PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF DIFFERENT SOLVENT EXTRACTS FROM Annona squamosa Linn. LEAVES

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AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration among all authors. Author MLMKA designed the study, carried out all the work, wrote the protocol, managed the analyses of the study, drafted the manuscript and carried out the corrections. Author FM performed the phytochemical study, statistical analysis and corrected the article. Author RM managed the literature searches and corrected the article. All authors read and approved the final manuscript.

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ABSTRACT
Plants are the chief source of natural compounds used for medicine, in which medicinal plants have attracted considerable interest and most attention for their wide variety of bioactive metabolites. Numerous studies have been carried out to screen extracts from medicinal plants for the presence of novel compounds and an investigation of their biological activities. Annona squamosa Linn. has extensively been used in the traditional and folkloric medicine and found to possess many biological activities. This study was carried out to evaluate the phytochemical screening and antioxidant activities of acetone, chloroform, hexane, methanol, Petroleum ether and aqueous extracts of Annona squamosa Linn. leaves. The antioxidant properties were determined by scavenging 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) method. Results of phytochemical analysis of extracts showed the presence of glycosides, saponins, tannins, flavonoids, phenols, etc. In-vitro antioxidant activities clearly suggest that methanol extract has higher antioxidant activity than the other extract due to a higher presence of phenolic and flavonoidal constituents in the methanol extract. These experimental findings would further establish the scientific basis of the traditional uses of the plant in the management of different conditions as well as control of different disease.

Keywords: Annona squamosa; antioxidant activity; phytochemical screening; free radicals.

1. INTRODUCTION
Antioxidants are compounds which inhibit or delay the oxidation of other molecules by both initiating and propagating oxidizing chain reactions [1]. They protect organisms against radicals that are important in neutralizing the damage triggered by radicals [2]. Reactive oxygen species (ROS) are small molecules derived from oxygen molecules including free oxygen radicals, such as superoxide (O₂⁻), hydroxyl (·OH), peroxyl (RO₂⁻), and alkoxyl (RO·) as well as hypochlorous acid (HOCI), ozone (O₃), singlet oxygen (¹O₂), and hydrogen peroxide (H₂O₂), which are non-radicals. These non-radicals are either
oxidizing agents or easily converted into radicals. Nitrogen-containing oxidants, such as nitric oxide (NO) peroxynitrite (ONOO•), nitrogen dioxide (NO2) are called reactive nitrogen species (RNS) [3,4]. They cause oxidative damage to macromolecules, including DNA, proteins, lipids, and small cellular molecules [5]. In addition to aging, free radicals is implicated in the treatment of other illnesses, including cancer, atherosclerosis, diabetes and neurodegenerative disorders [5]. Natural antioxidants are promising tools to protect against damage to cellular organelles caused by these free radicals. It has been documented that flavonoids and other phenolic compounds (proanthocyanidines, rosmarinic acid, hydroxycinnamic derivatives, catechins, etc.) of plant origin serve as scavengers and lipid peroxidation inhibitors [6].

Annona squamosa Linn. (Annonaceae) is a tiny tree or shrub well-branched with edible fruits called custard apple. Various sections of A. squamosa Linn. have been used in herbal medicine to cure different diseases such as dysentery, heart attacks, fainting, worm infections, constipation, hemorrhage, dysuria, nausea, fatigue, malignant tumors and ulcers and it is often an abortifacient [7,8]. Recently, A. squamosa Linn. peel extract was reported to have acaricidal, insecticidal and larvicidal effect, and was also used for palladium biosynthesis and silver nanoparticles [9,10]. Consequently, the present investigation was undertaken to study the phytochemical screening and antioxidant potential of various leaf extract solvents from A. squamosa.

2. MATERIALS AND METHODS

2.1 Chemicals and Solvents

All the chemicals used in this study were of analytical reagent grade from Xetra Biosolution. (Coimbatore, Tamilnadu, India).

2.2 Collection of Plant Material

The leaves of A. squamosa Linn. were collected from fields of Vaniyambadi, Tamilnadu, India, in December 2016. The leaves were identified and authenticated at the Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi. After identification, the plant material was processed for extraction procedure.

2.3 Preparation of the Plant Extract

The leaves of A. squamosa Linn. were thoroughly cleaned with water to remove dust particles and shade-dried at room temperature and reduced to coarse powder using a mechanical mixer. The powder was subjected to extraction by maceration using Acetone, Chloroform, Hexane, Petroleum ether, methanol and water to obtain their respective extracts. To 15 g of the powdered, 150 mL of solvent (Acetone, chloroform, Hexane, Petroleum ether, methanol and water) was added and stirred occasionally. The mixture was filtered through Whatman No.1 filter paper and the solvent was evaporated at 40°C to obtain a solid mass. Extract was stored at 4°C for further study.

2.4 Preliminary Phytochemical Screening

All extracts were subjected to preliminary qualitative phytochemical screening to detect the presence of glycosides, alkaloids, amino acids, flavonoids, carbohydrates, saponins, phenols, steroids, and tannins by standard methods [11,12,13].

2.4.1 Test for alkaloids

To 0.1 ml of each extract in the test tube, 2 – 3 drops of Dragendoff’s reagent was added. An orange red precipitate with turbidity denoted the presence of alkaloids.

2.4.2 Test for flavonoids

To 4 mg/ml of each fraction, a piece of magnesium ribbon was added followed by concentrated HCl drop wise. A colour change ranging from orange to red indicates flavones; red to crimson indicates flavonoids.

2.4.3 Test for glycosides

10 ml of 50% H2SO4 was added to 1ml of the filtrates in separate test tubes and the mixtures heated for 15mins followed by addition of 10 ml of Fehling’s solution and boiled. A brick red precipitate indicated presence of glycosides.

2.4.4 Test for reducing sugars

To 1 ml of each fraction in separate test tubes, 2.0 ml of distilled water were added followed by addition of Fehling’s solution (A + B) and the mixtures were warmed. Appearance of brick red precipitate at the bottom of the test tube indicates the presence of reducing sugar.

2.4.5 Test for saponins

Half gram of the powdered plant material was dispensed in a test-tube and 5.0 ml of distilled water was added and shaken vigorously. A persistent froth that lasts for about 15 minutes would indicate the presence of saponins.
2.4.6 Test for steroids

Two milliliters of the extracts were evaporated to dryness in separate test tubes and the residues dissolved in acetic anhydride followed by addition of chloroform. Concentrated sulphuric acid was added by means of a pipette via the side of the test tubes. Formation of brown ring at the interface of the two liquids and violet colour in the supernatant layer denotes the presence of steroids.

2.4.7 Test for tannins

Two ml of each extract was diluted with distilled water in separate test tubes, 2 – 3 drop of 5% ferric chloride (FeCl₃) solution was added. A green – black or blue colouration would indicate tannin.

2.4.8 Test for terpenoids

2 ml of the organic extract was dissolved in 2 ml of chloroform and evaporated to dryness. 2 ml of concentrated sulphuric acid was then added and heated for about 2 min. Development of a greyish colour indicates the presence of terpenoid.

2.4.9 Test for phenols

A fraction of the extracts was treated with aqueous 5% ferric chloride. Formation of deep blue or black colour.

2.4.10 Test for amino acids

2 ml of filtrate was treated with 2-5 drops of ninhydrin solution placed in a boiling water bath for 1-2 minutes. Formation of purple colour.

2.4.11 Test for quinones

A small amount of extract was treated with concentrated HCL. Formation of yellow precipitate (or colouration).

2.4.12 Test for carbohydrates

Crude extract was mixed with 2 ml of iodine solution. A dark blue or purple coloration indicates the presence of the carbohydrates.

2.5 DPPH Free Radical Scavenging Activity

The antioxidant activity of extracts was evaluated by 1,1-diphenyl 2-picrylhydrazyl (DPPH) radical scavenging assay which was described by [14] with slight modifications. 0.1 mM DPPH solution in methanol was mixed with 1 ml of animal extract solution of varying concentrations (5, 25, 50, 100, 125 and 150 μg/ml). Ascorbic acid was used as reference standard. Mixture of 1ml methanol and 1ml DPPH solution was used as control. The IC₅₀ values were determined as the concentration of the test mixture that gave 50% reduction in absorbance from that of the control blank. The reaction was carried out in triplicate and the decrease in absorbance was measured at 517nm after 30 minutes in dark using UV-Vis spectrophotometer (UV-VIS A&E Lab). The inhibition % was calculated using the following formula:

\[
\text{Percentage inhibition of DPPH activity} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100
\]

Where, Ac is the absorbance of the control, As is the absorbance of the sample.

2.6 Statistical Analysis

All the experiments were carried out in triplicate. Means and standard deviations were calculated using computer software Microsoft Office Excel 2013 (Microsoft, Redmond, Washington, USA). The results were expressed as percentage decrease with respect to control values and compared by one-way ANOVA analysis [15]. IC₅₀ values obtained by linear regression statistics based on least squares method. A difference was considered statistically significant if \( p \leq 0.05 \).

3. RESULTS

The phytochemical qualitative analysis revealed the presence and absence of Alkaloids, Flavonoids, Glycosides, Reducing Sugar, Saponins, Steroids, Tannins, Terpenoids, Phenols, Amino Acids, Quinones and Carbohydrates in different extracts as reported in Table 1.

The DPPH radical scavenging activity of A. squamosa leaf extract showed positive results against all the solvents aqueous, methanol, acetone, chloroform, petroleum ether and hexane. The highest DPPH scavenging activity was observed on methanal extract (Table 2). Other solvents like aqueous, chloroform, petroleum ether, acetone, and hexane also showed positive response towards antioxidant activity. The IC₅₀ value of Methanolic extract showed better value (96.09±1.3) followed by aqueous (148.09±1.2), chloroform (234.69±0.5), petroleum ether (361.22±0.7), acetone (396.43±0.9), and hexane (438.79±0.1). However, the standard ascorbic acid (23.23±1.7) showed higher antioxidant activity than the extracts.
Table 1. Phytochemical screening of leaf extract of *Annona squamosa*

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Acetone</th>
<th>Chloroform</th>
<th>Hexane</th>
<th>Methanol</th>
<th>Petroleum Ether</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. IC$_{50}$ values of various leaf extract of *Annona squamosa*

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Acetone</th>
<th>Chloroform</th>
<th>Hexane</th>
<th>Methanol</th>
<th>Petroleum Ether</th>
<th>Aqueous</th>
<th>Ascorbic acid (standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>12.15±1.3</td>
<td>32.22±1.9</td>
<td>13.67±1.4</td>
<td>42.55±1.6</td>
<td>18.75±2.1</td>
<td>31.66±1.3</td>
<td>49.67±2.4</td>
</tr>
<tr>
<td>100</td>
<td>17.35±2.4</td>
<td>39.17±2.4</td>
<td>17.22±2.5</td>
<td>48.69±1.9</td>
<td>23.42±2.4</td>
<td>40.10±1.7</td>
<td>57.39±3.1</td>
</tr>
<tr>
<td>200</td>
<td>23.14±2.9</td>
<td>45.44±2.9</td>
<td>25.35±3.0</td>
<td>56.48±2.4</td>
<td>28.21±2.9</td>
<td>46.46±2.2</td>
<td>67.69±3.2</td>
</tr>
<tr>
<td>400</td>
<td>29.77±3.2</td>
<td>51.64±3.3</td>
<td>29.78±3.3</td>
<td>65.09±2.9</td>
<td>34.61±3.4</td>
<td>55.89±2.8</td>
<td>75.51±2.9</td>
</tr>
<tr>
<td>600</td>
<td>35.89±3.6</td>
<td>59.93±3.5</td>
<td>33.14±4.1</td>
<td>74.04±3.4</td>
<td>39.48±3.9</td>
<td>66.46±3.3</td>
<td>84.58±2.6</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>396.43±0.9</td>
<td>234.69±0.5</td>
<td>438.79±0.1</td>
<td>96.09±1.3</td>
<td>361.22±0.7</td>
<td>148.09±1.2</td>
<td>23.23±1.7</td>
</tr>
</tbody>
</table>
4. DISCUSSION

Natural products play a major role as active substances, model molecules for the discovery and validation of drug targets. Plants are the chief source of natural compounds used for medicine, in which medicinal plants have attracted considerable interest and most attention for their wide variety of bioactive metabolites. The preliminary phytochemical tests revealed that the leaves of the plant possess alkaloids, glycosides, flavonoids, tannins etc. The bioactivity of plant extracts was attributed to the presence of phytochemical constituents; for example, plants rich in saponins have immune-boosting, anti-inflammatory [16], antiviral, and antibacterial properties [17]. Similarly, tannins have been reported to have antioxidant potential due to their basic character that allows them to react with proteins to form stable water-soluble compounds, thereby killing bacteria by directly damaging their cell membrane. Flavonoids are a major group of phenolic compounds reported for their antiviral, antimicrobial, and spasmylytic properties [16]. Alkaloids isolated from plants are commonly found to have antimalarial, antiasthma, anticancer and antimicrobial properties [18]. Plant glycosides are found to play essential role in the treatment of heart disorders and are known to possess beneficial effects on cardiac arrhythmias. Terpenoids have been reported to have anti-inflammatory, antimalarial, antibacterial, and antiviral activities and reported to inhibit cholesterol synthesis [19].

The DPPH free radical scavenging model can be used to evaluate the antioxidant activity in a relatively short time. DPPH is a stable free radical and accepts either an electron or OH* to become a stable diamagnetic molecule [20]. The decreased absorbance results 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 [21]. The color of the test solution then changes from yellow to different shades of green and blue [22]. The ability of these extracts to reduce Fe³⁺ may be attributed to the hydrogen donating effect of phenolic compounds [23].

The antioxidant activities of methanol, chloroform and aqueous extracts of A. squamosa Linn. was evaluated by [24]. Among the three accessions with different solvents used, the maximum antioxidant activity found the methanolic extract of A. squamosa Linn. leaves followed by other solvents. [25] in the DPPH radical scavenging test, the methanol-soluble extract proved to be the most powerful with an IC₅₀ value of 103.5 μg / ml. [26] assessed the potential for free radical scavenging from the leaves of the plant Annona squamosa Linn. using different antioxidant detection models. The Methanolic extract at 1000 μg/mL showed maximum scavenging activity. The increased radical scavenging activity of the leaf extract could be due to the high content of phenolic compound. It has been reported that phenic compounds were the main antioxidant components, and their total contents were directly proportional to their antioxidant activity [27]. Our reports are coincides considerable DPPH scavenging activity which was better than the reports of earlier studies (Thakur and Singh 1965). Therefore, in this study, the presence of the flavonoids and phenols in all the tested extracts of A. squamosa Linn. leaves might have contributed to the antioxidant activity.

5. CONCLUSION

From the above results, it can be concluded that the methanol extract of the leaves of A. squamosa Linn. have antioxidant activities. Further studies are needed to isolate the active components of the extracts and to elucidate the exact mechanism of action of antioxidant activities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES