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Study of the Anticancer Activity of Annona squamosa Seeds

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REF cells

Abstract

The whole world is still searching for natural sources in various fields, especially natural alternatives for treating diseases and various health disorders, most notably in the treatment of tumors and cancers. The current study included an in vitro anticancer estimation of Annona seed extract on two cell lines of breast cancer (MCF-7& MDA-231) and one normal cell line represented by REF. Active constituents in the seeds were extracted by maceration with 70% ethanol. General phytochemical tests were conducted to investigate the active constituents of the extracted seeds. The phenolic compounds and flavonoids in the extracted residue were estimated via HPLC in addition to the nonpolar substances in the seed residue. The three cell lines (MCF-7, MDA-231 & REF) were subjected to different concentrations of seed extract for the anticancer assay. Results showed that the ethanolic extract residue was 8.4 g from 90 g of powdered plant seeds, representing 9.34% W/W, and the seed residue contained many active constituents. The HPLC results indicated that the seed extract was rich in gallic acid, caffeic acid, pyrogaloll, cinnamic acid, p-coumaric acid and, to a lesser extent, chlorogenic acid, which are simple phenolic compounds. Luteolin, kaempferol, and apegnine and lower amounts of quercetin, rutin and catechin, which are flavonoids, were also detected in the residue extracted from the seeds. For the nonpolar seeds, the following components were detected at a decreasing level: β-myrcene, p-cymene, α-pinene, eugenol, and β-pinene. The anticancer activity of the seed extract at several concentrations differed across the three types of cell lines in the present study.

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Introduction

Annona fruit is a plant from the Annonaceae family that grows on palm trees, which are grown in a number of tropical regions worldwide (1). Its cultivation is widespread in Sudan and Yemen, where it is known as the Indian quince or Indian pineapple, and it is scattered in the areas of Wadi Aslam, Mahbashiyah and western slopes. Some trees are also planted in Gaza and Egypt in the governorates of Alexandria Eastern (Anshas), Giza (Pyramid), Minya and Aswan, and these fruits are usually dry. It is characterized by its rubber texture and sweet flavor (2). A. muricata has a number of medicinal uses that have been reported across the globe, ranging from the use of leaves, bark, roots, and fruits to the use of seeds(3). It is a functional food and is traditionally used as a tea for therapeutic purposes. The most widely used preparation in traditional medicine is the decoction of bark, roots, seeds or leaves, but the applications of these methods vary. In a number of tropical sub-Saharan countries, such as Uganda, all parts are used to treat malaria, stomachache, parasitic infections, diabetes and cancer (48-). These areas are rich in nutrients that are important for health, in addition to having multiple pharmacological applications in the traditional medicine of India, including its popular use for the treatment of cardiometabolic disease, which has been validated in experimental models (9),(10). In addition to the several biological activities of extracts from Annona squamosa, including anticancer, antidiabetic, antiobesity, and C.N.S. effects (11- 15), the plant can act as a lipid-lowering agent, and hepatoprotective functions have been described (16), (17). The plant is rich in natural phytochemicals such as alkaloids, phenols, acetogenins, flavonoids, and vitamins (18- 20). These compounds demonstrate several biological activities, including antioxidant, antihypertensive, antibacterial, antidiabetic, and anticancer effects (21- 26). It is especially known for its hypoglycemic effect and anti-inflammatory activity in diabetic patients (27), (28). In previous studies, AME was shown to enhance hepatic energy metabolism and autophagy, which improved hepatic function in T2DM mice (29- 31). The predominant compounds are acetogenins, followed by alkaloids, phenols and other compounds (32). Leaves and seeds are the main plant organs studied, probably because they are the most traditionally used organs (33). Annona muricata is expected to be nutraceutical to attenuate diabetic complications (34). Given that it is convenient to experimentally validate the entire active ingredient and that a direct effect on smooth muscle contraction could explain its effect on blood pressure. The vasorelaxant effect of the extract could be partially attributed to kaurenoic acid and cyclopeptides (34), (35). There is evidence that fertility in diabetic mice can be treated with antioxidant agents (36), (37).

In this study, we will look for its biological effects on humans with regard to seed extract due to the lack of many scientific studies on its medical importance and because the recent period has witnessed the establishment of research centers in the country where the plant grows naturally for the scientific evidence regarding the plant and its use.

The aim of the current study is to identify the major components present in the Annona seeds and their biological importance in terms of plant waste that can play a role in biological effectiveness for humans, espically as anticancer agent

Methods

Plant Collection and Classification

The plant fruits were purshased from a local market, and the seeds were obtained to be cleaned, washed, dried well, and then kept in a dry, dark place.

- Preparation of seed ethanolic extract

Approximately 90 g of powdered seed material was macerated in 70% ethanol for one week in a cool, dark place and then filtered and dried with a rotary evaporator at 45°C. The yield weight was designated as crude residue, which was subjected to general tests and quantitative and qualitative analyses to detect the types of phenolic, flavonoid and nonpolar compounds that the seeds might be rich in (38).

- Phytochemical investigation of the seed ethanolic extract

To investigate the major active content in the extracted residue, about 250 mg was dissolved in 25 ml distilled water to obtain a concentration of 10 mg/ml. The chemical tests included the following: detection of tannins, detection of alkaloids (dragangroff test), detection of saponins, detection of flavonoids, detection of polyphenolic compounds and detection of reducing sugars (39).

Seed ethanolic extract analysis via high-performance liquid chromatography (HPLC)

a-Analysis of phenolic and flavonoid compounds by HPLC

A stock solution at a concentration of 50 mg/10 ml was prepared (5 mg/ml) from the residue. A Shimadzu (Japan), HPLC apparatus with the conditions illustrated in Table (1) was used to identify and quantify some simple phenolic compounds and flavonoids from the seed extracts:

Table (1): HPLC conditions for ethanolic extracts

Phenolic compounds	Flavonoids	Phenolic acids
Instrument	Shimadzu, Japan	Shimadzu, Japan
Mobile phase	A= Acetonitrile: 80% 0.5% Formic acid 20% B= Acetonitrile: 30% 0.5% Formic acid 70%	A= 0.2% Acetic acid pH=4 B=Acetonitrile: Methanol (80:20) % A/B = 30%

Column	ODS_{C18} (250× 4.6 Id) mm/5µm partical size		ODS_{C18} (50× 4.6 Id) mm/13µm partical size	
Flow rate	0.8 ml/min		0.3 ml/min	
Injection Volume	20 µl		20 µl	
Concentration of sample	50 mg/1 ml		50 mg/1 ml	
Detection wave-length	UV–Vis at λ 338 nm		UV–Vis at λ 338 nm	
Column Tempera-ture	Room Temperature		Room Temperature	
Standards used	Flavonoid	Injection concentration (ppm)	Phenolic acid	Injection concentra-tion (ppm)
	Rutin	2.5	Pyrogallol	3
	quercetin	2.5	Gallic acid	15
	Luteolin	2.5	Cinnamic acid	3
	Apigenin	2.5	Chlorogenic acid	2
	catechin	2.5	p-Coumaric acid	3
	Coumarin	2.5	Caffeic acid	10
	Isorhamnetin	2.5	Ferulic acid	5
	kaempferol	2.5		

b-Analysis of nonpolar substances via HPLC

HPLC conditions for analysis of the nonpolarity present in the alcoholic extract of the seeds were estimated via an HPLC apparatus according to the conditions in reference (40), as shown in Table (2).

Table (2): HPLC conditions for nonpolar compounds

Parameters	Conditions
Instrument	Shimadzu, Japan
Mobile phase	Acetonitrile : H2O (40%)
Column	reversed-phase C18 column (250 x 4.6 mm i.d.; 5 µm).
Flow rate	1.0 ml/min
Injection Volume	20 µl
Concentration of sample	4 mg/1 ml
Detection wavelength	UV–Vis at λ 254 nm
Column Temperature	Room Temperature
Standard used	**β- Pinene** (1µg/ml)
	α- Pinene (1µg/ml)
	Eugenol(1µg/ml)
	β- myrcene(1µg/ml)
	p-cymrne(1µg/ml)
	Limonene(1µg/ml)

Anticancer activity

This study investigated the cytotoxic effects of different concentrations of seed extracts on the breast cancer cell line MCF-7 (passage 6), the breast cancer cell line MDA (passage 25) and rat embryo fibroplast cells, which represent normal cells (passage 5).

All the cell lines were supplied by the Biotechnology Research Center/Al-Nahrain University and included the following: **Breast cancer cell line (MDA-231):** The 25-pass cancer line, a universal epithelial breast cancer cell line, was generated from the epithelial cells of a 51-year-old Caucasian woman who developed metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories (41). **MCF-7** cells constitute a breast cancer cell line that was isolated in 1970 from a 69-year-old Caucasian woman (42). MCF-7 is the acronym for the **Michigan Cancer Foundation-7**, which refers to the Institute in Detroit, where the cell line was established in 1973 by Herbert Soule and coworkers (43).

The following protocol was performed in this assay (44). All the cell lines were activated and grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 IU/ml penicillin to obtain a monolayer in special tissue culture dishes under standard conditions (37°C, 5% CO2, humidified atmosphere). Each confluent monolayer was detached with 0.05% trypsin/0.02% EDTA solution to transfer or passage the cell lines.

Preparation of the seed ethanolic extract test solution

Approximately 100 mg of the crude residue was redissolved in 50 ml of distilled water to obtain a stock solution (2 mg/ml), which was sterilized with a Millipore 0.22 mm filter unit into sterile tubes and then stored at 4°C in a refrigerator prior to use. The sterile ethanolic extract of the seeds was diluted in a series of dilutions with the aid of sterile RPMI medium to obtain different concentrations ranging from 1 to 0.000488 mg/ml for the next assay. To investigate the cytotoxic effects (anticancer activities) of the plant extracts at different concentrations, the following steps were applied.

Seeding: All cell lines were suspended in growth media and then (100 µl) were seeded in a 96-well microtiter plate for each line in a separate tissue culture plate to get (5X104) cells/well. All the plates were incubated for 24 hours at 37°C until full cell attachment and to obtain monolayers. For the treatment, 100 µl of each tested solution of seed extract was added in triplicate, and 8 untreated wells, which contained only suspended cells in 100 µl of serum-free RPMI 1640 medium, were used as controls. The plates were incubated for 20 hours at 37°C.

-Recovery times and results reading: At the end of the exposure time, the medium from all the wells was decanted off, the attached cells were gently washed with PBS twice, and finally, 50 µl of MTT dye (2 mg/ml) solution was added to all the wells. The plates were incubated at 37°C for an additional 4 hours, after which 50 µl of DMSO was added to the plate to solubilize the violet crystals formed by the mitochondria of the living cells only. The color intensity was read by microplate reader at 492 nm, which was directly proportional to the number of living cells.

The percentage of cell inhibition (%IR) was calculated according to the following equation:

%IR=(Control average reading –Sample average reading/ Control average reading)X 100 (45).

Statistical analysis:

The Statistical Analysis System- SAS (2018) program was used to detect the effects of different factors on the study parameters. Least significant difference (LSD) tests were used to compare the means (two-way ANOVA) in this study (46).

Results

The ethanolic extract yield of seeds

Each 90 gm sample of powdered seeds extracted with ethanol yielded 8.4 g of residue or approximately 9.34% w/w residue.

-Phytochemical investigation of extracted seed residue

The results of the phytochemical tests of the major active components in the seed extracts are shown in Table (3).

Table (3): Phytochemical Tests of Annanna Seeds

Test	Result	Comments
Tannins test with lead acetate 1%	+	White precipitate
Reducing sugar with Benidect's test	Trace	Orange–Red precipitate
Alkaloids by Dragendroff's test	+++	Orange–brown precipitate
Saponines by foam formation test	++	Foam formation
Flavonoids by NaOH solution test	+	Bright yellow
Polyphenols by $FeCl_3$ 3% solution test	+	green p.p.t

Analyzing the seeds ethanolic extract by high performance liquid chromatography (HPLC)

a-Analysis of phenolic and flavonoid compounds in the seed extract residue

As shown in Figs. (1), (2), (3) and (4) and Tables (4) and (5), the plant seed extract was rich in different compounds in different quantities. Compared with standard phenolic compounds and flavonoids, the results showed that the plant extract rich with these substances and their amounts were expressed as percentages, which were calculated as follows:

Each phenolic compound or flavonoid concentration (mg/ml) can be calculated as the area under the curve for a sample

phenolic compound or flavonoid or the area under the curve for its standard phenol or standard flavonoid X standard concentration.

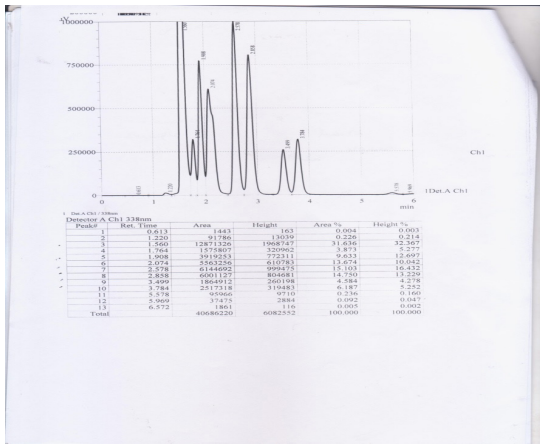

Figure 1: HPLC chromatograms of standard phenols

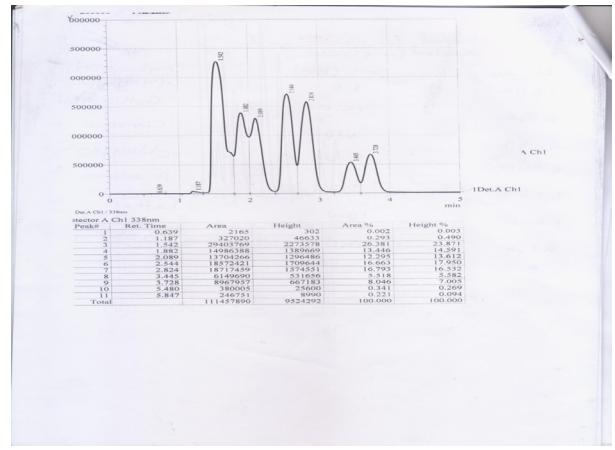

Figure 2: HPLC chromatograms of extracted phenols

Table (4): HPLC analysis results for standards and the extracted phenolic compounds

Phenolic compound	***Standard***	***Standard***	***Standard***	***Sample***	***Sample***	***Conc.µg/ml***	***Conc.mg/g***
	Rt. Min.	Conc. mg/l	Area	Ret.Time	Area	plant Extract	plant Extract
Pyrogallol	1.542	3	29403769	1.56	12871326	1.313	24.51
Gallic acid	1.882	15	14986388	1.908	3919253	3.92	73.173
Cinnamic acid	2.098	3	13704266	2.074	5563256	1.2178	22.73
Chlorogenic acid	2.544	2	18572421	2.578	6144692	0.662	12.36
p-coumaric acid	2.824	3	18717459	2.858	6001127	0.962	18
Caffeic acid	3.445	10	6149690	3.499	1864914	3.03	56.56
Ferulic acid	3.728	5	8967957	3.784	2517318	1.4035	------

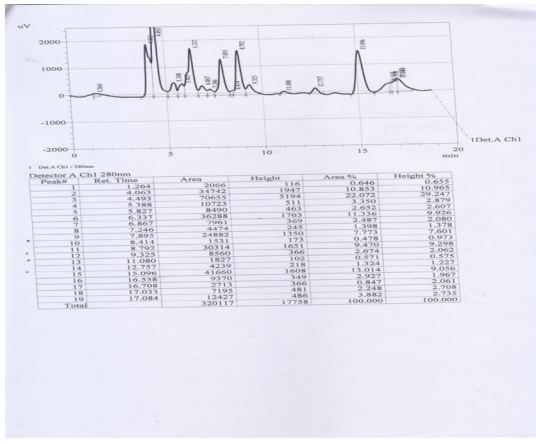

Figure (3) Standard Flavonoid HPLC Chromatogram

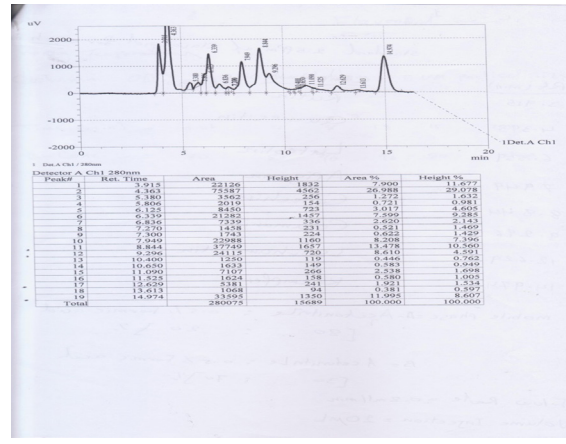

Figure (4) Chromatogram of Total Flavonoids in Plants

The retention times of the standard flavonoids and the amount of each of the total plant flavonoids are shown in Table (5).

Table (5) Retention Time in Minutes and Area Under the Peak for Each Flavonoid in Standard and Sample Solutions

Flavonoid	***Standard***	***Standard***	***Sample***	***Sample***	***Conc.µg/ml***	***Conc.mg/g***
	Ret.Time	Area	Ret.Time	Area	plant Extract	plant Extract
Rutin	3.915	22126	4.063	34742	2.657	22.32
Quercetin	4.363	75587	4.493	70655	2.85	23.94
Luteolin	6.339	21282	6.337	36288	3	25.2
Apegnine	7.949	22988	7.895	24882	2.91	24.45
Catechin	8.844	37749	8.792	30314	2.5	21
Coumarin	9.296	24115	9.325	8560	1.27	10.668
Isorhamnetin	12.629	5381	12.757	4239	2.26	18.98
Kaempferol	14.974	33595	15.096	41660	2.98	25.032

b-Analysis of nonpolar substances via HPLC

The nonpolar components in the alcoholic seed extract estimated via HPLC are shown in Figures (5) and (6), and the retention time and concentration of each compound are listed in Table (6).

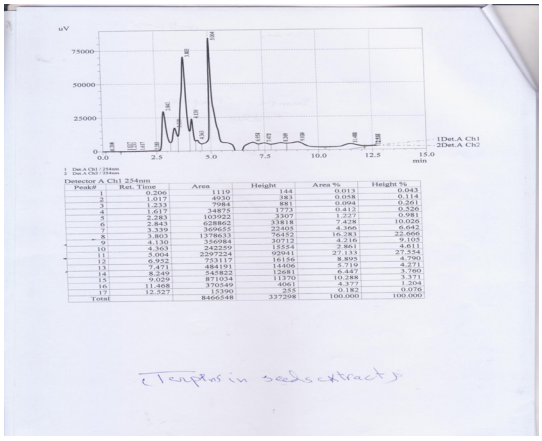

Figure (5): HPLC chromatogram of standard terpenes

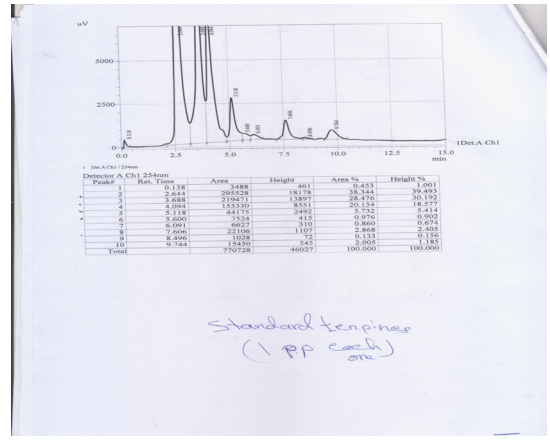

Figure (6): HPLC chromatogram of extracted terpenes of plant seeds

Table (6): HPLC analysis results for standards and the extracted phenolic compounds

Non polar terpines	***Standard***	***Standard***	***Standard***	***Sample***	***Sample***	***Conc.µg/ml***	***Conc.mg/g***
	Ret.Time	Area	***conc. (µg/ml)***	Ret.Time	Area	plant Extract	plant Extract
β- pinene	2.644	295528	1	2.843	628862	2.13	39.76
α-pinene	3.688	219471	1	3.803	1378633	6.3	117.6
Eugenol	4.049	155330	1	4.130	356984	2.3	43
β – myrcene	5.118	44175	1	5.004	2297224	52	970.7
p-cymene	7.606	22106	1	7.471	484191	22	410.7
Limonene	9.744	15450	1	Not found	---	Not found	

Anticancer activity

This study compared the cytotoxic effects of different concentrations of seed extract on the breast cancer cell lines MCF-7 and MDA-231 with those of the normal rat embryo fibroblasts, as shown in Table (7) and Figure (7).

Table (7): Cytotoxic effects of plant extracts at different concentrations on the breast cancer cell lines MCF-7 and MDA compared with those of the normal rat embryo fibroblasts REF

Concentration (mg/ml)	IR% for MCF-7	IR% for MDA-231	IR% for REF	L.S.D. value
Control(0)	0.00 ±0.00 F a	0.00 ±0.00 D a	0.00 ±0.00 D a	NS 0.00
0.0005	0.00 ±0.00 F a	0.00 ±0.00 D a	0.00 ±0.00 D a	NS 0.00
0.001	7.00 ±0.38 F a	0.00 ±0.00 D b	0.00 ±0.00 D b	4.57 *
0.002	20.00 ±1.08 E a	0.00 ±0.00 D b	0.00 ±0.00 D b	6.25 *
0.004	9.00 ±0.74 F a	4.00 ±0.26 CD b	0.00 ±0.00 D b	4.74 *
0.0078	34.00 ±2.17 D a	7.00 ±0.38 BCD b	31.00 ±1.68 C a	7.21 *
0.015625	32.50 ±1.89 D a	5.00 ±0.32 BCD b	36.00 ±2.54 C a	7.68 *
0.03125	34.50 ±2.52 D b	10.40 ±0.64 BC c	52.00 ±2.19 B a	8.02 *
0.0625	50.00 ±3.04 AB a	8.00 ±0.57 BC b	54.00 ±2.63 B a	7.59 *
0.125	52.30 ±2.36 A b	4.00 ±0.26 CD c	70.00 ±3.67 A a	8.77 *
0.25	50.00 ±3.04 AB b	11.30 ±0.76 B c	70.00 ±3.67 A a	8.36 *
0.5	39.00 ±2.19 CD b	10.00 ±0.64 BC c	66.00 ±3.71 A a	8.94 *
1	43.00 ±2.02 BC b	26.50 ±1.26 A c	55.00 ±2.47 B a	8.17 *
L.S.D. value	9.05 *	7.33 *	9.16 *	---

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* (P≤0.05).

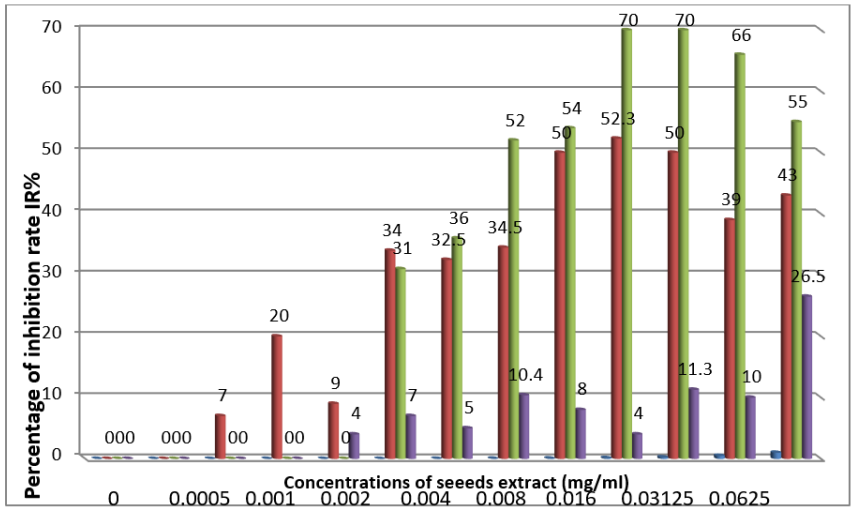

Figure (7): Histogram representing the percentage of the inhibition rate for different plant seed concentrations. The green color represents the IR% for normal REF cells, the blue color represents the IR% for the MDA-231 cell line, and the red color represents the IR% for MCF-7 cells.

Discussion

Annona squamosa is an edible fruit also known as sugar apples or sweetsops. The plant is known for its energy content due to its richness in sugars, important elements and vitamins, which has led to its popular and medical use in many cases of illness and physical ailments. This study focused on the seeds of this plant and investigated the most important active compounds it may contain, relying on it being a natural source of waste that can be recycled industrially and the possibility of extracting compounds with important biological effects that may be used as a treatment for many human diseases and disorders. The most important active compounds in the seed extract confirmed that these seeds are rich in alkaloids, saponins and some flavonoids. The total contents of phenols and flavonoids which the seeds enriched that had been highlighted in this study. The results revealed that the plant seed extracts contained different compounds with different quantities of phenolic compounds and flavonoids. All these phenolic compounds, including gallic acid, caffeic acid, pyrogallol, cinnamic acid, p-coumaric acid and chlorogenic acid, were investigated in the seed extract. Moreover, the seeds appeared to be rich in Luteolin, Kaemperol, and Apegnine and had relatively low amounts of Quercetin, Rutin and Catechin. These results emphasize that plant seeds represent a good source of phenols and flavonoids. The extracted seed residue showed potent toxicity toward breast cancer cells (MCF-7), as it did toward normal rat embryonic fibroblast (REF) cells. The cancer (MDA-231) cells were less affected by the concentrations used in the study. This may be due to the high content of alkaloids in the extract. The concentrations of plant materials, regardless of their source, are not completely safe for use. Rather, caution should be taken, and more experiments and studies should be conducted to determine a safe drug dose that has a medical effect as an anticancer agent or for any treatment, especially for humans. The results of the present study showed that the MDA cancer line is less affected by all concentrations of the alcoholic seed extract of the plant, such that it may require concentrations higher than 1 mg/ml to be a lethal concentration for the cells of this cancer line.

A study by Bader and co.2023, which was conducted in Saudi Arabia and compared the anticancer effects of the seeds and fruit extracts of Annona muricata, revealed that these extracts had a more potent effect on MCF-7 breast cancer cells than on MDA-MB-231 cells in a dose-dependent manner, with lower toxic effects on normal cells (47). This study revealed that the extract of the leaves or fruits had a more notable toxic effect than the other parts of this plant. In addition, a variety of various bioactive components result from seasonal variations, soil, climate, etc., and the varied potencies of the plant extracts might cause variations in the levels of their active components, which could lead to different biological effects.

The toxic effect of the seed extract on normal cells, which was clearly demonstrated in the present study, may be attributed to the presence of various phytoconstituents, including alkaloids, carbohydrates, coumarins, flavonoids, phenolics, proteins, sapponins, steroids and terpenoids, at different levels, each of which has a specific role in their biological activity. This may lead to variations in toxicity depending on the levels of these active ingredients in each part of the plant, in addition to the mechanism by which these components affect the type of living cells included in the study (48).

Conclusion

Despite the effects of different plant secondary metabolites, which might constitute promising resources for exploring new drug options for treating various disorders and diseases, including cancer, investigating the toxicity of plant compounds might involve in vitro and in vivo studies. This is a necessity that cannot be ignored. Although the extract of the seeds of this plant is effective against breast cancer, the extract is not free of toxicity toward normal cells, which makes it very important to research and study this plant, especially its seeds, in a way that ensures the safety of normal cells.

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Conflicts of interest

There are no conflicts of interest regarding the publication of this manuscript.

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Author Contribution

All the authors confirm their contributions to the paper, including the study conception; the study design; and the data collection, analysis, and interpretation of the results. All the authors reviewed the results and approved the final version of the manuscript.

Data availablility statement:

The corresponding author will provide the data upon reasonable request.

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