Assessment of preliminary phytochemical screening, polyphenol content, flavonoid content, and antioxidant activity of custard apple leaves (*Annona squamosa* Linn.)

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**Abstract.** *Annona squamosa* Linn. (custard apple) has been used extensively in India traditional medicine for treatment of dysentery, cardiac problems, fainting, worm infections, constipation, hemorrhage, dysuria, fever. This study assessed the phytochemicals present in the leaves of *A. squamosa*. The phytochemical was extracted separately with distilled water and 96% ethanol. A wide variety of pharmacologically active compounds such as alkaloids, coumarins, tannins, cardiac glycosides, flavonoids, carbohydrates, phenols, and saponins were found to present in the leaves of *A. squamosa*. However, terpenoids and phlobatannins were absent in this plant. This study also assessed the contents of phenolics and flavonoids for their in vitro antioxidant activity. The total polyphenol content of ethanol extract of *A. squamosa* measured by Folin-Ciocalteau reagent in terms of gallic acid equivalent achieved 242.88±6.13 mg GAE/g. The flavonoid content of the plant sample as quercetin equivalent achieved 82.61±0.82 mg QE/g. The antioxidant activity of the ethanol extract of *A. squamosa* was correlated with total phenolic and flavonoid content with values IC50 of 132.96±1.33 µg/ml, 64.74 ±0.52 µg/ml for DPPH and ABTS scavenging activity, respectively.

1. **Introduction**

Nowadays, natural products become more and more popular in many topics of scientific researches due to their chemical composition, following with two main reasons: applications as natural options for food ingredients and significant influences on human health based on their antioxidant characteristics. Moreover, medical plants play a vital role in the health preservation and care worldwide [1-7]. *Annona squamosa* L. (Sugar apple, custard apple, sitaphal) belongs to the Annonaceae family including about 135 genera and 2300 species. *Annona. squamosa* is an evergreen plant mainly found in tropical and subtropical regions such as Malaysia, Thailand, Laos, and Vietnam. In recent years, cultivating *Annona squamosa* L. have been receiving a great deal of public attention.
due to the essential oil extracted from its flowers and leaves. The previous phytochemical investigations made on the plant have shown that they possess a wide variety of compounds like diterpenes (DITs), alkaloids (ALKs), and cyclopeptides (CPs)[1-2]. Numerous study projects on A. squamosa have discovered that it has antioxidant, antiparasitic, insecticidal, and so on. [7]. Moreover, A. squamosa peel extract has been described to have larvicidal, acaricidal, insecticidal activity, and it has also used for biosynthesis of silver nanoparticles palladium [8]. Previous studies have demonstrated the function of the aqueous leaf extract of A. squamosa to ameliorate hyperthyroidism [9]. In natural, there are three main types of plant chemicals including alkaloids, terpenoids, and phenolic metabolites. Among these three groups, phenolic compounds play a vital role in dietary applications and extensively researched. Discovering new and safe antioxidants from natural sources become a great interest for applications in functional foods. Antioxidants play an essential role in the human protection body against free radical disorders acting as radical scavengers. Phenolics belongs to a class of chemical compounds including simple phenols and polyphenols. Polyphenols can reduce and prevent damage to the human body due to free radicals promote. Flavonoids can produce mechanisms that may inhibit invasion and kill tumor cells. The present study was carried out to evaluate phytochemical screening, total polyphenol, flavonoids content, and antioxidant activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-and-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) of the extract of A. squamosa.

2. Materials and methods

2.1. Sample collection and preparation
Annona squamosa L. seeds were collected from Duyen Hai district, Tra Vinh province, Vietnam in January 2019. First, the seeds were removed. They then were washed with water and kept on absorbent paper towels at room temperature to dry to moisture contents of 10% and ground to powder.

2.2. Qualitative Phytochemical Analysis
About 25g dried powder of the leaves was extracted with different solvents of increasing polarities: ethanol 96% and water. Plant extracts hexane, ethyl acetate, methanol, and methanol water were subjected to chemical tests for the presence of sterols, triterpenoids, carotenoids, tropolone, quinones, alkaloids, and flavonoids [9-12].

2.3. Quantitative Phytochemical Analysis

2.3.1. Determination of total phenolic content (TPC)
First, the 1mL extract was pipetted into a test tube containing 1 mL Folin-Ciocalteu reagent 10% (v/v). After 5 minutes, 1 mL Na2CO3 20% (w/v) was added to the sample. Next, the mixture was vigorously shaken and incubated for 30 minutes in the dark. Finally, the absorbance was spectrophotometrically measured at 765 nm, and the results were shown in mg of gallic acid equivalents per gram of sample (mg GAE/g) [13-15].

2.3.2. Determination of total flavonoid content (TFC)
Based on the aluminum chloride colorimetric method, the total flavonoid content was determined [13-15]. Mixing 0.5 mL the extract with 0.15 mL 5% NaNO2. After 5 minutes, mixing with 0.3 mL 10% AlCl3. Then, 1mL 1M NaOH and 2 mL distilled water was added and vigorously shaken. The absorbance was spectrophotometrically measured at 510 nm.
2.3.3. **Determination of antioxidant capacity**

**DPPH**

The antioxidant activity of the individual essential oil was tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Analytical chemistry laboratory - University Nguyen Tat Thanh). 600 µL DPPH (OD 517 nm = 0.0403 ± 0.013) into 500 µL solution sample. The sample solution with pre-concentration and the mixed the stable at room temperature in the dark within 37 min. The optical measurement of the mixture by UV/VIS - 1800 Shimadzu Spectrometer at 517 nm. Blank sample, but 500 µL solution replaced EtOH 99.7%. Standard sample: Vitamin C (0.1g ÷ 0.01) was dissolved EtOH 99.7% into volume flask 100mL, in the dark (C = 100 µL/mL). The percent DPPH scavenging effect was calculated by using the following equation [16-18]:

\[
\text{% Scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

**ABTS**

Based on Thaipong and Kamonwannasit, ABTS scavenging activity was used [14-16]. First, adding 10 mL of 2.6 mM K₂S₂O₈ in 10 mL of 7.4 mM ABTS solution in 15 hours. Next, preparing the working solutions by putting 1ml of stock solution into 60 mL of methanol to take the absorbance value of 1.1 ± 0.02 at 734 nm. Then, 0.5 mL of sample was added with 1.5 mL of the working solution for 30 minutes RT. Using UV-VIS spectrophotometer measured the mixture at 734 nm. The percentage of ABTS decolorization of the sample was determined according to the equation:

\[
\text{% Decolorization} = [1-(\text{ABS}_{\text{sample}}/\text{ABS}_{\text{control}})] \times 100
\]

### 3. Results

#### 3.1. Qualitative Phytochemical Analysis and percentage yields

Table 1 shows the result of phytochemical constituents of *A. squamosa* leaves in water and 96% ethanol. Evaluation of chemical components of *A. squamosa* leaves revealed the presence of alkaloids, coumarin, tannin, cardiac glycosides, flavonoids, carbohydrates, phenols.

The previous study demonstrates the phytochemistry of Annona squamosa leaf including alkaloids, flavonoids, phenols, saponins, glycosides in water, methanol, chloroform, and petroleum ether extracts [19]. Moreover, the previous study demonstrated the phytochemical studies of the different extracts solution, including water, methanol, chloroform, petroleum ether, and hexane. The methanol and water extracts of seed and leaf had more positive results for alkaloids, oils, tannins, phenols, and flavonoids [20]. The thin layer chromatography scanning of the *Annona squamosa* done by Jayshree et al and the literature survey showed that chief phytoconstituent of this plant is anonaine and some biological compounds (Linalool, Borneol, Eugenol, Farnesol, and Geraniol) [21].

**Table 1:** Phytochemical constituents of *A. squamosa* leaves in different solvent extracts.

| No | Compounds                  | Water | Leaves | Ethanol |
|----|---------------------------|-------|--------|--------|
| 1  | Alkaloids                 | ++    | ++     |        |
| 2  | Saponins                  | ++    | +      |        |
| 3  | Coumarins                 | +     | +      |        |
| 4  | Flavonoids                | ++    | ++     |        |
| 5  | Carbohydrates             | +     | +      |        |
| 6  | Cardiac Glycosides        | +     | +      |        |
| 7  | Phlobatannins             | -     | -      |        |
| 8  | Terpenoids                | -     | -      |        |
| 9  | Phenols                   | ++    | ++     |        |
| 10 | Tannins                   | ++    | +      |        |

+++ = Strong positive test, + = Weak positive test, - = Negative tests
3.2. Quantitative Phytochemical Analysis

Total polyphenol and flavonoid content

Phenolic compounds extracted from plants have been receiving a great deal of public attention during recent years. In facts, phenolic compound effects on diet health interaction in the human body. Polyphenols are the dominant plant compounds with antioxidant activity. Phenolic compounds belong to antioxidants, which acts as free radical terminators. The free radical scavenging activity frequently correlates with the total phenolic content in plants [18]. Total polyphenol content was performed as mg gallic acid equivalents per gram of dried sample (mg QE/g). Total polyphenol content was 242.88±6.13 mgGAE/g extract (figure 1). The total flavonoid content was performed as mg quercetin equivalents per gram of dried sample (mg QE/g). Flavonoid contents were 82.61±0.82 mg QE/g dry weight ethanol extract in A. squamosa leaves (figure 2). The results greatly recommend that the phenolics are essential components of this plant, and some of the pharmacological effects could be attributed to the presence of this valuable component. The literature survey showed that various extracts of seed, leaf, root and other parts of Annona squamosa have flavonoids and phenols [19]. We have recently reported that the total flavonoids of Annona squamosa were estimated for different extracts using quercetin as standard, among which water extract of leaf showed the high level of flavonoids of about 9.28 mg/g, followed by water and methanol extract of seed [20]. Moreover, in the total phenolic content estimation by folin assay, among the various extracts of leaf showed high phenolic content of about 13.0098 mg/g of extract, followed by the water and methanol extract of seed [20].

![Figure 1. Standard gallic acid solution (µg/ml)](image1)

![Figure 2. Standard quercetin solution (µg/ml)](image2)

DPPH

There are different techniques for estimating the antioxidant activity of both synthetic compounds and natural. The DPPH assay was a rapid and low-cost method, which usually used for evaluation of the antioxidative potential of different natural stocks. The DPPH scavenging assay is broadly applied to assess the free radical scavenging of plant extracts thanks to its sensitive, simple, rapid. Antioxidants can remove the radical by hydrogen donation, which results in a decrease of DPPH absorbance at 515 nm. The IC50 value was the concentration of the sample which inhibited percentage reaches 50%. Therefore, IC50 values are negatively correlated to the antioxidant activity, the lower IC50 value means the highest antioxidant activity of the tested sample. Table 2 shows the DPPH radical scavenging activity of ethanolic extract of A. squamosa. Ethanol extract (IC50 achieved 132.96±1.33 µg/ml) showed potent antioxidant activity. This activity might be due to the presence of phenolic compounds. The IC50 value of standard ascorbic acid was 2.64±0.02 µg/ml. The previous study showed the antioxidant analysis done for different extracts by FRAP assay, among which water extract of seed showed the high level of antioxidant of about 14.16 mg/g, followed by water and methanol extract of leaf. The amount of antioxidant present in 1 mg of extract was represented [20].
Table 2. DPPH radical scavenging activity of ethanolic extract of *A. squamosa* and ascorbic acid

| Concentration (µg/ml) | DPPH%  | Concentration (µg/ml) | DPPH%  |
|----------------------|--------|-----------------------|--------|
|                      | A. squamosa | ascorbic acid          |
| 24                   | 11.56 ± 0.33 | 19.42 ± 0.24          |
| 48                   | 17.66 ± 0.31 | 36.59 ± 0.26          |
| 72                   | 27.70 ± 0.45 | 55.72 ± 0.31          |
| 96                   | 35.96 ± 0.36 | 74.54 ± 0.46          |
| 140                  | 52.65 ± 0.44 | 96.68 ± 0.34          |

**ABTS**

Proton radical scavenging is an essential characteristic of antioxidants. ABTS acts as a protonated radical, which has a characteristic maximum at 734 nm. ABTS plays a vital role in determining the antioxidant capacity of hydrogen-donating antioxidants. The ABTS scavenging ability of *A. squamosa* with value IC<sub>50</sub> of 64.74 ±0.52 µg/ml, and ascorbic acid with an IC<sub>50</sub> value of 2.64±0.02 µg/ml (Fig 3). The previous literature survey showed in vitro antioxidant studies of *A. squamosa* leaves in quenching ABTS with an IC value of 40 µg/ml [21].

![Graph](image)

**Fig. 3.** ABTS scavenging activity of ethanolic extract of *A. squamosa* and ascorbic acid.

**Conclusion**

*Annona squamosa* Linn. (custard apple) is a precious medicinal plant that has been used for a long time in Vietnam. Previous studies have shown that *Annona squamosa* Linn. has the same pharmacological effects as a large succulent, aromatic perennial herb. *Annona squamosa* Linn. was subjected to phytochemical research resulted in the isolation of several flavonoids. Moreover, the plant exhibited antioxidant, anti-inflammatory, cytotoxic and antimicrobial activities. This study is to verify the anti-oxidant potential and to evaluate total polyphenol, flavonoid contents in *Annona squamosa* Linn. leaves. The phenolic content was found 242.88±6.13 mg GAE/g extract. Flavonoid content was 82.61±0.82 mg QE/g dry weight ethanol extract in *Annona squamosa* leaves. The antioxidant activity of the ethanol extract of *A. squamosa* was correlated with total phenolic and flavonoid content with values IC<sub>50</sub> of 132.96±1.33 µg/ml, 64.74 ±0.52 µg/ml for DPPH and ABT S scavenging activity, respectively.

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