Phytochemistry and Cytotoxic Activity of *Annonasquamosa* L. Fruit Pulpagainst Human Carcinoma Cell Lines

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Abstract: It was argue that the different parts of *Annonasquamosa* L. (English: sugar apple, Arabic: gishta) have been used for the treatment of various types of cancer however; surgery, chemotherapy as well as radiotherapy still remain the basis of treatment. The aim of the present study was to determine the phytochemistry and anticancer potential of ethanolic extract of fruit pulp of *Annonasquamosa* against cervical (HELA), liver (HEPG2), prostate (PC3), lung (H1299 & A549), larynx (HEP2), breast (MCF7) and intestine (CACO) cell lines. Qualitative phytochemical screening was carried out using the crude fruit pulp aqueous extract. Data revealed the presence of glycosides, alkaloids, saponins, phytosterols, phenols, flavonoids, terpenoids and tannins as well as acetogenins. The extract exhibited a strong significant anticancer activity against H1299 (IC_{50}= 9.96), CACO (IC_{50}= 25.32), HEP2 (IC_{50}= 26.13) and PC3 (IC_{50}= 26.66). Moderate action was exhibited for HELA (IC_{50}= 38.09) and A549 (IC_{50}= 39.60) while a weak achievement was recorded for MCF7 (IC_{50}= 351.73) and HEPG2 (IC_{50}= 587.96). The study may conclude that A. *squamosa* fruit pulp may be applied as food supplement in some cancer treatment protocols.
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1. Introduction

Herbal medicines are gaining popularity on account of their lesser harmful side effects on non-targeted human cells and biological environment **(Wang *et al*., 2014).** Annonaceae remain a hot family for the discovery of new anti-cancer drugs as contains a class of chemicals called acetogenins, which to be 300 times more potent than standard anti-cancer drugs **(McLaughlin, 2008)**. *Annona* species are tropical trees; evergreen or semi-deciduous. Theyare native to tropical regions like tropical America, eastern Africa, subtropical or tropical highland conditions; like East Africa and Asia, also, few species found in temperate regions. Nowadays it is cultivated in almost all Arabian regions like Egypt, Lebanon, Sudan, K.S.A., Oman, Jordan and Palestine. Leaves of *A. squamosa* are 5 to 12 cm long, simple, ovate-lanceolate to elliptical in shape, glabrous on the ventral surface, Flowers are fragrant (emerges from the leaf axils) and possesses a short peduncle, 2.5 cm in length, solitary or in fascicles with 2 to 4 flowers, The fruit is normally heart-shaped, conical, oval or somewhat irregular in form. The fruit surface is covered with small conical protuberances over the carpel. The fruit rind is fragile and thin, and is greenish-yellow when ripe. The white, flesh has a fragrant, delicate flavor. The fruit has numerous seeds (21 to 41 seeds/fruit), which are black and small size **(Orwa *et al*., 2009)**. *A. squamosa* cultivations noware difficult and relatively rare fruit crop in Egypt (**Moustafa *et al*., 1971)**. Two cultivars are growing at the Sabahia Experiment Station, Alexandria; Beni Mazarand Abd El Razik **(Morton, 1987)**.

The phytochemical screening and anticancer potentiality of the fruit pulp of *A. squamosa* against eight cancerous cell lines was the main objective of the current study.

2. Material and Methods

Preparation of Aqueous and Ethanolic Extract of *A. squamosa*

Fruits of *A. squamosa* were purchased from fruit store and identified according to **Morton (1987).** The fruit pulp was separated from the fruits andallowed to dry in an oven at 65°C for ten daysand immediately extracted.

The dried powder was extracted with distilled water and ethanol. For aqueous extraction, ten grams of the powder was mixed with 100ml distilled water, boiled for two hours and filtered. However, ethanol extract was prepared by mixing ten grams of powder with 100ml of ethanol in mechanical shaker for 48 hours at room temperature. Extracts were filtered, concentrated, dried and were stored in the refrigerator at 4°C for upcoming use. For acetogenins determination, 100g of the dried powder of *A. squamosa* fruit pulp was accurately weighed and extracted by a supercritical fluid extractor (SFE) under optimized conditions (extraction pressure: 30 Mpa; extraction temperature: 35°C; extraction time: one

hour; 20 ml 95% ethanol modifier) (21). After evaporating ethanol to dryness by a rotary evaporator, residue was dissolved in methanol in a 25 ml flask, and then filtrated through a 0.45 micro-m millipore filter before HPLC injection. Three aliquots of the solution (20 µl) were injected to RP-HPLC-DAD system.

Phytochemical Analysis

Qualitative phytochemical screening was carried out using the crude fruit pulp aqueous extract to determined glycosides, alkaloids, saponins, phytosterols, phenols, flavonoids, terpenoids and tannins **(Raaman, 2006)**. Likewise, quantitative determination of acetogenins was performed in ethanolic extract according to **Sun*et al*. (2001) and Yang and Li (2008).**

Measurement of Potential Cytotoxicity by Sulfo-Rhodamine-B stain (SRB) assay

Eight cancer cell lines were selected to test the anticancer potentiality of the ethanolic extract of *A. squamosa* fruit pulp. These were: breast cancer (MCF7), cervical (HELA), intestine (CACO), larynx (HEP2), liver cancer (HEPG2), lung cancer (H1299, A549) and prostate cancer (PC3).

Potential cytotoxicity was tested using the method of **Skehanand Storeng (1990).** Cells were plated in 96-multiwell plate (104cells/well) for 24 hrs before treatment with ethanolic extract of fruit pulp of *A. squamosa* to allow attachment of cell to the wall of the plate. Different concentrations of the extract (0, 5, 12.5, 25, 50 and 100µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the extract under test for 48 hrs at 37ºC and in atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with SRB. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer, color intensity was measured in an ELISA reader. The relation between surviving fraction and tested extract concentrationswas plotted to get the survival curve of each tumor cell lines. This experiment was established at the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt.

3. Results

Phytochemical Analysis

Table 1 represents the various phytochemicals present in fruit pulp extracts of *A. squamosa*. Data revealed the presence of eight dominant compounds viz. glycosides, phytosterols, alkaloids, saponins, phenols, flavonoids, terpenoids and tannins. The phytochemical screening of the two extracts concludedthat water and ethanolic extracts had positive results for all constituens except phytosterols which are absent in aqueous extract. Notably, aqueous extract attained the prominent presence for all secondary metabolites except phytosterols compared to ethanolic extract. On the other hand the content of acetogenins determined in the present study was about 2.75±0.12 mg/g plant powder.

Table 1: Phytochemical screening of *Annona squamosa*fruit pulp.

Phytochemicals		**GLY**	**PHT**	**ALK**	**SAP**	**PHE**	**FLV**	**TER**	**TAN**
Extract	**Aqueous**	+++	-	+++	+++	+++	+++	+++	+++
	Ethanolic	+	+	++	++	+	+	+	+

GLY: Glycosides PHT: Phytosterols ALK: Alkaloids TAN: Tannins
SAP: Saponins PHE: Phenols FLV: Flavonoids TER: Terpenoids
+++ indicates presence in good amount, ++ indicates presence in fair amount, + indicates presence in weak amount and - indicates absence.

The Anti-Proliferative Activity of *A. Squamosa* Ethanolic Extract

For graphic determination of the IC_{50} (drug concentration that yields 50% less cells than the drug-free control), a bar (P) parallel to the *x*-axis and intersecting the point 50% on the *y*-axis was constructed. In the next step, a bar was plotted parallel to the *y*-axis that starts from the point of intersection of P with the dose-response plot. The IC_{50} could then be directly determined at the point of intersection with the *x*-axis.

The anti-proliferative activity of *A. squamosa*fruit pulp ethanolic extract was performed on eight different cell lines by SRB assay **(Figure 1).** Dose response curves constructed for Sulfo-Rhodamine –B (SRB) method between the range of 20- 120 µg/ml. Results showed that the incubation of the extract with lung carcinoma cell line (H1299) strongly inhibited cell proliferation with IC_{50} value of 19μg/ml**.** Noticeably, the anti-proliferative activity against prostate (PC3) and intestine (CACO) cancer cell lines pointed up strong values of IC_{50} =34 and 35μg/ml respectively. On the contrary, the second type oflung carcinoma cell line (A549) showed a medium decline in the surviving fractions with LC_{50} = 51μg/ml in view of the influence of the extract**.** Likewise, cervical (HELA) and larynx (HEP2) carcinoma cell line was moderatelyenhanced by the extract (IC_{50}=50 and 56 μg/ml respectively**)**. Obviously, the extract had weak influenceon the second type of breast (MCF7) as

well liver (HEPG2) carcinoma cell lines with IC_{50} =98 and 99μg/ml.

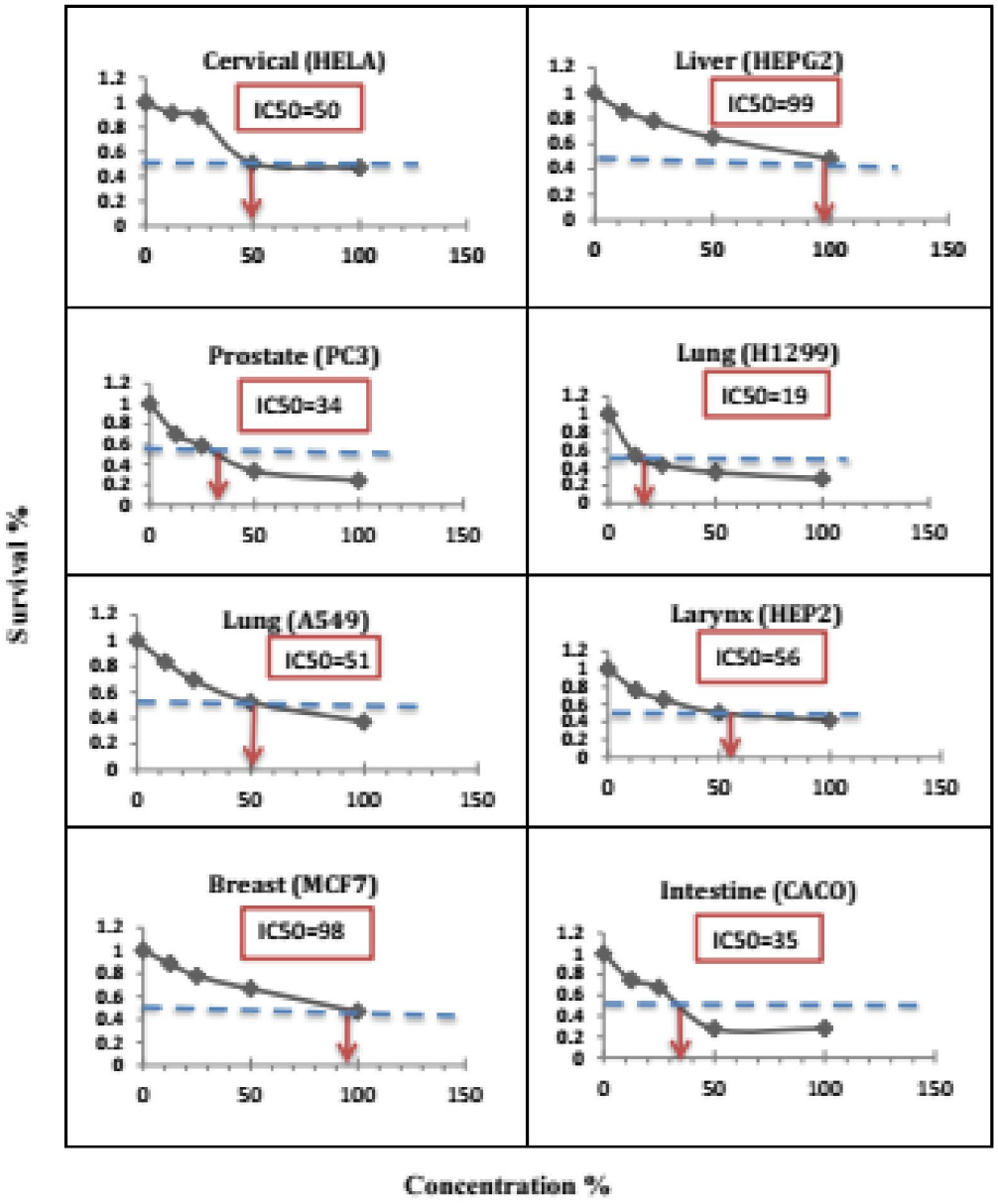

Figure 1. Effect of *A. squamosa* ethanolic fruit pulp extract and eight carcinoma cell lines.

4. Discussion

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Medicinal plants have been used to treat illness and diseases for thousands of years. Even now they are economically important, being used in the pharmaceutical, cosmetic, perfumery, and food industries (**Gowdhami *et al.*, 2014**). Cancer is a term describing conditions characterized by uncontrolled cellular proliferation and dedifferentiation **(Ponder, 2001).** Continuing oxidative damage to lipids, proteins, DNA, and the other molecules may contribute to the development of cancer, cardiovascular disease, and possibly, to neurodegenerative diseases **(Halliwell, 1996).**

Plant extracts can be regarded as chemical libraries of structurally diverse compounds, therefore constituting a promising approach in drug discovery. Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural products **(Réthy, 2007)**.

Annona squamosa is a multipurpose tree with edible fruits and is a source one of the medicinal and industrial products. *A. squamosa* is used as an antioxidant, anti-diabetics, hepatoprotective, cytotoxic activity, gene toxicity, anti-tumour activity, anti-lice agent. It is related to contain alkaloids, carbohydrates, fixed oils, tannins and phenolic compounds. Earlier works on the phytochemisrty of *A. squamosa* leaf, reported the presence of alkaloids, falvonoids, phenols, saponins, glycosides etc in water, methanol, chloroform, and petroleum ether extracts **(Saha, 2011).**

Fruits of *A. squamosa* are normally eaten fresh and possesses several medicinal properties. They are considered as a good tonic; enrichesblood, used as expectorant, increases muscular strength, cooling, lessens burning sensation, sedative to heart and relieves vomiting. Moreover, it is maturant and the mixture along with salt is usedagainst malignant tumors to hasten suppuration **(Ranjan, 1999)**. The vitamin C content is appreciable (35-42 mg/100 g) and slightly higher than in grapefruit as wellthe nutrient value of thiamine, potassium and dietary fibre is also significant **(Saha, 2011).**

In the present study the fruit pulp of *A. squmosa* containsa wide diversity of compounds, especially secondary metabolites, that have anticancer effect (*in vitro*). Distinguished examples of these compounds include flavonoids, phenols, saponins, glycosides, alkaloids, terpenoids andphytosterols. Comparatively the same data were obtained by **Anonymous (1986).** In addition, acetogenins also evaluated from the fruit pulp of the current work which was found to be selectively cytotoxic to certain human tumours **(Yang *et al.*, 2009)**.

As far as anti-tumor activity is concerned, results clearly indicated the cytotoxic effect of *A. squamosa* fruit pulp beyond eight tested cell lines for antiumoural activityresulted in promising starting sources for further investigation. Lung carcinoma cell line (H1299) was strongly (LC_{50}=19μg/ml) affected by the extract while the other type of lung cacenoma cell line (A549) was moderately (LC_{50}=51μg/ml) affected. Similarly, **Zhang *et al*. (2014)** reported that *A. squamosa* fruit pulp extract showed inhibition of cell proliferation of lung carcinoma cell line H1299 (LC_{50}=30μg/ml). To go through with this, the study revealed that prostate (PC3) and intestine (CACO) (LC_{50}= 34 and 35 μg/ml respectively) cancer cell lines were strongly affected upon applying the fruit extract. These findings are in a high accordance with **Bohlooli *et al*. (2012)** whichshowed remarkably higher sensitivity of the gastric cancer cells (AGS) to freeze-dried aqueous extract of *Ecballium elaterium* fruit. Furthermore, fruits of *Cucurbitapepo*displayed an anti-tumor effect against intestinal carcinoma (CAco) **(Badr *et al*., 2011**).

Additionally, a moderate influence was accentuated for cervical (HELA) and larynxcancer cell line (HEp-2) with IC_{50} value of 50 and 56μg/ml respectively. On the other hand, weak evident effect on breast (MCF 7) and liver (HEPG 2) cancer cell line (HEp-2) was detected. These observations suggest that the induction of apoptosis by *A. squamosa* fruit pulp extracts can be selective for certain types of cancerous cells **(Ashok *et al*., 2005).**

Cell cycle arrest caused by the treatment of plant extracts might be associated with its phytochemical compounds; terpenoids, phenolics and saponins. The anti- proliferative activity may be attributable to the existence of saponins. In fact, saponins, triterpenes and polyphenolic compounds were suggested to be responsible for the cytotoxicity against cancer cell lines **(Armania *et al.*, 2013).** Saponins have haemolytic property, induced cytotoxic effect, antitumor and anti-mutagenic activities and can lower the risk of human cancers, by preventing cancer cells from growing and has the ability to enhance the toxicity of certain ribosome-inactivating proteins synergistically at submicellar concentrations **(Sood *et al*., 2012)**. Annonaceous acetogenins are a new class of compounds that have been reported to have potent pesticidal, parasiticidal, antimicrobial, cell growth inhibitory activities. Strictly, DNA fragmentation confirmed that aqueous extrats of *A. squamosa* seeds extracts induced apoptosis in tumour cells through induction of reactive oxygen species (ROS) generation and reduced intracellular glutathione levels **(Ashok *et al*., 2004).**

Conclusion

A survey of the literature data revealed that the antitumour activities of the two cultivars of *A. squamosa* in Egypt had not been reported earlier. Extracts which exhibited substantial antiproliferative activity may represent a source for novel natural anticancer entities. Further evalution need to be carried out on *A. squamosa* in order to explore concealed areas and their practical clinical application (*in vivo*), which can be used for the welfare of the mankind.

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