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ANTIPROLIFERATIVE EFFECT AND CHEMICAL CONSTITUENTS OF *ANNONA SPECIES*

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Abstract

Many researchers are focused their work on medicinal plants for their efficacy, safety and quality to discover novel agents that may be benefit for treatment of many diseases. Different extracts of the three studied *Annona* species were screened for their effect on HTC116 (colon), (PC3) prostate, HepG2 (liver) and MCF7 (breast) cancer cell lines referring to RPE1 cell line as a normal. Some phenolic compounds as gallic, catechin and syringic acids of *A. squamosa* bark and leaves were recorded using HPLC technique. The essential oil obtained from the fresh leaves of the three *Annona* species (*A. squamosa*, *A. cherimola* and cultivar *Annona* Abdel Razek) showed high cytotoxic activity against the HCT116, PC3 and HePG2 cancer cell lines.

Keywords: *Annona species,* phenolics, alkaloids*,* anti-proferlative agent.

Introduction

Recently, many peoples prefer to return to original natural resources, using vast array of valuable natural drugs. Additionally, the side-effects of the compounds in natural remedies are considered low than synthetic ones (Beenaand Remani, 2008). The medicinal importance of the fruiting trees are due to the presence of vitamins, nutritional compounds, beside some special secondary metabolites like alkaloids (Johns *et al.*, 2011; Hassan *et al.* 2007), glycosides, terpenes, cyclopeptides, flavonoids, resins, volatile oils, tannins and acetognins (Neha and Dushyant 2011; Hassan *et al.* 2015, 2016). Several biological activities of *Annona* trees were recorded to the presence of secondary plant metabolites that had been reported as antioxidants (Saija *et al.* 1995; Kotkar *et al.* 2002), cytotoxic (Shok *et al.*, 2005), antithyroidic (*Sanjiv et al.* 2010), molluscicidal activity (Magadula *et al.*, 2009), antiplatelet (Yang *et al.* 2002), anti-inflammatory, antiviral, anti-diabetic and anti-HIV (Dash *et al.* 2001).The seeds of *Annona squamosa* Linn. have been used as a folk remedy to treat cancer in South China (Miao *et al.,* 2016). The phytochemical investigation of the ethanol fraction of *A. squamosa* seeds led to the isolation of new annonaceous acetogeninie compounds (Miao *et al.,* 2016). Therefore, the present work is designed to study the chemical composition of the most common *Annona* species that grown in Egypt and its antiproferative activity on different cancer cell lines.

Material and Methods

Plant materials and preparation of different extracts

Different parts of the three *Annona* species, leaves, fruits, bark and seeds were obtained from a private farm at Mansoriya region, Giza governorate, Egypt and identified by Dr. M. Gibali, Department of Taxonomy, Faculty of Science,Cairo University. Voucher specimens were deposited at the National Research Centre Herbarium under numbers 521, 522, 523 and 524, respectively.

Powdered *Annona* species were extracted exhaustively with EtOH (100, 80 than 50%) as promising extract by soaking at room temperature. The different combined alcoholic extracts were concentrated under reduced pressure at 45 °C using rotary evaporator. The crude residue was dissolved in hot water, left overnight, filtered using whatman No. 54 and successively partitioned with CH_2Cl_2, EtOAc and *n*-butanol (BuOH).Leaves of the three *Annona* species collected during November, 2017 were used for the determination of volatile oil contents. The volatile oil of each fresh sample was extracted by the water distillation method (for 3 hrs.) in a Clevenger's apparatus (Guenther, 1953). The resulted essential oil of each treatment was separately dehydrated with anhydrous sodium sulphate and kept in deep freezer until GC/MS analysis. Each sample was done in triplicate and the mean values of the oil content (%) were recorded.

Determination of total flavonoids, total phenolics and total alkaloids in different plant parts

Total flavonoids content was determined according to the method described by Singleton and Rossi (1965). The concentration was plotted from a standard curve of rutin. The mean of three readings was calculated and expressed as mg of rutin equivalents /100 g of air dried sample.

Folin–Ciocalteu method was used to determine total phenolic content according to the method described by Singleton and Rossi (1965). The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents /100 g of air dried sample.

Total alkaloids were determined according to Kam *et al.* (1999) and El-Gengaihi *et al.* (2013).

High performance liquid chromatography (HPLC)

The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler (G1329B), quaternary pump and a diode array detector. The measurements were integrated by Chemstation chromatographic software Computer Program. The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm I.D., 5 µm, USA) (De Brum *et al.,* 2013).

Cytotoxic effect on human cell lines

The cytotoxic activity test (*In vitro* bioassay on human tumor cell lines) was conducted and determined by the Bioassay-Cell Culture Laboratory, Pharmaceutical and Drug

Industries Research Division, National Research Centre, Dokki, Cairo, Egypt.Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983). All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium [for HepG2 (human hepatocellular carcinoma), PC3 (prostate carcinoma), MCF-7 (breast carcinoma), HCT-116 (colon carcinoma)– DMEM (Dulbecco's Modified Eagle Medium) for A549 and PC3], 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000 µg/ml Streptomycin Sulfate and 25 µg/ml Amphotericin B) and 1% L-glutamine at 37 ºC under 5% CO_2.Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ug/ml). After 48 h of incubation, medium was aspirated, 40µl MTT (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200µl of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37ºC. A positive control, composed of 100µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions (Thabrew *et al.* 1997; Bassem *et al.,* 2010). The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wave length of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

((Reading of extract / Reading of negative control) -1) × 100

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

Results and Discussion

The essential oils

Table (1) compiled the volatile oil content of the three Annona species fresh leaves. Annona Abdel Razik contains the highest amount of the essential oil amounted to 0.45 compared with 0.096 and 0.156 ml respectively for *A. squamosa* and *A. cherimola*, Mohamed *et al.* (2016).

GC/Mass analyses of the three volatile oils recorded the identification of β-pinene, α-copaene and isocaryophyllene in Abdel Razik while α-copaene and caryophullene are found in *A. cherimola* Table (2). The difference in chemical constituents between the three studied *Annona* volatile oils may be attributed to genetic factors, Mohamed *et al.* (2016) and Meiraa *et al.* (2015).

Table 1 : The concentration of volatile oil from fresh leaves of *Annona* species (ml/ 25g fresh)

Months	*A cheirmola*	*A squamosa*	*A* Abdel Razik
Jan.	0.008^{f}	0.002^{f}	0.012^{f}
Mar.	0.015^{e}	0.018^{e}	0.125^{e}
Jun.	0.048^{d}	0.024^{d}	0.16^{d}
Aug.	0.08^{c}	0.03^{c}	0.28^{c}
Oct.	0.158^{a}	0.096^{a}	0.48^{a}
Dec.	0.098^{b}	0.05^{b}	0.36^{b}

- Statistical analysis is carried out by one way analysis of variance (ANOVA), Co-stat Computer Program.
- Unshared letters between brackets are significant values between groups at p **>0.0001**

Table 2 : The Major constituents of the essential oil of *Annona* sp.

Peak No.	[a] Identification constituents		% components in *Annona* sp.		
		RT	Abdel Razek	*A. squamosa*	*A. Cherimola*
2	α-Pinene	4.84	9.96	2.13	6.47
4	β-Pinene	6.05	12.90	-	8.2
18	2-Carene	19.82	8.27	11.92	2.44
24	Isocaryophillene	22.72	0.16	27.59	1.61
26	Caryophyllene	23.36	5.34	0.86	13.99
42	α-copaene	25.93	17.06	-	21.78
45	α-Selinene	26.28	3.09	0.7	4.52
61	γ-Elemene	29.02	5.84	-	1.35
77	tau.-Cadinol	32.37	1.02	5.54	1.75

Total phenolics content

As shown in Table (3) the total phenolics in bark, fruit, and seed extracts were lower than their contents in leaves of the three *Annona* species; the highest concentration of total phenolics was found within butanol and total alcohol extract of leaves of *A. Cherimola*, *A. squamosa* and cultivar Abdel Razek (21.45, 15.36 and 20.36 mg/g, respectively).

The data revealed that, using different solvents during the extraction changed the yield of total phenolics according to the nature of each solvent because the yield of total phenolics is solvent dependent. So, the highest extractable values have been attained using alcohol compared with the other used solvents. Cultivar Abdel Razek seeds may be considered the poorest source of the total phenolics compared with the other species. Also, clear differences could be observed among leaves, bark, fruit and seeds extracts for all solvents investigated. *A. cherimola* leaves are considered the highest source of total phenolics (21.45 mg/g) rather than those of *A. squamosa* and cultivar Abdel Razek for all solvents used herein. For example, total phenolic contents in *A. cherimola* leaves extracted by butanol reached 20.68 mg/g and increased to 21.45 mg/g by alcohol before fractionation. These results may throw some lights on the polar properties of the phenolics characterized in *Annon*a species, and this may be confirmed by the less efficiency of chloroform for extracting phenolics. The extractive capacity of phenolic components from *Annona* depends on the type of solvents. The best extraction efficiency was achieved by ethanol 100%, then 80% followed by 50%.These results go parallel with the data obtained by Lapornik *et al.* (2005) and Tomar and Sisodia (2013). They found that the extraction of phenolic compounds from a plant depends on the methods and type of extracting solvent. A high yield of phenolics can be extracted from sorghum leaf using water (Agbangnan *et al.,* 2012), while extraction of the most phenolics from wheat bran requires 80% ethanol (Verma *et al.,* 2008). In another investigation dealing with effect of different solvents on extraction of phenolics from aerial parts of *Potentilla atrosanguinea* showed that 50% ethanol was more efficient than pure or 50% forms of methanol and acetone (Kalpana *et al.,* 2008). Also, Rodtjer*et al.* (2006) reported that the 70% EtOH extracted phenolics more efficiently than the pure solvent extracts did.

Table 3 : Total Phenolics content (mg/g) in different *Annona spp*. using different extracting solvents

Different plant Parts		Total Phenolics			
		Chloroform	**Ethyl Acetate**	**Butanol**	**Total alcohol**
A. cherimola	Leaves	2.36	16.68	20.68	21.45
	Bark	1.68	14.68	15.64	9.85
	Fruit	0.05	8.68	12.98	10.98
	Seed	1.03	10.75	8.32	9.68
A. squamosa	Leaves	0.6	9.69	12.87	15.36
	Bark	0.4	8.36	9.15	10.03
	Fruit	0.3	6.68	4.45	6.68
	Seed	0.3	3.45	3.36	7.15
A. cherimola x A. squamosa (abdelrazek)	Leaves	1.3	15.65	18.36	20.36
	Bark	0.9	13.36	15.36	12.87
	Fruit	0.2	8.96	6.32	5.65
	Seed	0.12	6.65	8.63	7.89

Total flavonoids

The yields obtained by using various solvents and the composition of total flavonoids are shown in Table 4. The highest flavonoid content was found in leaves of *A. cherimola* (4.6 mg/g) with total EtOH and the lowest one was from *A. squamosa* fruit and seeds (0.1 and 0.1 mg/g, respectively) in case of extraction with chloroform. These results go in parallel with the results obtained by Ghasemzadehand Jaafar (2011). They found that, the methanol extracts contained higher amounts of total flavonoids than acetone and chloroform extracts from *Z. officinale* leaves. Anand *et al.* (2015) reported that acetone was superior to aqueous and methanol for extraction of the flavonoids from *Camellia sinensis*, green tea.

Table 4 : Total flavonoids content (%)in different parts of *Annona* sp. parts using different extracting solvents.

Different plant parts		Total Flavonoids			
		Chloroform	**Ethyl Acetate**	**Butanol**	**Total alcohol**
A. cherimola	Leaves	0.07	1.24	2.9	4.6
	Bark	0.03	0.99	1.2	1.5
	Fruit	0.02	0.63	1	1
	Seed	0.04	0.45	0.26	0.36
A. squamosa	Leaves	0.05	0.88	2	2.7
	Bark	0.02	0.78	1.36	3.2
	Fruit	0.01	0.32	0.78	0.8
	Seed	0.01	0.22	0.29	0.25
A. cherimola x A. squamosa (abdelrazek)	Leaves	0.07	1.02	3.2	4.2
	Bark	0.04	0.98	1.86	0.98
	Fruit	0.02	0.78	2.32	1.9
	Seed	0.02	0.45	0.87	0.6

Total alkaloids

The results in table (5) showed that the presence of the total alkaloids extracted by different solvents were very low (0.01%). Moreover, no significant difference was observed between the two solvents EtOAc and BuOH in all solvents used. It appeared that the higher total alkaloid was in the bark than in the seeds of cultivar Abdel Razek (0.08 and 0.06%, respectively) which are more than in other two species.

Table 5: Total alkaloids content (mg/g) in different *Annona* sp. using different extracting solvents.

Different plant parts		Total Alkaloids			
		Chloroform	**Ethyl Acetate**	**Butanol**	**Total alcohol**
A. cherimola	Leaves	0.01	0.001	0.009	0.03
	Bark	0.02	0.002	0.01	0.05
	Fruit	0.01	0	0.008	0.02
	Seed	0.02	0.001	0.007	0.03
A. squamosa	Leaves	0.01	0.001	0.003	0.016
	Bark	0.02	0.002	0.004	0.02
	Fruit	0.03	0	0.009	0.03
	Seed	0.03	0.002	0.004	0.02
A. cherimola x A. squamosa (abdelrazek)	Leaves	0.01	0.001	0.009	0.05
	Bark	0.02	0.001	0.02	0.08
	Fruit	0.01	0.001	0.01	0.05
	Seed	0.02	0.002	0.009	0.06

High performance liquid chromatography

The total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts (Katsube *et al.,* 2004; Wu *et al.,* 2004). HPLC is the preferred technique for both separation and quantification of phenolic compounds (Naczk and Shahidi, 2004). Various factors affect HPLC analysis of phenolics, including sample purification, mobile phase, column types and detectors (Stalikas, 2007). Table (6, 7) reveals that, the alcoholic extract of the three *Annona* species through growth season contain the highest amounts of gallic, catechin, rutin and coumaric acid in May, 2017. These acids are the highest in leaves than bark in the three species followed by the values obtained in August, 2017. The quantitative extractable individual phenolics varied from one species to another and also according to the type of solvent employed. Thus the choice of the proper solvents and type of *Annona* may depend on the desired phenolics needed. The following examples may confirm the above mentioned results. Butanol extract of the three leaves of *Annona* gave the highest values of rutin [46.02 in Abdel Rezek (LA), 50.26 in *A. squamosa* (LB) and 42.81 mg/100g in *A. cherimola* (LH)] and catechin [9.12 in LA, 4.54 in LB and 8.64 in (LH) mg/100g], while EtOAc extract of leaves contained the highest portion of rutin in an amount of 2.61 in LA, 3.76 in LB and 0.25 in LH mg/100g. Catechin values were 0.953 in LA, 1.002 in LB and 0.14 in LH mg/100g. The differences obtained in the present investigation data may be an indicator to difference in species studied or to seasonal growth of the trees. These findings are in agreement with Mariod *et al.* (2012) who indicated that the alcoholic extract of *Annona squamosa* leaves are the best solvent to extract phenolics. Ethyl acetate is the solvent of choice to extract the phenolics in the present investigation. The extractable material is the highest by ethyl acetate solvent. The phenolics determined by HPLC and extracted by ethyl acetate fractionate many compounds than that extracted by BuOH (Table 5).

Gallic, catechin, and syringic acids were determined by HPLC in the bark of *A. squamosa* with the amounts of 38.56, 2.03, 6.32 mg/100g of extract, while these acids amounted 21.66, 1.002 and 1.65 mg/100g of extract in the leaves of *A. squamosa*, respectively. In this respect, isolation and characterization of such phenolics may be useful in production of natural antioxidant substances from a waste material of *Annona* trees which may add an economical value to *Annona* fruits.

Cytotoxic effect on human cell lines

The cytotoxic activity of the alcoholic extract of different *Annona* species against certain cancer cell lines as HCT116 (colon cancer), PC3 (prostate cancer), HepG2 (liver cancer) and MCF7 (breast cancer) comparing with RPE1 (retina normal cell) is shown in table 6. Incubation of cell lines with DMSO (negative control) didn't show any toxicity on the cells during the incubation period. The alcoholic extract of leaves from the three species of *Annona* trees induced weak activity against the tested cell lines. However *A. cherimola* showed a mild antiproliferative effect against prostate cell line with an IC_{50} of 66.8 µg/ml. Evaluation of the antiproliferative activity of alcoholic extract of seeds from different *Annona* species showed that *Annona squamosa* seeds have a potential cytotoxic activity on HCT116, HePG2 and MC7 with $I.C_{50}$ of 3.5 µg/ml, 7.1 µg/ml and 0.7 µg/ml, respectively. *Annona cherimola* seeds came in the second efficacy rank and had less cytotoxic effects than *A. squamosa* on HCT116, HePG2 and MCF7 cell line with $I.C_{50}$ values represented by 15.9 µg/ml, 19.8 µg/ml and 12.5 µg/ml, respectively Table (8).

While, the bark extracts of *Annona* species showed low antitumor activity against HCT116, PC3, MCF7 and HepG2 carcinoma, while fruit extract of the three *Annona* species had negligible cytotoxic activity.

The volatile oils extracted from fresh leaves of the three *Annona* species induced relatively high cytotoxic activity against the human carcinoma cell lines used in this study with IC_{50} values amounted 0.7 µg/ml, 0.7µg/ml and 2.1µg/ml for *A.* Abdel Razek, *A. squamosa* and *A. cherimola* against HCT116 cells, respectively. The IC_{90} were equal to 11.9 µg/ml, 31.1 µg/ml and 40 µg/ml for the three *Annona* species

in the same order against PC3 cells. The essential oils of the leaves of the three species showed markedly high cytotoxic effect against HepG2 cells with their IC_{50} values reached to 1.5 µg/ml, 3.7 µg/ml and 2.1µg/ml for *A.* Abdel Razek, *squamosa* and *cherimola*, respectively. In breast carcinoma cells, *A. squamosa* volatile oil proved the highest activity (IC_{50}=1.0 µg/ml), while *A. cherimola* and Abdel Razek essential oils showed less activity with IC_{50} of 4.5 µg/ml and 3.8 µg/ml, respectively.

A. cherimola volatile oil induced the highest anticancer activity (IC_{50}=0.7 µg/ml) on HepG2 carcinoma cell, however Abdel Razek and *squamosa* showed weak effect with IC_{50} of 1.5 µg/ml and 3.7 µg/ml, respectively Table (8).

Butanol extract had no cytotoxic activity, On the other hand, fractioned total alkaloids from *A.* Abdel Razek were relatively potent and showed high IC_{50} values of 66.5, 76.4 and 58.9 µg/ml against PC3, HepG2 and MCF7 carcinoma cell line, respectively. The cytotoxic effect induced may be partly attributed to alkaloidal component of *Annona* species.

The data obtained from this study showed antitumor activity of the alcoholic extracts obtained from the three *Annona* species and their parts. Differential activity of the tree parts against carcinoma cell lines were recorded.

Fruits, the edible part had no cytotoxic activity, while other parts as leave and bark had variable activity according to the active ingredient found in them. Acetogenins were found abundantly among the members of the *Annonaceae* family. These acetogenins are known to have potent antineoplastic, antiparasitical and antimicrobial activities as well as its cytotoxic effect to certain human carcinoma cell lines (Moghadamtousi *et al.,* 2015). Alcoholic extract from the seeds of *A. squamosa* possessed significant antitumor activity against AK-S tumor *in vitro* (Pardhasaradhi *et al.,* 2004). Another study reported that seed extract significantly induced antitumor activity *in vitro* against four hepatoma cells lines (Wang *et al.,* 2014).

Many reports published the antitumor activity of *Annona muricata* species. In Indonesia, Suyatmi *et al.* (2012) indicated the potential selective anticancer activity of ethanolic extract of *Annona muricata* leaves against Hela cervical cancer cell line. The IC_{50} value of the extract for Hela cell lines was 97 µg/ml, while the Vero cell line was 356µg/ml. Volatile oils obtained from the leaves of *Annona muricata* grown in Nigeria was studied for its cytotoxic activity by Owolabi *et al.* (2013). They found that the oil obtained by hydro-distillation was dominated by E-caryophyllene (38.9%) and eugenol (30.2%) with low amounts of α-humulene (4.3%), δ-cadinene (6.0%), and caryophyllene oxide (5%) which coincides with the results of Mohammed *et al.* (2016). This study also reported that the oil had notable *in vitro* cytotoxic activity (99.2%) on MCF7 cells at 100 µg/ml and the authors attributed the cytotoxic effect to the main components found in the volatile oil. These finding goes in parallel with the present study.

Table 6 : Concentration of phenolic compounds (as mg/100g) of different *Annona* species through growth seasons.

Mon.		Samples	Gallic Acid	Catechin	Caffeic Acid	Syringic Acid	Rutin	Coumaric Acid	Vanillin	Querectin	Cinnamic Acid
February	Leaves	LA	0.47	-	-	-	1.35	-	0.12		0.0021
		LB	-	-	-	-	3.85	-	0.37		-
		LH	-	-	-	-	1.69	-	0.05	0.006	-
	Bark	BA	-	-	-	-	-	-	0.08	0.077	-
		BB	0.57	-	-	1.25	-	-	0.06		0.01
		BH		-	-	-	0.02	-	0.12	0.005	-
May	Leaves	LA	9.98	1.50	-	-	1.61	0.07	-	-	-
		LB	43.07	0.82	-	-	7.31	0.05	-	-	-
		LH	-	1.21	-	-	8.20	-	-	-	-
	Bark	BA	-	-	0.07	-	0.03	-	0.11	-	-
		BB	-	0.01	0.03	-	0.09	-	0.08	0.007	-
		BH	-	-	-	-	0.02	-	-	-	-
August	Leaves	LA	-	1.04	-	-	3.54	0.03	-	-	-
		LB	-		-	-	7.47	-	-	-	-
		LH	-	0.71	-	-	4.41	-	-	-	-
	Bark	BA	-	-	-	-	0.06	-	-	-	-
		BB	-	-	-	-	0.05	-	-	-	-
		BH	-	-	-	-	0.04	-	-	-	-
November	Leaves	LA	-	-	-	-	1.12	-	0.07	-	-
		LB	-	-	-	-	2.71	-	0.21	-	-
		LH	-	0.12	-	-	1.84	-	0.07	-	-
	Bark	BA	-	-	-	-	0.01	-	0.1	-	-
		BB	-	-	0.01		0.03	-	0.04	-	-
		BH	-	-	-		0.001	-	0.02	-	-
	Seeds	SA	-	-	0.08	4.13	-	-	-	-	0.14
		SB	-	-	-	-	-	-	-	-	0.32
		SH	-	-	-	-	-	-	-	0.08	0.09
	Fruits	FA	9.55	-	-	-	-	-	-	-	-
		FB	20.40	-	-	-	-	-	-	-	-
		FH	13.67	-	-	-	-	-	-	-	-

(-) not found

Table 7: Concentration of phenolic compounds (mg/100g) of different extracts of *Annona* species.

Fractions		Samples	Rutin	Querctin	Catechin	Vanillin	Gallic Acid	Caffeic Acid	Syringic Acid	Coumaric Acid	Cinnamic Acid
EtOAc	Leaves	LA	3.52	0.47	0.953	-	-	0.28	-	-	0.06
		LB	2.61	0.044	1.002	0.144	21.66	0.44	1.65	0.032	
		LH	3.76	0.097	0.14	0.119	-	0.36	1.31	0.037	0.068
	Bark	BA	0.25	0.28	0.54	0.538	-	0.21	3.54	0.049	0.002
		BB	0.76	0.27	2.03		38.56	-	6.32	0.344	0.042
		BH	0.319	0.19	1.16	0.779	17.72	0.12	4.04	0.137	0.019
	Seeds	SA	-	0.59	0.97	-	-	-	5.64	-	0.136
		SB	-	8.96	-		-	-	-	-	0.971
		SH	-	1.24	1.53	0.231	-	-	4.73	-	0.328
	Fruits	FA	0.092	0.024	0.03	0.118	-	0.13	4.87	-	0.002
		FB	0.103	0.027	2.05	0.105	-	0.31	1.75	-	0.022
		FH	0.141	-	0.97	0.065	7.63	0.22	2.023	-	0.023
BuOH	Leaves	LA	46.02	-	9.12	-	-	-	-	-	0.251
		LB	50.26	-	4.54	-	-	-	-	-	-
		LH	42.81	-	8.64	-	-	-	-	-	-
	Bark	BA	2.14	-	-	-	-	-	-	-	-
		BB	1.44	-	-	-	-	-	-	-	-
		BH	2.27	-	-	-	-	-	-	-	-
	Seeds	SA	-	-	-	-	-	-	-	-	-
		SB	-	-	-	-	-	-	-	-	0.028
		SH	-	-	-	0.142	-	-	-	-	-
	Fruits	FA	-	-	-	-	-	-	-	-	-
		FB	-	-	-	-	-	-	-	-	-
		FH	-	-	-	-	-	-	-	-	-

(-) not found

LA = Leaves Abdel Razek, BA = Bark Abdel Razek, SA = Seed Abdel Razek, FA = Fruit Abdel Razek
LB = Leaves *A. squamosa*, BB = Bark *A. squamosa,* SB = Seed *A. squamosa*, FB = Fruit *A. squamosa*
LH = Leaves *A. cherimola*, BH = Bark *A. cherimola,* SH = Seed *A. cherimola,* FH = Fruit *A. cherimola*

Table 8 : Antiproferlative effect of different extract of Annona species.

		Antiproferlative effect														
Samples		HCT116			PC3			HePG 2			MCF7			RPE1 (Normal cells)		
		100 µg/ml (%)	IC_{50}	IC_{90}	100 µg/ml (%)	IC_{50}	IC_{90}	100 µg/ml (%)	IC_{50}	IC_{90}	100 µg/ml (%)	IC_{50}	IC_{90}	100 µg/ml (%)	IC_{50}	IC_{90}
Abdel Razek	Leaves	30.30	-	-	60.40	-	-	26.10	-	-	40.90	-	-	76.60	68.3	111.1
A. squamosa		20.40	-	-	57.30	-	-	29.80	-	-	31.50	-	-	100	43.1	68.2
A. Cherimola		24.80	-	-	66.80	66.8	81.02	30.20	-	-	12.10	-	-	82.30	61	100.8
Abdel Razek	Seeds	100	40.8	65.6	83.90	60.3	99	100	38.7	62.5	100	38.3	61.7	100	29.1	49.1
A. squamosa		100	3.5	6.1	54.20	-	-	100	7.1	14.5	100	0.7	8.6	100	100% up to 6.25µg/ml	
A. Cherimola		98.50	15.9	24	61.50	-	-	100	19.8	32.1	100	12.5	26.2	100	11.6	24.2
Abdel Razek	Bark	52.10	88.3	142.9	75.30	65.5	110.1	90.30	50.8	86.8	75.50	59.5	107.9	0	-	-
A. squamosa		93.10	58.4	92.5	70.10	75.8	120.5	97.10	46.7	74.3	68.10	73.5	122.7	100	45.1	71.4
A. Cherimola		19.50	-	-	62.40	-	-	24.10	-	-	0	-	-	54.40	-	-
Abdel Razek	fruit	9.20	-	-	60.40	-	-	6.20	-	-	13.20	-	-	82.30	-	-
A. squamosa		12.10	-	-	66.30	80.9	125.7	21.80	-	-	2	-	-	0	67	106.3
A. Cherimola		0	-	-	54.30	-	-	0	-	-	16.20	-	-	0	-	-
Abdel Razek	Volatile oil	100	0.7	6.1	100	11.9	25.3	100	1.5	5.9	100	4.5	8.2	100	0.5	6.1
A. squamosa		100	0.7	4.03	97.60	31.1	58.1	100	3.7	7.7	100	1	6.6	100	0.9	6.6
A. Cherimola		100	2.1	6.9	92.90	40	73.4	100	0.7	4.1	100	3.8	7.2	100	1.8	6.8
Total Flavonoids		22.10	-	-	55.50	-	-	11.50	-	-	44.00	-	-	29.30	-	-
Total Alkaloids		0	-	-	82.60	66.8	104.5	72.60	76.4	118.5	71.30	58.9	114	100	45	70.8
DMSO		1	-	-	1	-	-	1	-	-	3	-	-	0	-	-
Negative control		0	-	-	0	-	-	0	-	-	0	-	-	0	-	-
Positive control Doxorubicin µM		-	37.6	65.1	-	23.8	41.1	-	21.6	37.8	-	26.1	45.02	-	23.1	41.2

From the previous literature it was proved that α-copaene possesses a non-genotoxic/mutagenic feature, weak antioxidant and cytotoxic activity *in vitro* for normal and cancer cell lines. α-Copaene might be a new agent which can inhibit proliferation in N2a-NB cells in a dose-dependent manner and activates the caspase-3 enzyme (Turkez *et al.,*

2014). Caspase-3 is an executioner enzyme in a caspase-dependent apoptosis cascade. Induction of caspase-3 activity in turn leads to chromatin condensation, degradation and dissolution (Dahham *et al.,* 2015). α-Pinene; a major component of different volatile oils was able to induce apoptosis that evidenced by early disruption of the mitochondrial potential, production of reactive oxygen species and increase in caspase-3 activity and heterochromatin aggregation(Matsuo *et al.,* 2011).

Recently, Moghadamtousi *et al.* (2015) reviewed the traditional uses of the isolated acetogenins from *Annona muricata* and their biological activities. Annona activated P21 and arrested cancer cells at a growth static G1 phase and caused more significant cytotoxicity to cancer cells in growth of S phase cells and enhance the expression of bax/bad.

Conclusions

The three *Annona* species through growth season contain the highest amounts of gallic, catechin, rutin and coumaric acid in May 2017. These acids are the highest in leaves than bark in the three species followed by the values obtained in August 2017. The highest concentration of total phenolic was found in butanol and total alcohol extract of leaves of *A. Cherimola*, *A. squamosa* and cultivar Abdel Razek (21.45, 15.36 and 20.36 mg/g, respectively).The highest flavonoid content was found in leaves of *A. cherimola* (4.6 mg/g) with total EtOH and the lowest one was from *A. squamosa* fruit and seeds (0.1 and 0.1 mg/g, respectively). While, the higher total alkaloid was recorded in the bark than in the seeds of cultivar Abdel Razek (0.08 and 0.06%, respectively) which are more than in the other two species.

The volatile oils extracted from fresh leaves of the three *Annona* species induced relatively high cytotoxic activity against the human carcinoma cell lines used in this study with IC_{50} values of 0.7 µg/ml, 0.7µg/ml, and 2.1µg/ml for *A.* Abdel Razek, *A. squamosa* and *A. cherimola* against HCT116 cells, respectively. The IC_{90}were also found to be 11.9µg/ml, 31.1 µg/ml and 40 µg/ml for the three *Annona* species in the same order against PC3 cells. The essential oils of the leaves of the three species showed markedly high cytotoxic effect against HepG2 carcinoma cells with their IC_{50} values of 1.5 µg/ml , 3.7 µg/ml and 2.1µg/ml for *A.* Abdel Razek, *squamosa* and *cherimola*, respectively.

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Ethics approval and consent to participate

The study was complied with the ethical guidelines of Medical Ethical Committee of the National Research Center in Egypt.

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