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Original Article

# Phytochemical Screening and Cytotoxic Activity of Two Extracts from Seeds of Lebanese *Annona squamosa* L

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ABSTRACT

Two types of extracts were prepared, methanolic and aqueous. Then, astandardphytochemical screening has been done to cast the light on its secondary metabolites. Also, biological activities were studied starting with the antioxidant activity using free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), followed by an antidiabetic using the method of glucose uptake by yeast cells, last but not least the anti-proliferative activity on two types of cancer cells (Jurkat and MDA)using the neutral red assay. Obtained results revealed that seeds of*Annona squamosa* due to its content in some secondary metabolites such as flavonoids, phenol and alkaloids presentedan antioxidant capacity reaching a maximum of 61 % at the concentration 0.5 mg/mL for extracts, an antidiabetic property (96 %) at glucose concentration 10 mM and an anti-proliferative capacity. These results indicated that seeds could bea good source of element that may be used to help human health and to prevent some diseases.

**Key words:** *Annona squamosa,* secondary metabolites, phytochemical screening, biological activities

## 1. INTRODUCTION

As technology advances, the pharmaceutical industry is increasingly focusing on plants as a source of medicine, relating to how diseases are controlled and managed. Humans have utilized plants for wide range of applications including cosmetics, food industries, and biomedical systems. Thus, with the investigation of medical plants for

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antimicrobial activities and phytochemicals, new avenues to find new compounds for therapeutic purpose are under debate.

It is tough that up to 80 % of the world population rely on medicinal plants to satisfy their needs in maintaining good health [1]. The reason behind the great demand on medicinal plants is their richness in secondary metabolites. In opposite to primary metabolites, which play a vital role in housekeeping function as taking lipids, sugar and nucleic acids,they are not produced in all kinds of plants, however, both types can undergo the same metabolic pathway [2].

Currently, the Mediterranean basin is considered as an ultimate area for the conservation of plant biodiversity, due to its unique climate and habitat heterogeneity. Among various countries in this area, Lebanon was chosen as a promising side, due to its richness in 236 therapeutic plants, one of them is the *Annona squamosa*; plant of Annonaceae family, whose all parts have been proved to possess medicinal property[3–5].

The phytochemical screening of *A. squamosa*'s fruit showed that methanolic and aqueous extracts contain alkaloids, flavonoids, saponins, carbohydrates, phenols, glycosides, but no significant evidence showed the existence of triterpenoids or steroids [6]. Thus, *A. squamosa* can be involved in treating or preventing several diseases such as tumor, renal failure and diseases associated with ROS generation, such as Oxidative stress [7]. Although seeds of *A. squamosa* were proved to have natural medicinal behavior, yet toxicity assays have to be carried out. Aneela*et al.*[8] studied the effect of high concentrations up to 2000 mg/mL which proved to reduce food intake and body weight.

Our work was intended to discover for the first time, the chemical composition and some biological importance likeantioxidant, antidiabetic and antiproliferativecapacities of two extracts from the Lebanese*Annona squamosa*'s seeds.

## 2. MATERIAL AND METHODS

### Plant collection and preparation of powder

Fresh plants were gathered from South Lebanon in 2014-2015. The biological authentication was carried out by the Professor George Tohme, the president of the Lebanese C.N.R.S.

Fresh seeds obtained from the fruits were washed well then were cut into small pieces and dried in the shade at room temperature, away from the sunlight. During the drying process, the seeds were turned over repeatedly for homogeneous drying. After that, the dried seeds were grinded by a grinder. The obtained powder was then preserved in a container away from light, heat, and moisture for later use. Then, 200 g of powder were blended with two solvents, water and methanol (750 mL) with agitation at room temperature for 48 hours. After that the macerateswere taken and filtered using filter paper and each filtrate was evaporated using rotary evaporator at 37 °C under reduced pressure. Filtrates were put in the refrigerator at -20 °C and then lyophilized to obtain powders.

### Phytochemical screening

In order to study the chemical composition of the different extracts, qualitative tests have been done according to Nasser et al.[9] to detect the presence of primary and secondary metabolites as shown in

Table **1**. These tests are useful to estimate some biological activities might be due to the presence of some secondary metabolites in the seeds of the studied fruit.

**Table 1: Detection of primary and secondary metabolites in the seeds of *A. squamosa***

| Metabolites | Added reagent | Expected result |
|---|---|---|
| **Alkaloids** | Dragendorff reagent | Red or orange precipitate |
| **Tannins** | $FeCl_3$ (1%) | Blue coloration |
| **Resins** | Acetone + water | Turbidity |
| **Saponins** | Agitation | Formation of foam |
| **Phenols** | $FeCl_3$ (1%) + $K_3(Fe(CN)_6)$ (1%) | Green-blue coloration |
| **Terpenoids** | Chloroform + $H_2SO_4$conc | Reddish brown coloration |
| **Flavonoids** | KOH (50%) | Yellow color |
| **Carbohydrates** | α-naphtol + $H_2SO_4$ | Purple ring |
| **Reducing sugar** | Fehlings (A+B) | Brownish-red precpitate |
| **Quinones** | HCl conc | Yellow precipitate |
| **Sterols & Steroids** | Chloroform + $H_2SO_4$conc | Red color (surface) + fluorescence Greenish-Ring |
| **Cardiac glycosides** | Glacial acetic acid + $FeCl_3$ (5%) + | |
| **Diterpenes** | Copper acetate | Green color |
| **Anthraquinones** | HCl (10%) + Chloroform + | Pink color |
| **Proteins &aminoacids** | Ninhydrin 0.25% | Blue color |
| **Lignins** | Safranin | Pink color |
| **Phlabotannins** | HCl (1%) | Blue color |
| **Anthocyanins** | NaOH (10%) | Blue color |
| **Flavanones** | $H_2SO_4$conc | Bluish-red color |
| **Fixed oils and fats** | Spot Test | Oil stain |

### Anti-oxidant activity:

The antioxidant activity of the seeds of *A. squamosa* has been evaluated using the method of Rammal et al. [10,11]. Mother solution (1 mg/mL) was prepared and followed by serial dilution in order to obtain all increasing concentration needed (0.1, 0.2, 0.3, 0.4, 0.5 mg/mL) from each extract (aqueous and methanolic). 1 mL of each prepared diluted extract was added to 1 mL of DPPH (0.15 mM). The solutions were then incubated for 30 minutes at room temperature in the dark, and the absorbance was measured at 570 nm.The antioxidant activity was calculated according to the following formula:

**% Antioxidant activity = [(Abs control – Abs sample) / Abs control] x 100**

Control was the mixture of 1 mL of DPPH with 1 mL of the solvent (water and methanol) and the blank was formed by 1 mL of the solvent.

**Anti-diabetic activity**

**Glucose uptake in Yeast cells:** Commercial baker's yeast was washed by repeated centrifugation (3000g for 5 minutes) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1–5 mg) were added to 1 mL of glucose solution (5, 10 and 25 mM) and incubated together for 10 minutes at 37 °C. The reaction was started byadding 100 µL of yeast suspension, vortexed andfurther incubated at 37 °C for 60 minutes. After this period, the tubes were centrifuged (2500 g for 5 minutes) and glucose was estimated in the supernatant[12]. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

**Increase in glucose uptake(%) = [(Abs sample – Abs control) /Abs sample] / * 100**

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample.All the experiments were carried out in triplicates.

**Anti-proliferative activity:**

**Cells and cell culture**:Jurkatand MDA cells were cultured in 96-WellELISA plates containing each100 µL equivalent to 20000 cells.

Mother solution of each extract was prepared using 100 mg of powder in 100 µL of DMSO.In order to prepare extract that will be added to Jurkat cells, from the mother solution decreasing concentrations were prepared (200, 100, 50, 25, 5 mg/mL). 2 µL of each diluted solution were added to 100 µL of RPMI medium containing Jurkat cells in Eppendorf's tubes. Seeding and treatment were done at the same time, 100 µL of mixture prepared were placed in a well in triplicates. For MDA cells, same concentrations were prepared but in Eppendorf's tubes containing 100 µL of DEME medium. Three ELISA plates were prepared to study the activity at 24, 48 and 72 hours.

**Neutral Red Analysis**: Cell viability was performed using Neutral Red assay. Neutral red, a chromogenic dye, is an indicator of lysosomal activity. Live cells demonstrate a chromogenic change with neutral red that is detected spectrophotometrically. Briefly, cells were detached from the tissue culture flask with 2 mL of trypsin solution. The cell pellet was obtained by centrifugation (1.000 rpm for 5 minutes). The density of the viable cells was counted by the trypan blue exclusion in a haemocytometer. Cells were then plated in 96-Well microtiter plate, at a concentration of $1\times10^4$cells/well and incubated in a humidified 37°C, 5%$CO_2$incubator that allows the cells to adhere. After 24 hours, the cells were treated with five different concentrations (200, 100, 50, 25, 5 mg/mL)of aqueous and methanolic extractseach being tested in three replicates. The plates were incubated for 24, 48 and 72 hours at 37°C in a 5% $CO_2$incubator. The untreated cells were regarded as a negative control, whilst cells incubated only with methanol (0.5%, v/v) were used as a vehicle control. No effect due to the methanol was observed. Arsenic was used as the positive control. At 24, 48 and 72 hours, the old medium was replaced with 100 $\mu$L of fresh medium containing 40 $\mu$g/mL neutral red and incubated for 3 hours. This is to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. Then, the media was discarded and cells were washed twice with 100 $\mu$L of 1X PBS. The intracellular accumulation of neutral red dye was extracted in 200 µL of a 50% ethanol-1% acetic acid lysing solution.

Theoptical density (OD) of the eluted dye was read at 490 nmusing a microplate reader. The percentage of inhibition of each of the test samples was calculated according to the following formula using the OD values obtained:

**Percentage of inhibition (%) =[(OD control − OD sample)/OD control]× 100**

## 3. RESULTS AND DISCUSSION

**Phytochemical screening**

The results presented in Table show that both extracts from seeds of *A. squamosa* were rich in secondary metabolites such as phenolic compounds, saponins terpenoids flavonoids etc…. Also methanolic fraction contained more of alkaloids, resins and carbohydrates than aqueous fraction.The only metabolites present only in the aqueous fraction were saponin and proteins. Tannin, coumarin, and volatile oil were similarly absent in both fractions.

**Table 2: Phytochemical screening of the methanolic and aqueous extracts of seeds of *A. squamosa*.**

| | Methanol | Water |
|---|---|---|
| **Alkaloids** | +++ | + |
| **Tannins** | - | - |
| **Resins** | +++ | + |
| **Saponins** | - | + |
| **Phenols** | +++ | +++ |
| **Terpenoids** | +++ | ++ |
| **Flavonoids** | +++ | +++ |
| **Carbohydrates** | +++ | - |
| **Proteins** | - | + |
| **Coumarone** | - | - |
| **Volatile oil** | - | - |

**Antioxidant activity**

The results presented in Fig show that the antioxidant activity increased with the concentration of the extracts to reach a maximum of 61 % at 0.5 mg/mL for both extracts, but the activity of methanolic extract was always higher than that of the aqueous extract.

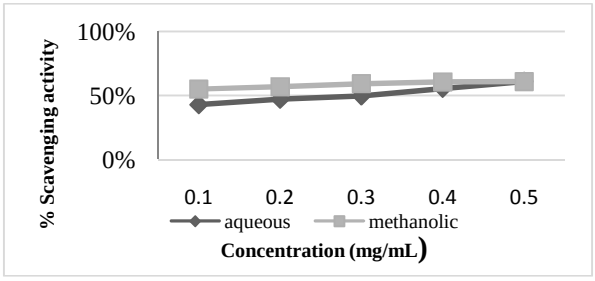



**Fig 1: Variation of the percentage of antioxidant activity in function of different concentrations for the two studied extracts.**

According to the phytochemical screening results, the seeds of this plant is rich in phenol and flavonoids and these tow metabolites have an antioxidant activity by inducing free radical scavenging[13,14] thus can be used in drugs to prevent and cure degenerative diseases.

Literature mentioned that leaves of *A. squamosa* have an important antioxidant activity due to its richness in phenolic compounds [15].

**Anti-diabetic activity**

The rate of glucose transport across cell membrane in yeast cells system was determined.Glucose uptake by the yeast cells is indicated by the amount of glucose remaining in the medium. The methanolic extract exhibited significantly higher activity than aqueous extract at all used concentrations. However, the highest uptake of glucose was seen in 5 mM glucose concentration (Figure 2).

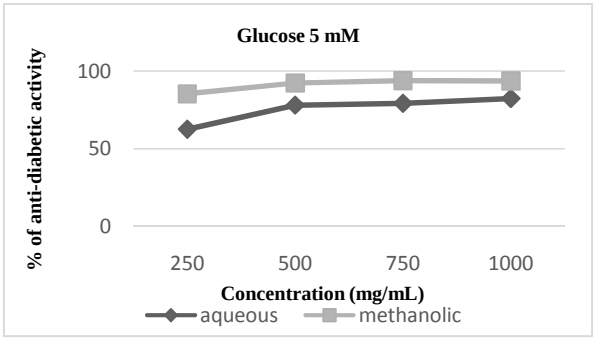
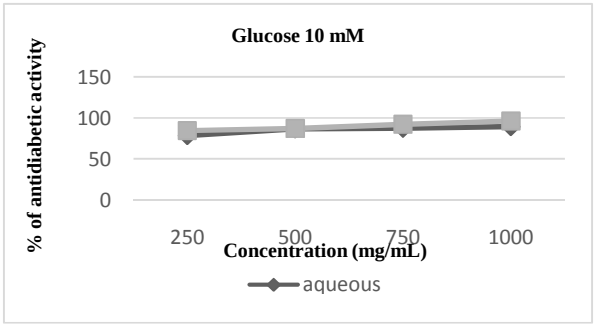



**Fig 2: Variation of % of anti-diabetic activity of aqueous and methanolic extracts in function of different concentration at 5 mM of glucose.**




**Figu 3: Variation of % of anti-diabetic activity of aqueous and methanolic extracts in function of different concentration at 10 mM of glucose.**

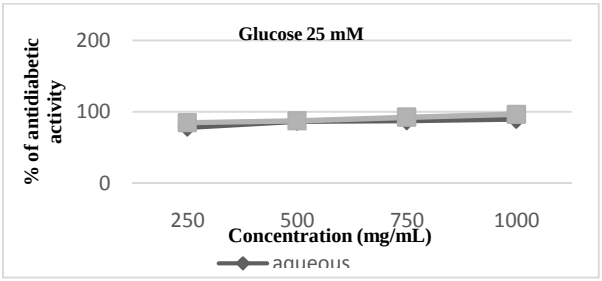



**Fig 4: Variation of % of anti-diabetic activity of aqueous and methanolic extracts in function of different concentration at 25 mM of glucose.**

These results show that the antidiabetic activity of *A. squamosa*seeds was always higher in the methanolic extract then in the aqueous one. And even within the methanolic fractions, when the concentration of glucose was higher the activity was higher. The activity increased to attend the highest value (96 %) at the concentration 1000 mg/mL of the methanolic extract for the glucose 10 mM.

As mentioned in the literature many plants have been used to treat diabetes due to the presence of secondary metabolites [16]such as flavonoids, alkaloids and steroids [17]. It is important to note that the effect of the plant is due to the combined effect of all these secondary metabolites together [16].This data is compatible with our phytochemical screening results which reveal that the methanolic extract of the *A. squamosa's* seeds was rich in flavonoids, tannins and steroids more than the aqueous extract which explains the higher activity of methanolic extract.

The mode of action of these secondary metabolites is by inducing β-cell of islets to release pancreatic secretion and/or by stimulating the transport of glucose from blood to peripheral tissue and the inhibition of production of endogenous glucose and the activation of gluconeogenesis in liver and muscles [18].

**Anti-proliferative activity**

Results of the anti-proliferative activity were reported using neutral red technique, and then raided by ELISA reader. Aqueous extract of the seeds of *A. squamosa* showed a stable and effective inhibition of MDA cells with highest inhibition at 48 hours using 50 mg/mL. At 24 hours the inhibition was the lowest and at 72 hours it decreased with the increased concentration (Fig ).

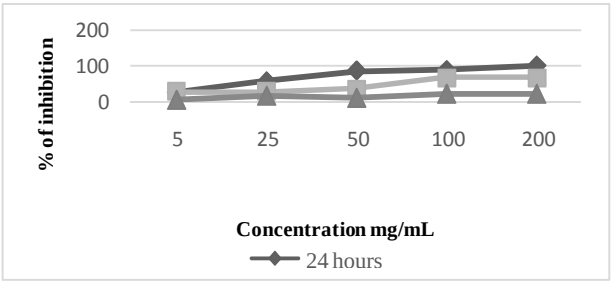



**Fig 5: Variation of % of inhibition of MDA cellsin function of different concentration of aqueous extract at 24, 48 and 72 hours.**

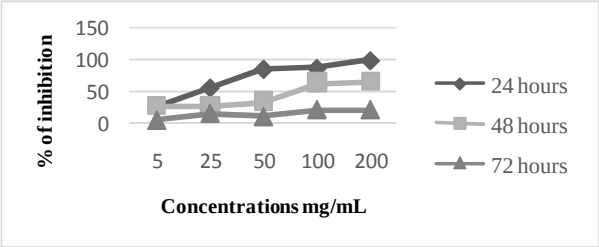



**Fig 6: Variation of % of inhibition of MDA cells in function of different concentration ofmethanolic extractat 24, 48 and 72 hours.**

Fig shows that methanolic extract of the seeds of *A. squamosa*increased in the percentage of inhibition at 24, 48 and 72 hours, with highest value at 48 hours atthe concentration 200 mg/mL.

The percentage of inhibition of Jurkat cells was increased at 48 and 72 hours but it was maximal at 48 hours. It increased to attend a maximal value of 90 % while at 24 hours there was no effect.

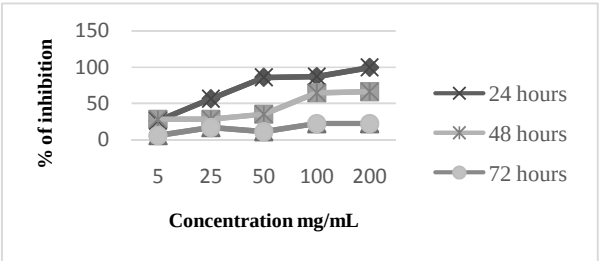



**Fig 7: Variation of % of inhibition Jurkat cells in function of different concentration of methanolic extract at 24, 48 and 72 hours.**

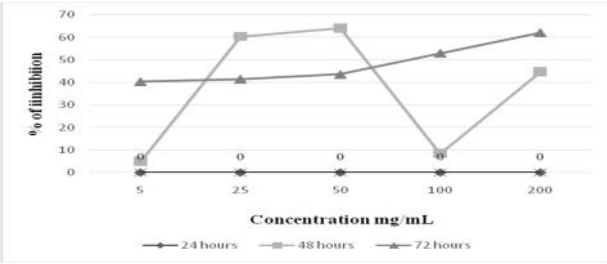



**Fig 8: Variation of % of inhibition Jurkat cells in function of different concentration of aqueous extract at 24, 48 and 72 hours.**

Aqueous extract from seeds didn't show any effect at 24 hours. While the highest percentage of inhibition was at 72 hours with a maximal value at 200 mg/mL.All these results showed that seeds of this plant present an anti-proliferative activity with the highest activity at 72 hours using methanolic extract on Jurkat cells. So we can conclude that metabolites extracted with methanol had a stronger effect on cells and especially on suspension cells.

Data revealed that alkaloids are responsible of anti-proliferative activity. It was also found in the literature that the anti-proliferative activity of alkaloids and their derivativeswas due to their high potency to induce apoptosis in cells and anti-invasive of collagen type 2[19].This data is compatible with phytochemical screening results which showed that seeds of this plant were rich in alkaloids might be responsible of this anti-proliferative activity.

## 4. CONCLUSION

The results of this study showed the pharmacological importance of the Lebanese *Annona squamosa* as a medicinal plant and the possibility of using it in order to produce drugs, especially due to its antioxidant, antidiabetic and anti-proliferative properties since it is rich in secondary metabolites responsible of these activities. So, it can be used to prevent some diseases related to oxidative stress, diabetes and as an alternative medicine in treating cancer.

Therefore, results obtained from this study were quite promising and comparable with other studies.It also warrants further investigation to isolate, specify and identify the secondary metabolites responsible of each activity in the plant to elucidate their mode of action. This further study makes *Annona squamosa* useful for the synthesis of drugs for several diseases.

## 5. ACKNOWLEDGMENTS

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**Conflict of Interest: None**

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