to destroy vermin. Ripe fruit made into paste with betel leaves accelerates suppuration in tumors [12]. Recently, *A squamosa* peel extract has been reported to have acaricidal, insecticidal and larvicidal activity, and it has also been used for biosynthesis of palladium and silver nanoparticles [13,14]. To our knowledge, there are no systematic studies relating to the *in vitro* free radical scavenging effect of *A squamosa* extract so far. Therefore, the present investigation was undertaken to study the antioxidant potential of two different fruit extracts of *A squamosa*.

2. Materials and methods

2.1. Plant material

Fresh *A squamosa* fruits were collected from a local market in Chennai, Tamilnadu, India. The fruits were identified by reference to Flora of the Presidency of Madras, by Gamble J.S., 1921 [15].

2.2. Extraction procedure

Methanol and aqueous extracts were selected because they have been reported to be among the best solvents for the extraction of antioxidant compounds. Fifty grams of the fruit of *A squamosa* was weighed accurately. The fruit paste was soaked in 50 mL of the two solvents separately and kept in a dark place for 3 days in a shaker. Carbon dioxide was released frequently. After 3 days, samples were filtered and the filtrates were kept in a water bath at about 40°C in order to concentrate them. The concentrated filtrates obtained were used for further studies at different concentrations.

2.3. Preliminary phytochemical screening

The methanolic and aqueous extracts of *A squamosa* were used to test the phytochemical compounds, including tannins, flavonoids, alkaloids, saponins, carbohydrate, phenols, triterpenoids, and steroids, in accordance with the methods of Trease and Evans, Harbourne [16,17], with a slight modification.

2.4. Determination of DPPH radical scavenging activity

A 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay was performed as described by Koleva et al [18]. Approximately 10 μL of different concentrations (15—500 μg/mL) of test sample solutions were added to 190 μL DPPH (150 μM) in ethanol solution. The solutions were later vortexed and incubated for 20 minutes at 37°C. The solvent alone acted as a blank. The decrease in absorbance of test mixtures (due to quenching of DPPH free radicals) was determined as being 517 nm, and the percentage of inhibition was calculated. Ascorbic acid was used as a standard. The IC_{50} values were determined as the concentration of the test mixture that gave 50% reduction in absorbance from that of the control blank.

2.5. Determination of lipid peroxidation inhibition

A 10% chicken liver homogenate was prepared using ice-cold potassium chloride (0.15 M) in a Teflon tissue homogenizer, and the protein content was adjusted to 500 mg/mL. In the control system, which comprised 1 mL of tissue homogenate, lipid peroxidation was initiated by adding 25 mM of ferrous sulfate, 100 mM of ascorbate and 10 mM of potassium dihydrogen phosphate. The volume was made up to 3 mL with distilled water and incubated at 37°C for 30 minutes. In the test system, homogenate was incubated with different concentrations of *A squamosa* extracts (15—500 μg/mL). The extent of inhibition of lipid peroxidation was evaluated by estimating the level of thiobarbituric acid reactive substances (TBARS) through measuring the absorbance at 532 nm [19]. Ascorbic acid was used as a positive control. The percentage of inhibition of lipid peroxidation was calculated by using the formula: % inhibition = (absorbance of control—absorbance of test sample)/absorbance of control × 100.

2.6. Scavenging of nitric oxide radical activity

Aqueous sodium nitroprusside at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite, the presence of which can be determined by the use of Greiss reagent. Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with 3 mL of different concentrations (15—500 μg/mL) of *A squamosa* extracts dissolved in methanol and incubated at 25°C for 150 minutes. The samples were then allowed to react with Greiss reagent. The absorbance of the

chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was measured at 546 nm [20]. Ascorbic acid was used as a positive control. The experiments were repeated in triplicate. The percentage scavenging of NO radical activity was calculated using the formula: % inhibition = (absorbance of control−absorbance of test sample)/absorbance of control × 100. The results were then analyzed.

2.7. Determination of O_2^- radical scavenging activity

The assay for O_2^- radical scavenging activity was based on a riboflavin−light−NBT system [21]. The reaction mixture contained 0.5 mL of phosphate buffer (50 mM, pH 7.6), 0.3 mL riboflavin (50 mM), 0.25 mL phenazinemethosulfate (PMS) (20 mM) and 0.1 mL nitrobuletetrazolium (NBT) (0.5 mM). Varying concentrations (15−500 μg) of 1 mL of *A squamosa* extract were added to this mixture. Reaction was initiated by using a fluorescent lamp to illuminate the reaction mixtures containing the different concentrations of *A squamosa* extracts. After 20 min of incubation, the absorbance was measured at 560 nm. The absorbance of the control was determined by replacing the sample with methanol. Ascorbic acid was used as a positive control. The percentage inhibition of O_2^- generation was calculated using the formula: % inhibition = (absorbance of control−absorbance of test sample)/absorbance of control × 100.

2.8. Determination of $OH^\bullet$ scavenging activity

$OH^\bullet$ scavenging activity of the *A squamosa* extracts was assayed using the method described by Halliwell and Gutteridge [22]. The reaction mixture contained 500 mL of 2-deoxyribose (2.8 mM) in a potassium phosphate buffer (50 mM, pH 7.4), 200 mL of premixed ferric chloride (100 mM) and ethylene diamine tetraacetic acid (100 mM) solution (1:1; v/v) and 100 mL of H_2O_2 (200 mM), either with or without the extract (100 mL) in different concentrations (15−500 μg). The reaction was triggered by adding 100 mL of 300 mM ascorbate, and the mixture was incubated for 1 hour at 37°C. A solution of thiobarbituric acid (TBA) in 1 mL (1%; w/v) of 50 mM sodium hydroxide (NaOH) and 1 mL of 2.8% (w/v; aqueous solution) trichloroacetic acid (TCA) was added. The mixtures were incubated for 15 minutes in a boiling water bath and then cooled. The absorbance was measured at 532 nm. The absorbance of the control was determined by replacing the sample with methanol. Vitamin C was used as a positive control. The $OH^\bullet$ scavenging activity of the extracts was calculated using the formula: % inhibition = (absorbance of control−absorbance of test sample)/absorbance of control × 100.

2.9. Determination of total antioxidant activity (TTA)

The total antioxidant activity was evaluated using the method described by Prieto et al [23]. An aliquot of sample solution/vitamin E (equivalent to 500 mg) was combined with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In the case of the control blank, methanol was used in place of the sample. The tubes were capped and incubated in a boiling water bath at 95°C for 60−90 minutes. The samples were then cooled at room temperature, and the absorbance was measured at 695 nm against the blank in a spectrophotometer. The total antioxidant activity was expressed as gram equivalents of vitamin E.

2.10. Determination of reducing power

The reducing power of extracts was determined according to the method of Yen and Chen [24]. Different amounts of each extract (25−800 μg/mL) in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferric cyanide [$K^3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, and the mixture was then centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The reducing power was expressed as gram equivalents of vitamin C.

2.11. Statistical analysis

The experimental results derived in the study were expressed as the mean from three parallel measurements. Linear regression analysis was used to calculate the IC_{50} values.

3. Results and discussion

Different concentrations of the methanolic and aqueous extract of *A squamosa*, ranging from 15 to 500 μg/mL, were tested for their antioxidant activity using different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the models.

3.1. Phytochemical screening

Phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, carbohydrates, phenols, steroids and glycosides. These are depicted in Table 1.

3.2. DPPH free radical scavenging property

The results pertaining to the free radical scavenging activity of the two different extracts, along with the standard reference vitamin C, are shown in Table 2. The model of stable DPPH free radicals can be used to evaluate the antioxidant activity in a relatively short time. The obtained data revealed that DPPH is a stable free radical and accepts either an electron or $OH^\bullet$ to become a stable diamagnetic molecule [25]. The DPPH free radical scavenging activity is a widely used model for evaluating the

Table 1 Preliminary phytochemical screening of methanolic and aqueous extracts of *A squamosa* fruit.

S. no.	Test	Methanol	Aqueous
1.	Alkaloids	+	+
2.	Flavonoids	+	+
3.	Saponins	+	+
4.	Carbohydrates	+	+
5.	Phenols	+	+
6.	Triterponoids	−	−
7.	Steroids	−	−
8.	Glycosides	+	+

free radical scavenging ability of various compounds [26]. The absorbance decreased, resulting in a color change from purple to yellow, as radicals were scavenged by antioxidants through the donation of hydrogen to form the stable DPPH molecule. The concentration of the sample necessary to reduce the initial concentration of DPPH by 50% (IC_{50}) under the experimental conditions was determined. A lower value of IC_{50} indicates higher antioxidant activity. The best free radical scavenging activity was obtained with the methanolic extract (IC_{50} 135.2 μg/mL), while the aqueous extract showed comparable levels of free radical scavenging activity with an IC_{50} value of 157.2 μg/mL. Several studies have evaluated the relationship between the antioxidant activity of plant products and their phenolic content. Substances that are able to perform this reaction can be considered as antioxidants and radical scavengers [27]. Higher total phenol and flavonoid contents lead to better DPPH scavenging activity [28].

3.3. Lipid peroxidation scavenging property

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation (LPO) by ferrous sulfate takes place either through a ferryl—perferryl complex [29] or through an OH radical by the Fenton reaction [30], thereby initiating a cascade of oxidative reactions. The efficacy of LPO scavenging activity of both the solvent extracts of *A squamosa* is shown in Fig. 1. In the present investigation, the methanolic fruit extract registered the highest LPO scavenging activity (70.98%) while the aqueous extracts of the samples showed a lower level of activity than that seen with the methanolic extract (53.89%). This may be because the antioxidants in the methanolic extract offer resistance to oxidative stress by numerous mechanisms, including scavenging free radicals and inhibiting lipid peroxidation, thereby preventing disease [31].

3.4. NO scavenging property

Similarly to reactive oxygen species, NO is also implicated in inflammation, cancer and other pathological conditions [32]. NO is a very unstable species under aerobic conditions [33]. The plant products have the property to counteract the effect of nitric oxide formation and, in turn, may be of considerable interest in relation to preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity of the plant products may also help to arrest the chain of reactions, initiated by excess generation of NO, that are detrimental to human health. The extracts exhibited moderate NO scavenging activity. Compared to aqueous extract (57.8%), the best NO scavenging activity was obtained with methanolic extract (79.25%) at a concentration of 500 μg (Fig. 2). The percentage of inhibition increased with increasing concentration of the extracts [32,34,35].

3.5. O_2^- scavenging property

Fig. 3 shows the O_2^- scavenging activity. The methanol extract displayed a greater degree of inhibition, at 69.28%, than did the aqueous extract, the figure for which was 57.21%. O_2^- radicals are formed by activated phagocytes, such as monocytes, macrophages, eosinophils, and neutrophils. The production of O_2^- radicals is an important factor in the killing of bacteria by phagocytes [36]. O_2^- radicals are biologically important active species because they decompose deleterious oxidative species, such as singlet oxygen and $OH^•$ [37]. In the PMS/NADHNBT system, the O_2^- derived from dissolved oxygen by the

Table 2 DPPH radical scavenging activity of methanolic and aqueous extracts of *A squamosa* fruit.

	% of inhibition		
Concentration (μg)	Methanol extract	Aqueous extract	Standard ascorbic acid
500	91.83	70.64	95.85
250	71.91	49.07	78.42
125	35.31	42.20	61.58
60	21.72	31.72	48.12
30	15.84	14.72	26.85
15	8.86	6.85	15.75
IC_{50} (μg)	135.2	157.2	124.75

Values are mean ($n = 3$).

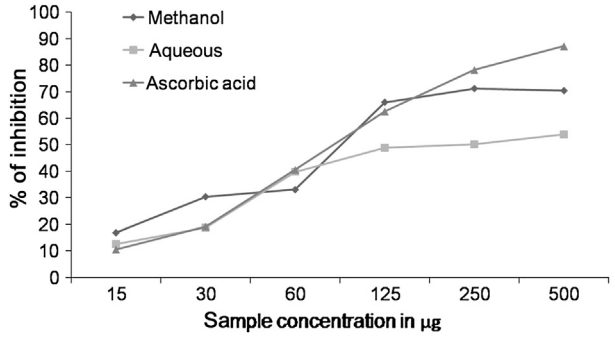

Figure 1 Lipid peroxidation scavening activity of methanol and aqueous extracts of AS and standard ascorbic acid. Values are mean ($n = 3$).

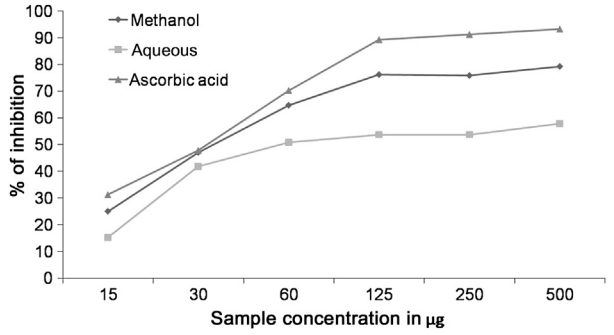

Figure 2 Nitric oxide (NO) scavening activity of methanol and aqueous extracts of AS and standard ascorbic acid. Values are mean ($n = 3$).

PMS/NADH coupling reaction reduces NBT, which absorbs at 560 nm. The decrease in absorbance in the presence of antioxidants indicates the scavenging of superoxide in the reaction mixture. It can be seen that superoxide scavenging activity increased as the concentration increased.

3.6. OH• scavenging property

Scavenging of OH• is an important antioxidant activity because of the very high reactivity of the OH•, which enables it to react with a wide range of molecules, such as sugars, amino acids, lipids, and nucleotides, found in living cells [38]. Thus, removing OH• is very important for the protection of living systems. The OH• scavenging potential of various solvents of *A squamosa* extracts has been shown in Fig. 4. The OH• scavenging activity increased with increasing concentration of the fruit extracts. In the present investigation, the OH• scavenging activity observed was in the range of 13.27—74.65% in methanolic extract and 8.92—60.56% in aqueous extract at a concentration of 15—500 μg. The OH• scavenging ability of the methanol extract was found to be higher than that of the aqueous extract.

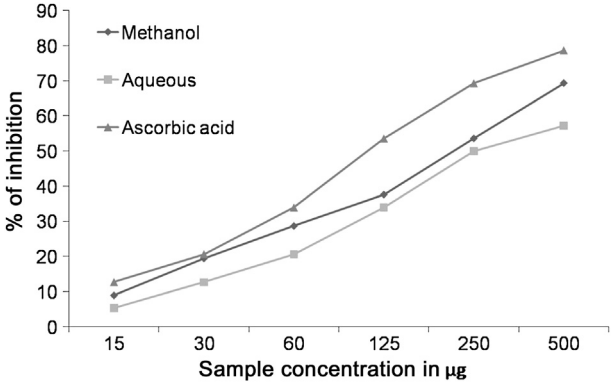

Figure 3 Superoxide radical scavening activity of methanol and aqueous extracts of AS and standard ascorbic acid. Values are mean ($n = 3$).

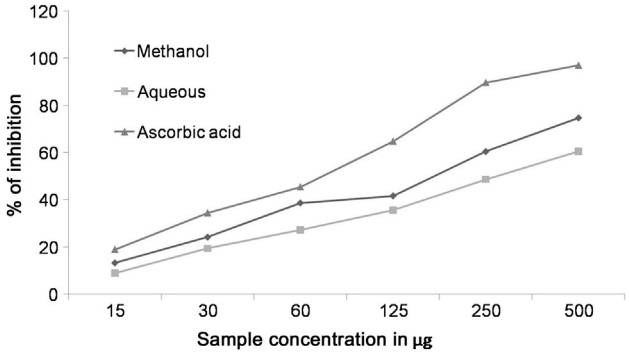

Figure 4 Hydroxyl radical scavening activity of methanol and aqueous extracts of AS and standard ascorbic acid. Values are mean ($n = 3$).

3.7. Reducing power property

Reducing power, which was used to measure the reductive ability of antioxidants, was evaluated by the transformation of Fe(III) to Fe(II) in the presence of the fruit extracts [39]. Antioxidants reduce the Fe^{3+} ferricyanide complex to the ferrous form by donating an electron. The color of the test solution then changes from yellow to different shades of green and blue [40]. The ability to reduce Fe(III) may be attributed to the hydrogen donating effect of phenolic compounds [41]. In the present study, 59 μg and 46 μg of the methanolic and aqueous fruit extracts showed antioxidant activity against the standard ascorbic acid, as is depicted in Table 3.

3.8. Total antioxidant property

Total antioxidant activity in the range of 245—376 mg ascorbic acid equivalents/g has been observed in *Phyllanthus* species [42]. The phosphomolybdenum method was used to evaluate the total antioxidant activity of the fruit fractions from *A squamosa*. The antioxidants can reduce Mo(IV) to Mo(V), and the green phosphate/Mo(V) compounds, which have an absorption peak at 695 nm, were generated subsequently [43]. The total antioxidant activity of *A squamosa* was measured by monitoring the absorbance of the reaction mixture at 695 nm. The total antioxidant activity of methanolic and aqueous extracts was 207 μg and 189 μg, respectively, against the standard α-tocopherol; this is shown in Table 3. A higher absorbance

Table 3 Total antioxidant activity and reducing power of methanolic and aqueous extracts of *A squamosa* fruit

Solvents	Total antioxidant activity (equivalent to μg of α-tocopherol)	Reducing power (equivalent to μg of ascorbic acid)
Methanol	207.0	59.0
Aqueous	189.0	46.0

Values are mean ($n = 3$).

value of the reaction mixture indicates stronger total antioxidant activity.

4. Conclusions

From the above results, it can be concluded that the methanolic extract of the fruit *A squamosa* showed more potent *in vitro* antioxidant activity, with higher percentage inhibition, than the aqueous extract of the fruit. Phytochemical analysis revealed the presence of phenolic and flavonoid compounds in the extracts. These compounds may be responsible for the free radical scavenging activity that was observed. Further studies are in progress to isolate the active principles from the extract and to elucidate the exact mechanism of action of the free radical scavenging effect.

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