**Reviewer’s Comments**



GC-MS Profiling and Antioxidant Activities of Essential Oils from Leaves of *Annona Squamosa*L.

**ABSTRACT:**Medicinal plants, their biological activities, and their phytochemical contents are important for finding safe and potent new compounds for therapeutic use. In order to investigate the chemical contents and to evaluate the storage effect on the antioxidant activity of Lebanese *Annona squamosa* leaf essential oil -, the current study was undertaken.The leaves of *Annona squamosa* taken from Batroun (Lebanon), were dried naturally and the essential oil (EO) was extracted by hydrodistillation. Gas chromatography-mass spectrometry (GC-MS) technique was used to analyze the composition of the EO. Concerning the antioxidant activity, two different methods namelyradical scavenging activity (DPPH test) and ferric reducing antioxidant power (FRAP) were used. The results show that the majority of the compounds identified in the samples of the EOs studied, belong to the sesquiterpenoids. On the other hand, the storage of the plant materials containing theEOs or the EOs themselves, leads to a loss in the volatile compounds, which is reflected in the bioactivity as shown in the results of the antioxidants assays.

**Key words:***Annona squamosa*, Essential oil, GC-MS, Antioxidant, FRAP, DPPH

INTRODUCTION

Medicinal plants are part of the history of all continents.Through the centuries, knowledge about plants has been organized, documented, and passed down across generations1. Herbal medicine is now used on a daily basis as prevention rather than therapy to protect our health.A resource containing phytochemicals such as flavonoids, polyphenols, alkaloids, tannins, terpenoids, coumarins, and others.One of these *Annona squamosa* (AS) edible fruit plants belonging to the *Annonaceae* family is commonly known as the sugar apple, custard apple, and sweetsop2. Research on this plant showedseveral medicinal properties such as cardiotonic, antimicrobial,insecticidal, and anti-cancerous activitivities3. With a traditional use widely spread in many countries mainly in Lebanon due to its geographical location, its Mediterranean climate where it adapts well it is a small, well-branched tree that grows at altitudes of 0 to 2,000 m and does well in hot, dry climates. The planthas been reported to possess a wide variety of pharmacological activities4. Essential oils (EO), volatile compounds extracted from plants, are complex compounds with strong odors, made up of various plant metabolites5. EOs are believed to have many different biological activities6. Furthermore, the composition of EOs in the same plant species is affected by several parameters, such as harvest time, extraction method, and protection7–9. ASEOs have showed a wide range of biological properties10–13.

The present study aimed to extract and identified the volatile organic components from dry leaves of AS using hydrodistillation and GC/MS, to study the changes inEOcomposition during storage of leaves in paper bags or the storage of EO samples, and measure the effect on the antioxidant activity through two *in vitro* antioxidant tests.

MATERIALS AND METHODS

Plant materials: Collection, Identification, and Preparation

The leaves of ASwere collected directly from the producer who owned the trees. The trees were grown in Batroun, a coastal city in northern [Lebanon](https://en.wikipedia.org/wiki/Lebanon)that rises 80 to 100 m from sea level. The plant has been identified and confirmations were done through the Flora of the presidency of Madras, by Gamble J.S. 192119. The voucher specimen (No. 1806) of the plant material is maintained in our laboratory.

AS leaves were collected in February and beginning of March 2019 from *Annona* trees. The leaves were shade dried for 3 weeks and then pulverized into fine powder.The powder was divided into two batches, the first being stored at room temperature in dark for further use in the next year, and the second part was undergone hydro-distillation extraction to yield the first EO sample (S1). The latter, in turn, was stored at – 18 °C, in stoppered glass vessels containing some air for one year yielding the second EO sample (S2) (March 2020). Finally, the third sample (S3) is the EO obtained after fresh extraction of the conserved leaves.

Extraction of essential oils of *Annona squamosa* (ASEO)

Foremost, 50 g ofAS powder were introduced into a 1000 mL round bottom flask containing 500 mL of distilled water. The hydro-distillation was performed in a Clevenger-type distillation apparatus designedaccording to British Pharmacopoeia specifications14. After 3 hours of distillation, the ASEO was collected in the receiver arm. For further use, the oils were sealed and maintained in amber glass vials at 4 °C.The percentage of ASEO content was calculated according to the following equation (1):

$Yield \% = \frac{V}{W} x $100 (equation 1)

Where *W* is the dry weight of the used matter and *V* is the volume of collected ASEO.

Analyses of volatile organic compounds

One microliter of ASEO sample was diluted (1:100) with hexane and injected into the gas chromatography‑mass spectrometry (GC-MS) system. GC SHIMADZU QP2010 system was used to analyze the volatile compounds in the *N. tabacum* extract (without derivatization). DB-5MS (5% Diphenyl / 95% Dimethylpolysiloxan) capillary column having (30 m length, 0.25 i.d., film thickness 0.28 µm) and helium as carrier gas (1 mL/min, constant flow) was used for compound separation. The oven temperature was programmed from 65°C (2 min initial time) increased to 300°C at 10°C/min (isothermal for the final time). The actual temperature in the MS source reached 230 ºC, and the MS was operated in the electron impact mode at 70 eV ion source energy. The injector temperature was 250°C, while the injection volume was 1 µL and a total run of one hour is performed, with mass detector scan range *m/z* = 50–550. Data receipt and processing were performed using Shimadzu GC-MS solution software. The detected compounds were tentatively identified, by MS spectral correlations using NIST08 (National Institute of Standards and Technologies, Mass Spectra Libraries), as well as published data.

*In vitro* antioxidant activities

The antioxidant activity was measured using two methodsnamelyDPPH free radical scavenging assay and reducing power assay.

DPPH assay

2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) method for determining antioxidant activity isa spectrophotometric method based on the hydrogen atom transfer and single electron transfer mechanisms. These assays included radical scavenging activity, based on the antioxidant reducing of the violet DPPH radical via a hydrogen atom transfer mechanism, resulting in the color change15. The violet DPPH radical is measured by a UV-Vis spectrophotometer at approximately 515 to520 nm.Several solutions of increasing concentrations varying from 0.81 ng.mL-1 to 10.81 ng.mL-1 of ascorbic acid were prepared in test tubes. About 1 mL of the ASEO solution of different concentrations was taken in test tubes, then 1 mL of the DPPH methanolic solution (81.15 μM) was added. Simultaneously, a control was generated by mixing 1 mL of DPPH solution with 1 mL methanol.After 30 min of incubation in dark at room temperature, 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 (equation 2):

%DPPH scavenging activity = $\frac{Acontrol-Asample}{Acontrol}×100$ (equation 2)

Where: - Acontrol is the absorbance of the mixture of MeOH and DPPH

 - Asample is the absorbance of the sample extract/standard mixed with DPPH.

Based on graphic values of the percentage of DPPH inhibition vs extractconcentration, the half-maximal inhibitory concentration (IC50) (the concentration of the sampleneeded to inhibit 50% of the DPPH) of each samplewas 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.

Reducing power assay

The reducing power assay method is designedbased on the reduction potential of the components by reacting with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+). The latter product mixed with ferric chloride form ferric–ferrous complex having an absorption maximum of 700 nm16.The reducing ability of ASEO was determined according to a method reported by Oyaizu17. The aliquots of different concentrations of the standard/test sample (10 to 100 μg.mL-1) in 1.0 mL of distilled water were mixed with 2.5 mL of (pH 6.6) phosphate buffer + 2.5 mL of (1%) potassium ferricyanide. Subsequently to a cooling step, the mixture was placed in a water bath at 50°C for 20 minutes. About 2.5 mL (10%) trichloroacetic acid aliquots were added to the mixture, which was then centrifuged for 10 minutes at 3000 rpm.The upper layer of solution of 2.5 mL was mixed with 2.5 mL distilled water and a freshly prepared 0.5 mL of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in a UV spectrometer. The solutions wereprepared on the day of the experiment and well protected from sunlight. Ascorbic acid at various concentrations (5 to 40 μg.mL-1) was used as standard. The sample was prepared using a similar procedure but by replacing the EO with an equal volume of methanol. The absorbance values were plotted against the concentration, and a linear regression analysis was carried out. The higher absorbance of the reaction mixture indicates a greater reducing power. All data were recorded as mean ± SD for three replicates.

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) and were calculated using Excel 2013 (Microsoft Corporation, Redmond, WA, USA). Linear regression analysis was used to calculate the IC50 values.

RESULTS AND DISCUSSION

Compound identification using GC-MS

The essential oilobtained by hydro-distillation from the leaves of *A. squamosa* were yellowish-green and the yield was 0.1% (v/w), based on dry weights. The chemical constituents of ASEO samples were analyzed by GC-MS (Figure 1). This led to the identification of different compounds that were determined by referring to previously published articles and referring to the suggestions of the NSIT Library. The chemical composition of the ASEO is shown in .The components are listed in order of their elution on the DB5MS column. The results showed that the EO of the three samples was mainly composed of the sesquiterpenoids. By comparing samples, S2 to S3, some of the sesquiterpenoids that were present in S3 were not found in S1. Besides, by comparing sample S2 with the results from the previous year (S1), the compounds that were present in S1 disappeared from S2.

A total of 19 (61.2%), 11 (51.07%), and 10 (43.33%) compounds were identified in the sample of S1, S2, and S3 respectively. ASEO was predominantly composed of sesquiterpenes (50.78%), and the remaindersare monoterpenes. Bicyclic sesquiterpenes comprised 19.66% of the sample. The three major constituents that were discovered were the δ-elemene (11%), carophyllene (10.15%), and β-elemene (14.14%). While β-elemene is absent, the two other compounds are in agreement with the results of Al-Nemari et al.18.EOs from the leaves of numerous *Annonaceae* genera, including *Annona*, have been discovered to include spathulenol and caryophyllene oxide, which could be used as chemotaxonomic identifiers for these genera19, 20.

Concerning the variation after storage the percentage of α-humulene increases from 0.6 to 2.57 in agreement with the work of Mockutë9, in contrast,although the amounts of compounds with caryophyllene (β‑caryophyllene + caryophyllene oxide) were nearly the same in fresh and stored EOs, caryophyllene oxide decrease from 8.15% (S1) and disappear in S2. On the other hand, the proportion of constituents with a low molecular weight (monoterpenes)significantly decrease, while the conditional percentage of larger molecular weight molecules having three isoprene units(sesquiterpenes) increased as a result of the above decrease. It's worth noting that the content in α-pinene, linalool, and thymol in S2 increases per the results of Baritaux et al.8. Other researchers have found lower amounts of monoterpenes and higher levels of certain sesquiterpenes in dill and ginger21, 22.



**Figure 1: GC-MS of different ASEOs**

**Table 1: Major compounds identified in the ASEOs obtained by hydrodistillation using GCMS**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| No | Compound | % Area | Formula | Classification |
| **S1** | **S2** | **S3** |  |  |
| 1 | α-pinene | 2.71 | 3.7 | - | C10H16 | Bicyclic monoterpene |
| 2 | β-Pinene | 0.9 | - | - | C10H16 | Bicyclic monoterpene |
| 3 | β-Myrcene | 0.18 | - | - | C10H16 | Monoterpene |
| *4* | *O*-Cymene | 0.75 | - | - | C10H14 | Monoterpene |
| 5 | D-limonene | 0.74 | - | - | C10H16 | Bicyclic monoterpene |
| 6 | 3-Carene | 0.24 | - | - | C10H16 | Bicyclic Monoterpene |
| 7 | Linalool | 2.29 | 3.2 | - | C10H18O | Monoterpene |
| 8 | Thymol | 2.6 | 2.8 | - | C10H14O | Monoterpene |
| 9 | δ-Elemene | 11 | 12.1 | 3.81 | C15H24 | Monocyclic sesquiterpene |
| 10 | α- Cubenene | 0.13 | - | 0.67 | C15H24 | Sesquiterpenoid |
| 11 | Copaene | 1.22 | - | 2.8 | C15H24 | Bicyclic sesquiterpene |
| 12 | β-Elemene | 14.14 | 11.5 | 11.39 | C15H24 | Monocyclic sesquiterpene |
| 13 | β-Caryophyllene | 10.15 | 11.3 | 15.56 | C15H24 | Bicyclic sesquiterpene |
| 14 | γ- Elemene*o*-Menth-8-ene | 1.39 | 1.65 | - | C15H24 | Monocyclic sesquiterpene |
| 15 | α-Humulene | 0.31 | 0.6 | 2.57 | C15H24 | Monocyclic sesquiterpene |
| 16 | Germacrene-D | 0.54 | 1.9 | 0.70 | C15H24 | Monocyclic sesquiterpene |
| 17 | Isoledene | - | - | 1.23 | C15H24 | Sesquiterpenoids |
| 18 | α-Amorphene | - | - | 3.23 | C15H24 | Sesquiterpenoids |
| 19 | α-Selinene | 3.66 | - | - | C15H24 | Sesquiterpenoid |
| 20 | Caryophylleneoxide | 8.15 | 2.13 | - | C15H24O | Bicyclic sesquiterpene |
| 21 | Sphathulenol | 0.14 | 0.19 | 1.37 | C15H24O | Bicyclic sesquiterpene |
|  | Total | 61.24% | 51.07% | 43.33% |  |  |

This profile variation of the samples can be attributed to the fact that EO components are known to easily convert into each other by oxidation, isomerization, cyclization, or dehydrogenation reactions induced either enzymatically or chemically, due to their structural link within the same chemical group23.

There have been many reports about the composition of EOs from the different parts of *A. Squamosa* (). For instance, the chemical profile of EO from the leaves of *A. Squamosa* growing in Badagary (Nigeria) was mainly composed of (E)-Ccryophyllene (38.9%), eugenol (30.2%)24.This research work aims to study the effect of the extraction factor on the volatile compounds occurring in AS. The GC-MS method highlighted the difference in the content of the EO whether it was extracted and conserved in the refrigerator or preserved in the leaves. The chemical composition of EOs and plant secondary metabolites, in general, is affected by different abiotic factors, namely climate, growing conditions, or harvest time are the most studied25.

**Table 2: Major components of Leaves from Annona species essential oils reported from some regions of the world**

|  |  |  |  |
| --- | --- | --- | --- |
| **Major compounds (%)** | **Geographic regions** |  | **References** |
| (E)-Caryophyllene (38.9), eugenol (30.2), δ-cadinene (6.0), caryophyllene oxide (5.0), α-humulene (4.3) | Nigeria | EOAM | [24] |
| β-Caryophyllene (24.5), β-Cubebene (13.0), β-Elemene (5.9), α-Cadinol (5.2), α-Terpinene (4.6) | Vietnam | EOAS | [26] |
| β-Caryophyllene (40), β-elemene (14.4), α-santalene (9.5), (Z)-hex-3-enol (5.2), δ-cardinene (4.8) | Cameroon | EOAM | [27] |
| Germacrene-D (22.01), trans-caryophyllene (12.12), bicyclogermacrene (2.80), α-copaene (2.12), and humulene (1.15), as well as phytol (2.22) and squalene (1.3). | Saudi Arabia | ASAS | [18] |
| Spathulenol (43.7), limonene (20.5), caryophyllene oxide (8.1) and a pinene (5.5) | Brazil | EO A. vepretorum | [19] |

Sesquiterpenes isolated from EOs are among compounds with promising antimicrobial activity28, 29. These encompass β-caryophyllene, a sesquiterpene extensively present in EOs, which possesses anti-inflammatory and anticarcinogenic activities30. Its oxygenated form caryophyllene oxide, present at 8.15% in the obtained EOAS, owns high antimicrobial properties31. Furthermore, β-elemene presents good antitumor and anti-inflammatory activities without obvious cytotoxicity or clinical side effects32.

DPPH assay

The three assessed EOs were able to reduce the stable, purple-colored radical DPPH**·** to yellow colored DPPH-H, thus samples S1, S2 and S3 had IC50 values of 6, 9, and 8 μg.mL‑1 respectively (). The positive control (ascorbic acid) had an IC50 value of 3 μg.mL‑1. Thehighest antioxidant activity was obtained with the sample with the lowest IC50.



**Figure 2: IC50 values of samples A and B compared with ascorbic acid (AA).**

Among the samples,S1 revealed the lowest IC50, this means that sample S1 has more ability to inactivate free radicals leading to more antioxidant activity. This could be explained by the fact that the number of secondary metabolites (such as phenolic compounds, terpenoids…) present in the ASEOwas influenced by several factors including duration of conservation, and chemical variability. The obtained IC50are in good agreement with those obtained for a different part of *A. squamosa*33. The IC50 value is lower than the 1.33 mg.mL-1 reported for seed oil34. In addition, the results showed that it outperforms EO from the leaf of its peers *Annona muricata* (244.8 μg.mL‑1)35.

FRAP method

The antioxidant potential of plant extracts or EOs may be determined by their reducing power36.The reducing power of ASEO was determined for samples S1, S2, and S3 at different concentrations ().It was observed that the absorbance of all samples gradually increases with the increasing concentration of oil. Also, the capacity of the extracts to reduce Fe3+ to Fe2+is lower than that of ascorbic acid, and sample S1 had the greatest reducing power. The results were consistent with DPPH results.



**Figure 3: Reducing power of the different samples in comparison with ascorbic acid as a reference.**

However, the reducing power of the three ASEOsis found lesser than the positive control compound. Our findings were in agreement with the observation obtained with AS leaves extracts37, 38. In other words, the reducing power of *A. squamosa* extracts referred to its electron transfer capacity in a redox reaction, leading to the neutralization of free radicals and forming stable products. Therefore, the reducing capacity of the extracts is another significant indicator of antioxidant activity. It has been reported that the reducing power of extracts probably depends on the hydrogen-donating ability present in terpenoids and phenolics compounds. As a result, antioxidants can be thought of as reductants, and the inactivation of oxidants by reductants can be thought of as redox processes in which one reaction species is reduced at the expense of the other's oxidation.

**Statistical analysis**

CONCLUSION

The phytochemical content and antioxidant activity of *A. squamosa* essential oils were influenced by the time of conservation. In this work, the phytochemical screening using GC-MS revealed that different oil of ASEO contain sesquiterpenoids. In addition, many compounds present in EO obtained from fresh samples disappeared with time even with conservation at -18°C. Same results were obtained with conserved plant materials. The reason behind that might be due to reactions between compounds, or a fragmentation process took place. The results of antioxidant activity showed that fresh prepared EO sample from fresh dried leaves had exerted the best antioxidant activity. The results were confirmed by using two antioxidant assays namely DPPH and reducing power assay. Further studies can be performed to reveal the reactions responsible for the biological activities present in the ASEOs. Evaluation of the anticancer activity of sample S1 against cell lines can be done in the future since antioxidant is correlated to anticancer activity.

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

AUTHOR CONTRIBUTIONS

Concept: A.J., G.I., E.C.; Design: A.J., G.I., E.C.; Control: A.J., E.C.; Materials: A.J.; Data Collection and/or processing: S H., A.J.; Analysis and/or interpretation: S.H., A.J., G.I., E.C.; Literature review: S.H., A.J.; Manuscript writing: S.H., A.J.

**LIMITATIONS OF THE STUDY**

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