INTRODUCTION
Free radicals are the inevitable by-products resulting from biological redox reactions. At high concentrations, they produce oxidative stress, a harmful process that can damage cell function and structures. They have been reported to inactivate enzymes and damage important cellular components causing tissue injury through covalent bond and lipid peroxidation [1]. Research in the medical field reports the implication of free radicals induced oxidative stress in the development of many diseases such as inflammatory diseases, atherosclerosis, aging, diabetes, and cancer [2-4]. The antioxidants are associated with the reduction of free radical generation. Thus, it may be beneficial to recover normal function and treat such diseases. Antioxidants are a crucial defense against free radical-induced damage and are critical in maintaining optimum health and wellbeing [5]. In recent years, there has been an increased interest in the therapeutic use of antioxidants in the treatment of diseases associated with oxidative stress. Antioxidant-rich diet and natural antioxidant supplements as part of a healthy lifestyle are now being recognized to protect health from oxidative stress [6]. Plants and other organisms have evolved a wide range of mechanisms to surmount this problem with a wide variety of antioxidants for molecules and enzymes [7, 8].

Annona squamosa Linnaeus (A. squamosa) is a small, well-branched tree of the family of Annonaceae. The plant is native to the tropical Americas and is cultivated mainly for its edible fruit commonly called “sugar apple”. It was later taken to the Philippines and Asia via West Indies. A. squamosa grows at altitudes of 0 to 2,000 m and does well in hot, dry climates; at much lower altitudes than many of the other fruit bearers in its family [9, 10]. In Lebanon, the A. squamosa crop is gaining in popularity and has been cultivated along the coastal zone at an altitude of 0-200 m above sea level [11]. All parts of A. squamosa tree are traditionally used to treat various diseases [12]. It is considered to be a good source of natural antioxidants for various diseases [13]. It has been reported to possess a wide variety of pharmacological activities [14-18]. Therefore, this investigation was carried out to study the phytochemical screening, evaluate the total phenol content and investigate the antioxidant potential of Lebanese A. squamosa. Another aim of this research was to compare the two solvent systems in terms of their extraction efficacy of potent antioxidants compounds from different parts of A. squamosa plant.

MATERIALS AND METHODS

Chemicals
Ethanol and methanol used in this study were analytical grade purchased from Sigma-Aldrich, Lebanon. All chemicals 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid (GA) powder, Folin-Ciocalteu’s Reagent (FCR), sodium bicarbonate, iron chloride, magnesium shavings, ammonium acetate buffer, sulfuric acid, hydrochloric acid used were purchased from VWR, Lebanon. The water used in all procedures was a distilled one and obtained from TKA MICROMED apparatus for water distillation.

Apparatus
All samples were weighed using a RADWAG XA 82/220/2X laboratory balance. The dried leaves and the dry bark were ground using a POLYMIX grind mill. The extracts were concentrated using a Ciocalteu’s Reagent (FCR), sodium bicarbonate, iron chloride, magnesium shavings, ammonium acetate buffer, sulfuric acid, hydrochloric acid used were purchased from VWR, Lebanon. The water used in all procedures was a distilled one and obtained from TKA MICROMED apparatus for water distillation.

Collection and preparation of plant material
The samples of A. squamosa plants studied in this work were collected directly from the producer who owned the trees. The trees were grown in Batroun, a coastal city in northern Lebanon, which rises 80 to
100 m from sea-level. The plant has been identified by Pr. Jean HABB (Professor of Pharmacognosy at the Lebanese University) and confirmations were done through the Flora of the presidency of Madras, by Gamble J. S. 1921 [19]. The voucher specimens (No. 1803-1804) are deposited at Pharmacognosy Department, Faculty of Pharmacy of the Lebanese University (fig. 1).

A. squamosa leaves and bark were collected in the month of February and the beginning of March 2018 from Annona trees. The fruit was collected in the month of January 2018. Different plant parts, namely bark and leaves, were isolated from A. squamosa tree. Different fruit parts, namely pulp, and pericarp were isolated. The leaves were either used as fresh or shade dried on a well-ventilated rack for 3 w. The bark was also shade dried for two months. The dried samples were ground separately into fine powder and stored at room temperature for further use.

Preparation of plant extracts

Two solvents systems were used for the extraction: 80 % methanol (v/v in water) and 80 % ethanol (v/v in water). A. squamosa plant extracts were obtained by macerating 20 g finely cut fresh leaves/20 g pulverized dried leaves/20 g pulverized dried bark/96 g pericarp, or 96 g pulp (filtrated using filter paper) with frequent agitation for 24 h with 100 ml of the solvent system. The obtained extracts were then filtered twice through Whatman’s filter paper No. 1 and the filtrates were concentrated under vacuum using a rotary evaporator and then filtered twice through Whatman’s filter paper No. 1 and the filtrates were stored at room temperature for further use.

Physicochemical screening

Prepared plant extracts were analyzed for the presence or absence of chemical constituents like hydroxytable and condensed tannins, flavonoids, and saponins. Alkaloids tests were performed directly on the plant material. The change of color or formation of precipitate was observed when the test reagent was added to the prepared sample for the physicochemical test. The result was recorded as present (+) or absent (-) depending on the outcome of the test. All the experiments were executed in triplicates.

Determination of total phenol content (TPC)

Total phenolic contents of A. squamosa parts were estimated using the Folin–Ciocalteu reagent based spectrophotometric assay [20]. Gallic acid is used as a standard for determining the phenol content of various extracts. For this purpose, 100 µl of different concentrations (0.05–0.88 g. ml⁻¹) of the test extract solutions were combined with 0.5 ml of Folin–Ciocalteu reagent (2 N) and the mixture was incubated for 5 min in the dark. Then 2 ml of sodium carbonate aqueous solution (7 % w/v) was added. Samples were incubated in darkness for 30 min at room temperature. The absorbance of the reaction mixture was measured at 760 nm against a blank using a UV–VIS spectrophotometer. Distilled water and 2 ml of Na₂CO₃ solution acted as a blank. A standard curve was plotted using different concentrations of gallic acid. The absorbance obtained was plotted against the acid concentration. The total phenolic content was calculated as mg of gallic acid extract (GAE) per g of dry A. squamosa extract using the following formula (1):

\[
GAE = \left( \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \right) \times \text{m (g)}
\]

Where: -GAE = mg equivalent of GAE per gram of dry extract used.
-\( C \) is the concentration of gallic acid (mg. ml⁻¹) calculated by the calibration curve regression equation.
-\( V \) is the volume of plant extract solution
-\( m \) is the mass of a plant extract used to prepare the test extract solution used.

Further calculations were done to obtain the total phenol content in mg GAE per gram of initial plant and fruit part used.

Determination of antioxidant activity

The antioxidant activities of A. squamosa dried leaves, fresh leaves, dried bark, pulp, and pericarp of the fruit were assessed using DPPH assay as described by Kumawat et al. [21]. 1 ml of the methanolic extract solutions of different concentrations (0.001–0.26 mg. ml⁻¹) was added to 1 ml of a methanolic solution of DPPH (81.14 µM) in a test tube, followed by vigorous stirring. After 30 min of incubation in the dark, the decrease in absorbance of each mixture (due to quenching of DPPH free radicals) was determined at 517 nm against a blank (methanol) using a UV-VIS spectrophotometer. The percentage scavenging activity of the DPPH radicals was calculated according to the following formula (2):

\[
\text{% DPPH scavenging activity = } \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \times 100
\]

Where: -\( A_{\text{control}} \) is the absorbance of the mixture of methanol and DPPH solution
-\( A_{\text{sample}} \) is the absorbance of the mixture of the sample extract/standard and DPPH solution

Based on graphic values of the percentage of DPPH inhibition vs extract concentration, the half-maximal inhibitory concentration (IC₅₀) (the concentration of the extract needed to inhibit the 50% of the DPPH) of each extract was estimated. The antioxidant activities of all the samples were compared to the antioxidant activity of ascorbic acid, i.e. ascorbic acid was used as a reference standard.

Statistical analysis

The experimental runs and the analyses were carried out in triplicate. The experimental results derived in the study were expressed as the mean±standard deviation (SD). Linear regression analysis was used to calculate the IC₅₀ values. Data were analyzed through independent samples t-test using SPSS statistics 9.0 software, the same software was used to test IC₅₀.

RESULTS AND DISCUSSION

Extraction yields

The extraction yield depends on the nature of the solvent, the time, the temperature and the chemical nature of the sample used [22]. It is calculated as % w/w (weight of extract/weight of plant material). In the present study, the obtained extraction yields for the two solvent systems used namely methanol 80 % and ethanol 80 % are listed in table 1. Although a number of factors have to be considered when selected the appropriate solvent, ethanol was investigated to test the possibility to replace methanol in order to reduce the gallic toxicity. Knowing that some conditions of time and temperature were applied for the same plant part used during extraction, methanolic system was more effective in extraction compared to ethanol in the case of fresh leaves and dried bark. While ethanolic system was the suitable solvent for the extraction of pericarp. No significant difference is detected in the case of dried leaves and pulp (P > 0.05).
So they act as reducing agents and antioxidants by donation of a hydrogen atom or chelating metals. Plants contain many phenolic compounds which interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. They act as reducing agents and antioxidants in oxidation reactions by donation of a hydrogen atom or chelating metals. The total phenol content (TPC) is expressed in mg equivalent G. A./g of plant part. The results showed that the total phenol content is expressed in mg equivalent G. A./g of plant part. The results showed that the total phenol content is expressed in mg equivalent G. A./g of plant part.

| Plant part     | Solvent        | Morphology | Extraction yield (%w/w) |
|----------------|----------------|------------|-------------------------|
| Dried leaves   | Methanol 80%   | Powder     | 10.46                   |
|                | Ethanol 80%    | Powder     | 10.57                   |
| Fresh leaves   | Methanol 80%   | Semi-solid | 7.00                    |
|                | Ethanol 80%    | Semi-solid | 3.30                    |
| Dried bark     | Methanol 80%   | Powder     | 14.80                   |
|                | Ethanol 80%    | Powder     | 10.92                   |
| Pulp           | Methanol 80%   | Powder     | 19.67                   |
|                | Ethanol 80%    | Powder     | 19.25                   |
| Pericarp       | Methanol 80%   | Powder     | 17.99                   |

Results of phytochemical screening

The results of the phytochemical qualitative analysis are reported in Table 2 and Table 3. The screening test showed the presence of flavonoids and alkaloids in all studied extracts of *A. squamosa*. Also, hydrolyzable tannins were absent in all studied extracts. Condensed tannins were absent in pulp extract, but were present in all other extracts and mostly abundant in fresh and dried leaves extracts. Dried bark and leaves were rich in alkaloids. Besides the fact that ethanol is not more polar than methanol, it is worth noting that no difference was revealed in terms of families of secondary metabolites extracted. In addition, our results are in good agreement with the experimental data of Sabbah et al. [23] and Mahawar et al. [24].

| Plant part     | Alkaloids screening of *A. squamosa* plant. |
|----------------|-------------------------------------------|
| Dried leaves   | +++ (Dragendorff reagent) ++ (Buchardart reagent) |
| Dried bark     | ++                                    |
| Pulp           | +                                     |
| Pericarp       | +                                     |

+++: Strong intensity reaction, ++: Medium intensity reaction, +: Weak intensity reaction

| Plant part     | Hydrolyzable tannins | Condensed tannins | Flavonoids | Saponins |
|----------------|----------------------|-------------------|------------|----------|
| Dried leaves   | -                    | +++               | +          | ++       |
| Fresh leaves   | -                    | ++++              | +          | -        |
| Dried bark     | -                    | +                 | +          | -        |
| Pulp           | -                    | +                 | +          | -        |
| Pericarp       | -                    | +                 | +          | -        |

++++: Strong intensity reaction, ++: Medium intensity reaction, +: Weak intensity reaction, -: absent

Total phenol content (TPC)

Plants contain many phenolic compounds which interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. So they act as reducing agents and antioxidants [25]. Fig. 2 shows the total phenol of methanolic and ethanolic extracts of *Annona squamosa*, leaves, bark, pulp and pericarp. The total phenol content is expressed in mg equivalent G. A./gram of plant part. The results showed that the different plant extracts contain relatively high contents of total phenols ranging between 1.12 and 117.19 mg GAE/g plant part.

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![Fig. 2: Total phenol content of different extracts, (values are expressed as means±standard deviation (n=3), values marked by the same letter are not significantly different (P < 0.05))](image-url)
The highest amounts of total phenolic content were 117.2, 112.9, 82.2 mg gallic acid extract/g plant part and they correspond respectively to A. squamosa dried leaves methanolic extract, dried leaves ethanolic extract and bark methanolic extract. The bark ethanolic extract as well as the methanolic and ethanolic extract of A. squamosa fresh leaves, showed moderate amounts of TPC of 47.02, 48.05, and 17.96 mg gallic acid extract/g plant part respectively. The lowest amounts of TPC recorded were 7.7, 7.0, 1.1, 1.2 mg gallic acid extract/g plant part for methanolic, ethanolic extracts of pericarp and pulp, respectively.

The conducted work showed no significant difference in the total phenol content between methanolic and ethanolic extract of A. squamosa dry leaves, dry bark, pulp and pericarp (P > 0.05). In these cases, ethanol can effectively substitute methanol in the extraction of polyphenols from these plant parts. However, extraction solvents, methanol and ethanol, significantly affected the amount of total A. squamosa fresh leaves phenolic compounds. Methanol was found to be more efficient in the extraction of phenolic compounds from fresh leaves than ethanol (P < 0.05).

Radical scavenging activity

DPPH radical scavenging activity assays of the 10 different extracts of A. squamosa plant parts extracted by both ethanol and methanol showed significant DPPH radical scavenging activity in a concentration-dependent manner. The results revealed that the antioxidant activity increased with the concentration of the extracts. DPPH free radical scavenging assays were repeated in triplicate. The half-maximal inhibitory concentration (IC₅₀) values were determined as the concentration of the test mixture that gave 50% reduction in absorbance from that of the control and the mean was calculated (fig. 3).

The potential free radical scavenging activity was exhibited by the methanolic extract of dried leaves (IC₅₀ = 13.61 µg/ml), whereas the ethanolic extract exhibited relatively less free radical scavenging activity (IC₅₀ = 15.97 µg/ml).

The IC₅₀ values of the fresh leaves ethanolic and methanolic extracts were 20.75 µg/ml, 27.35 µg/ml, respectively. The IC₅₀ values of the pericarp ethanolic and methanol extracts were 76.47 µg/ml, 70.91 µg/ml respectively. The poorest free radical scavenging activity was recorded for the methanolic extract (IC₅₀ = 871.33 µg/ml) and the ethanolic extract (IC₅₀ = 659.68 µg/ml) of pulp. However, ascorbic acid showed the highest DPPH radical scavenging activity (IC₅₀ = 6.62 µg/ml). The conducted work proved no significant difference in scavenging activity between methanol and ethanol extract of A. squamosa dry and fresh leaves, pericarp and pulp (P > 0.05). However, A. squamosa bark methanol extract exhibited significantly higher scavenging activity than ethanol extract (P < 0.05).

To the best of our knowledge, there is no study available that has focused upon different parts of A. squamosa grown in Lebanon. On the other hand, only few studies compared the TPC of different parts extracts of A. squamosa cultivated in other places in the world. Our results are very consistent with a result reported by Montero Fernandez et al. [26] which states that A. squamosa pericarp extract is more rich in TPC than pulp. In another work, Mariod et al. evaluated the total phenol content and antioxidant activities of A. squamosa leaves and bark methanolic extracts and determined by the DPPH technique IC₅₀ values of 7.81 and 125.0 µg ml⁻¹, respectively. This result is close to the one found in this work in the case of the leaves methanolic extract, but greater than that found in the present study for the bark methanolic extract [27].

The results were also expressed as ascorbic acid equivalent antioxidant capacity (AEAC) (table 4) i.e. mg ascorbic acid equivalents/mg dry wt, which was calculated as follows: [28]

\[
\text{AEAC (mg AAE/mg dry wt)} = \frac{\text{IC}_{50} \text{ sample (mg AAE/ml)}}{\text{IC}_{50} \text{ sample (mg AAE/ml)}} \times \frac{\text{mg ascorbic acid equivalent}}{\text{mg dry wt}}
\]

where IC₅₀ ascorbic acid and IC₅₀ sample are the effective concentrations of the ascorbic acid and the sample respectively. The higher the AEAC value, the greater is the antioxidant activity.

| Table 4: Ascorbic acid equivalent antioxidant capacity AEAC (mg AAE/mg dry wt) |
|-----------------|-----------------|-----------------|-----------------|
|                | MeOH            | EOH             |
| Dried leaves   | 0.233           | 0.198           |
| Fresh leaves   | 0.116           | 0.153           |
| Dried bark     | 0.082           | 0.057           |
| Pulp           | 0.004           | 0.005           |
| Pericarp       | 0.045           | 0.041           |

The methanolic dried leaves extract showed the highest AEAC value of 0.233 mg AAE/g dry wt of extract. Polyphenolic compounds have been reported to have antioxidant activities due to the reactivity of the phenolic moiety, scavenging free radicals via electron donation or hydrogen donation [29]. Hence, the antioxidant activities of dried leaves come to confirm these reports. IC₅₀ and AEAC values obtained
REFERENCES
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