DOI: https://doi.org/10.52756/ijerr.2023.v30.024 ***Int. J. Exp. Res. Rev.*, Vol. 30: 264-275 (2023)**

Original Article | *Peer Reviewed* | *Open Access*

International Journal of Experimental Research and Review (IJERR)

ISSN: 2455-4855 (Online)
www.iaph.in
ISSN 2455-4855

In vitro antioxidant and anticancer potential of *Annona squamosa* L. Extracts against breast cancer

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Article History:

Received: 02th Feb., 2023
Accepted: 13th Apr., 2023
Published: 30th Apr., 2023

Keywords:

Annona squamosa L., anticancer activity, antioxidant activity, phytochemical screening, MTT assay

Abstract: Various ailments have long been treated with plant-based remedies. This research aims to determine which compounds are present in *Annona squamosa* L. leaves and seeds extracts, as well as their antioxidant and anticancer potential. *Annona squamosa* L. hexane, methanol, and aqueous extracts were analysed for their phytochemical content, and results showed the existence of many useful compounds. The DPPH assay was used to measure the antioxidant activity. In particular, the low IC50 values revealed that the plant extracts under study possessed remarkable antioxidant activities. Antioxidant phytochemicals such alkaloid, flavonoid, phenol, oil, phytosterol, coumarin, and saponins may be responsible for these benefits. This research shows that extracts of *Annona squamosa* L. have a high antioxidant activity and promise as a dietary supplement and possible medicinal agent. MTT assay was used to test for anticancer characteristics. Cell viability was assessed for each extract using a negative control and a positive control (Cisplatin). Both the *Annona squamosa* L. (seeds) methanolic extract and the *Annona squamosa* L. (leaves) water extract showed substantial efficacy in this investigation against the MCF-7 breast cancer cell line. It follows that extracts of *Annona squamosa* L. (leaves) and (seeds) could be used to create a natural medicine for breast cancer treatment.

Introduction

Antispasmodics, emetics, cancer treatments, and antibiotics are only a few drugs originating in plants (Shehata et al., 2021; Al-Nemari et al., 2022). The purported antimicrobial properties of medicinal herbs are utilized by indigenous communities throughout the world (Al-Nemari et al., 2020). Traditional medicine has traditionally relied on plant remedies to address a wide range of conditions. The *Annona squamosa* L. plant is a noteworthy member of the Annonaceae family, which includes many other edible plants (Leatemia et al., 2004). There are between 2300 and 2500 species and more than 130 genera in the Annonaceae family of flowering plants. These include trees, shrubs, and lianas. Only a small number of species inhabit temperate regions, with the vast majority inhabiting tropical ones. It is estimated that 900 species are native to the Neotropics, 450 to the Afrotropics, and the remaining species to the Indo-Malayan region. The Annona family of plants is also widely dispersed in India. The most drought-resistant species of Annonaceae is *Annona squamosa* L., which grows poorly in humid environments. Optimal conditions

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for growth include an average annual rainfall of more than 700 mm (Bhattacharya et al., 2016).

Taxonomical classification

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida (Dicotyledons)
Subclass: Magnoliidae
Order: Magnoliales
Family: Annonaceae (Custard-apple family)
Subfamily: Maloideae
Tribe: Abreae
Genus: *Annona* L.
Species: *Annona squamosa* L.

As per the Botanical description *Annona squamosa* L. is a monoecious plant that grows as a well-branched shrub/small tree among thin grey bark (Vyas et al., 2012). The crown is a sphere or flattened ball-shaped. The leaves are oblong-lanceolate in shape, 10 to 15 cm long and 3 to 5 cm broad, and alternately placed on short petioles (Kumar et al., 2005). Flowering happens in persistently humid areas from spring through early summer. Flowers are bracteate, protogynous, pedicillate, spirocyclic, actinomorphic and bisexual (Vithanage et al., 1984). They are commonly found in groups of 2 to 4, and each bloom is about 2.5 cm long, with oblong green outer petals and a purple base. Inner petals are reduced to minute scales or are completely missing. Fruits by white pulp are edible and come in various shapes (including rounds and hearts) and scents. With a length between 1.3 and 1.6 centimetres, slight swelling at the hilum or albumen packed by many transverse, brown lines of clefts, and a smooth, shiny blackish or dark brown exterior, each carpel contains one rectangular, smooth, glittering seed (Vyas et al., 2012).

Annona squamosa L. has several therapeutic effects, including pain relief and wound healing, and is consequently employed in traditional medicine. It's also used for its anti-inflammatory, anti-viral, anti-bacterial, anti-fungal, anti-cancer, and anti-parasitic properties (Pandey et al., 2011). Their root is a powerful purgative, and root bark scrapings are used to treat toothaches. It also contains anticonvulsant properties. This plant's leaves are used to treat hysteria. Wounds boils, and gastritis can all benefit from a decoction of the leaves, while crushed leaves can be applied directly (Eva, 2006). The fruit has been shown to help lower blood sugar. Its juice can be used to alleviate fever and chills. They found that the pulp caused mutations in lab mice. The fruit is astringent when unripe but helpful in treating malignant tumours; it also has many seeds. The oil content of the seed averages 30%. *Annona squamosa* L. and other plant-based pesticides have great promise for use in environmentally responsible farming (Singh et al., 2019).

Considering the therapeutic relevance of the said plant, the phytochemical screening, antioxidant activity and anticancer activity were performed on several extracts of *Annona squamosa* L. Overall six extracts were prepared using two parts (Seed, Leaf) of *Annona squamosa* L. Plant and three solvents namely 'hexane', 'methanol', and 'aqueous'.

Materials and Methods

Plant collection and identification

During the month of October 2021, fresh *Annona squamosa* L. leaves and Fruit (for seeds) were collected from the Danta Taluka of Banaskantha district in Gujarat, India. Dr. Hitesh Solanki, Professor, Department of Botany, Bioinformatics & Climate Change Impact Management, University School of Sciences, Gujarat University, Ahmedabad has authenticated the plant collected.

Processing of the plant

The processing of the plant is divided into subsequent steps like washing, drying, grinding and storage. The obtained healthy leaves were washed with water to eliminate the adhering unwanted particles. Drying plant material reduces the moisture content of fresh materials. It is an important step in preparing dried material for further processing. Drying conditions, on the other hand, have been found to have a significant influence on sensory quality, bioactive component stability and activity. For 7-15 days, the plant material was dried in the shade. Grinding is done to create a homogeneous sample and to increase the sample's surface contact with the solvent solution. Finally, the powder of leaves and seeds is kept at a lower temperature (Dave et al., 2021).

Physicochemical Parameters

The various physicochemical properties specified by The Unani Pharmacopoeia of India. The following parameters were included: odour, content, ash value, insoluble ash value (Sasidharan et al., 2011).

Determination of Moisture (Loss on drying)

One and a half grammes of powdered leaves were weighed and placed in a flat, narrow porcelain plate. It was baked between 100 and 105 degrees Celsius to dry it out. Desiccators are used to remove moisture from substances by causing them to lose weight.

Ash values

Air-dried samples were analysed for their acid-insoluble ash, total ash and water-soluble ash values.

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Total ash value

In a formerly burnt and tarred silica crucible, weigh about 2 gm of the air dried material. Spread the material out evenly and gradually raise the temperature to 500 - 600°C until it is white, indicating the absence of carbon. Allow the remnants to cool in a desiccator for 30 minutes before weighing with no time interval. Percentage of total ash was calculated using air-dried material standards.

$$\text{Total ash} = \frac{(Z-X)}{Y}$$

X= empty dish weight

Y= drug taken weight

Z= weight of dish + ash (after complete incineration)

Acid insoluble ash

Boil 25 mL of 2N HCl in a crucible containing all of the ash for 5 minutes while covering it with a watch glass. Cauldron: 5 mL of hot water is used to rinse the watch glass. The insoluble components can be filtered out of the liquid with the help of hot water and ash-free filter paper. Filter paper remnants that don't dissolve should be transferred to the first crucible, dried on the hot plate, and burned until their weight remains constant. The leftovers should be placed in desiccators to cool for 30 minutes before being weighed. Learn how many milligrammes of acid-insoluble ash are in one gramme of air-dried material.

Water soluble ash

For 5 min, bring 25 mL of water and all ash in the crucible to a boil. Use a glass melting pot to combine the insoluble ingredients. Fire it up in an oven preheated to no more than 450^0Cfor 15 minutes after a wash in hot water. Besides reducing trash weight, it also reduces the amount of debris in the ash. Find milli grammes of water-soluble ash per gramme of air-dried substance. Ash that has been air-dried was used to calculate how much of it dissolves in water.

Preparation of plant crude extracts

The plant juices were extracted via maceration. Powdered plant materials (30 gm) were soaked in Hexane (30 mL), Methanol (30 mL), and water (30 mL) for 6 days at room temperature to produce Hexane, Methanol, and Aqueous extracts, respectively. The aqueous, methanol, and hexane extracts were separated and concentrated in a water bath at 70°C and 50°C, respectively, to produce semisolid products. The freeze-dried extract was stored in a sealed container at 4 degrees Celsius until further analysis (Naz et al., 2012).

Table No. 1 displays the results of calculating the extractive yield as a percentage using the formula given below.

$$\%\ \text{Extractive yield (w/w)} = \frac{\text{weight of dried extract}}{\text{weight of dried leaves}} \times 100$$

Phytochemical Screening

Extracts were subject to preliminary phytochemical testing to determine the presence of different phytochemical constituents (Parekh et al., 2007).

Test for Total Phenol

In 5 mL of distilled water, 50mg of extract was dissolved. A few drops of neutral 5% ferric chloride solution were added to this. The presence of phenolic compounds was indicated by the presence of a dark green colour.

Test for Flavonoid

An aqueous extract sample was treated with a 10% ammonium hydroxide solution. The presence of flavonoid was confirmed by yellow fluorescence.

Test for Alkaloids

A total of 0.5g of concentrated extracts was mixed with 5 mL of 1% aqueous HCL acid. The mixture was gently heated for 20 minutes, cooled and filtered. The filtrate was used for following tests:

Wagner's Test

A few drops of Wagner's reagent were added by the side of the test tube to a few mL of extract. The presence of a reddish brown precipitate confirms the test as positive.

Wagner's Reagent

Iodine (1.27g) and potassium iodide (2g) are dissolved in 5 mL of water and made up to 100 mL with distilled water.

Hager's Test

The presence of alkaloids was confirmed by the yellow coloured precipitate when mL of the extract was treated with Hager's reagent.

Hager's Reagent

Saturated aqueous solution of picric acid.

Test for Tannin

The 50mg extract was dissolved within water, followed by 3 mL of 10% lead acetate solution.The presence of tannin was detected by a bulky white precipitate.

Oil test

A small amount of extract was passed between two filter papers. The presence of oil was indicated by an oil stain on the paper.

Test for Phytosterol

50mg extract was dissolved in 2 mL acetic anhydride. To this, one or two drops of concentrated sulphuric acid were added slowly along the side of the tube. An array of color change showed the presence of phytosterol.

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Test for Saponin

50 mg extract was combined with 20 mL of distilled water and agitated in a graduated cylinder for 15 minutes. The formation of foam indicates Saponin.

Test for Coumarin

Take 5 mg of plant extract in 10 mL of distilled water. Take 2-3 mL of plant extract from it in a test tube and add a few drops of $FeCl_3$. Then after, add a few drops of HNO_3. Yellow color indicates the presence of Coumarin.

Test for Carbohydrates

Filtrate (1 mL) was mixed with Barfoed's reagent (1 mL), which was then cooked in boiling water bath for 2 min. Carbohydrates were present because of the red precipitate.

Test for Protein

To 2 mL filtrate, added only some drops ninhydrin reagent. The presence of protein was indicated by blue color formation.

Antioxidant activity by DPPH test

DPPH free radical scavenging test was used to assess the antioxidant activity of the extracts. Plant extracts of various concentrations were made, such as 100µg/mL, 250µg/mL, 500µg/mL, 750µg/mL and 1000µg/mL for each plant. DPPH solution was prepared with a concentration of 0.004% in methanol. As a positive control, ascorbic acid was utilized. Each sample was combined with 1 mL of DPPH solution. The resulting mixture was vortexed, incubated for 30 minutes at room temperature in a reasonably dark environment and then measured at 517nm with a spectrophotometer. The blank comprised 80% methanol (v/v). Measurements were taken in triplicate. After adding DPPH solution to 1 mL of methanol, a negative control was taken. Higher radical scavenging activity is indicated by lower absorbance of the reaction mixture. The DPPH scavenging effect (%) was calculated using the formula (Mahdi-Pour et al., 2012):

DPPH Scavenging effect (%)={(Ao-A)/Ao}×100

Where Ao is the absorbance of negative control (0.004% DPPH solution) and A is the absorbance in presence of extract. The result was reported as IC50 values.

Anticancer activity against Breast cancer

Cell viability assay

The plant growth-promoting properties of extracts were tested using MTT assay (Sneeha et al., 2016).

Cell lines and culture medium

The National Centre for Cell Science in Pune is where we obtained our MCF-7 breast cancer cell lines. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum with penicillin-streptomycin at 37 degrees Celsius in a humidified environment of 5% carbon dioxide.

Sample preparation

Each plant extract was dissolved in dimethyl-sulfoxide to create a stock solution, which was then diluted using complete culture media to the necessary quantities (5, 10, 20, 40 and 80 µg/mL).

Steps

A 96-well microtiter plate was used to seed the cells (density: 4 $x10^5$ cells/well) and sufficient media was added in each well. It was incubated for 24 hours at 37˚C and 5% CO_2.

When the cell density reached 80%, the culture medium was discarded and 1 mL of fresh medium added. Treatments included CLH, CLM, CLW, CSH, CSM, CSW, Cisplatin (positive control), and a control group receiving no therapy for 24 hours at 5, 10, 20, 40 and 80 µg/mL concentrations.

Ten µl of MTT (0.5 mg/mL) was added to each well once dosing period ended. Another three to four hours were spent incubating the plate at ambient temperature. The absorbance was measured at 570 nm after adding 50 µl of dimethyl sulfoxide using a spectrophotometer (Al-Nemari et al., 2022; Kumar et al., 2021).

Statistical analysis

The data was presented using the mean and ± SD (standard deviation) calculated with SPSS (Statistical Package for the Social Sciences) version 21. Anticancer activity of plant extracts was analysed statistically using student's t-test. A p-value below 0.05 was judged important when compared to the respective control, whereas a p-value above 0.05 was deemed inconsequential.

Results and Discussion

Physico-chemical parameters of the selected plant samples are tabulated as below.

Table 1. Physicochemical properties of Leaves and seeds of *Annona squamosa* L.

Test Parameters	*Annona squamosa* L.(Leaves)	*Annona squamosa* L.(Seeds)
Color	Dark Green	Brown
Odour	Specific	Specific
Taste	Bitter	Slightly Bitter
Moisture (%)	4.35±0.33	4.75±0.48
Total Ash (%)	6.75±0.12	2±00
Acid Insoluble Ash (%)	1.25 ±0.12	0.42 ± 0.001
Water Soluble Ash (%)	6.5 ±00	1.35±0.045

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Table 1 represents the physicochemical properties of the selected plant samples (leaves, seeds) of *Annona squamosa* L. Percentages for moisture, acid-insoluble ash, ash total ash, and ash soluble in water were calculated using triplet analysis. The mean and standard deviation for all three values obtained were calculated and shown in the table above.

The moisture content of the selected plant samples was found to be less than 5% using the loss-on-drying method. Total Ash values for the leaves sample were 6.75% and for the seeds was 2%, which were found to be below 10%. The acid-insoluble ash of *Annona squamosa* L. leaves and seeds were 1.25% and 0.42%, respectively, whereas the water-soluble ash of selected plant sample powder was 6.5% (leaves) and 1.35% (seeds). Color and odour were examined and reported in Table 1 before being utilized for future study.

Table 2. Extraction yield and physical properties of extracts

Name of plant	Physical characteristics and % Yield	Methanol	Hexane	Aqueous
Annona squamosa L. (Leaves)	Colour of extract	Dark Green	Dark Green	Brown
	Sense of touch	Sticky	Sticky	Sticky
	% Yield	12.15± 0.32	2.94± 0.05	37.00± 0.1
Annona squamosa L (Seeds)	Colour of extract	Dark Green	Yellow	Brown
	Sense of touch	Sticky	Oily	Sticky
	% Yield	11.96± 0.45	21.41± 0.48	11.33± 0.45

The table illustrates the physical characteristics of plant extracts from the two plant samples selected using three solvent systems, as well as the extraction yield of all six plant extracts. The extraction yield (%) of each plant extract is shown as the mean of three replicates with standard deviation. All plant extracts were found to be sticky to the touch. The colour of extracts for the *Annona squamosa* L. (Leaves) was observed as Dark Green in Methanol and Brown in Aqueous solvent systems. The colour of *Annona squamosa* L. (Seeds) extracts was dark green, Yellow and Brown in different solvent systems of Methanol, Hexane and Aqueous.

The amount of extract obtained differs really depending on the solvent used and the plant substance extracted from. The extraction yields of *Annona squamosa* L. (Leaves) in Methanol, Hexane and Aqueous solvent systems were 12.15%, 2.94% and 37%, respectively. Aqueous extract yielded a higher extraction yield, while Hexane extract yielded a lower yield. The extraction yields for *Annona squamosa* L. (Seeds) were 11.96%, 21.41% and 11.33% in Methanol, Hexane and Aqueous solvent systems, respectively. The Aqueous extract yielded less extraction (11.33%), whereas the Hexane extract yielded a higher extraction yield (21.41%).

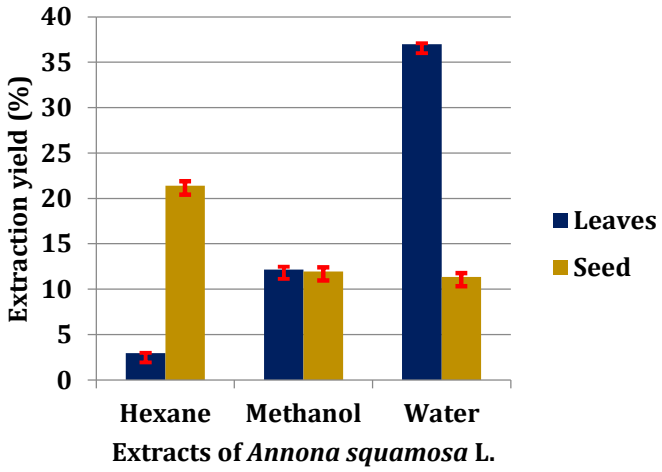

Figure 1. Comparison of extraction yield

Figure 1 depicts the extraction yield comparison of Leaves and Annona squamosa L seed extracts. In the case of Methanol extracts, the extraction yield for both plant samples was found to be almost the same. Still, a considerable difference was observed between the extraction yields of hexane and aqueous extracts of both plant samples. The extraction yield of *Annona squamosa* L. Seeds sample remains close to 11% in Methanol and Aqueous extracts, whereas it is 21.41% in Hexane extract. *Annona squamosa* L. leaves samples were extracted using a variety of solvents, with the aqueous extract yielding the highest percentage (37%) and the hexane extract yielding the lowest percentage (2.94%).

Phytochemical Screening

Phytochemical screening was performed for six plant extracts and the results were reported as positive (+) or negative (-). Based on the concentration amount, the Positive test result was classified as low (+), moderate (++), or high (+++).

Table 3 describes the phytochemical screening for *Annona squamosa* L. leaves and seeds extracts. The phytochemical analysis revealed occurrence of Phenol,

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Flavonoid, Alkaloid, Tannins, Oil, Phytosterol, Coumarin and Carbohydrates in the *Annona squamosa* L. leaves sample. While the seeds sample of *Annona squamosa* L. revealed the presence of Flavonoid, Alkaloid, Tannins, Oil, Phytosterol, Saponins, Coumarin, Carbohydrates and Protein.

Table 3. Phytochemical screening of extracts of *Annona squamosa* L.

Annona squamosa L.	Methanol		Hexane		Aqueous	
Test	**Leaves**	**Seeds**	**Leaves**	**Seeds**	**Leaves**	**Seeds**
Phenolic compound	+++	+	+	-	+++	-
Flavonoid	+++	++	+	-	++	-
Alkaloid	+++	+++	++	+++	+++	+++
Tannin	++	+	+	-	+++	++
Oil	+	+++	++	+++	++	++
Phytosterol	+++	+++	+++	+++	+++	+++
Saponins	+	++	-	++	+	+++
Coumarin	++	++	+++	+++	++	+++
Carbohydrates	+++	++	+	-	+++	++
Protein	+++	+++	-	-	-	+++

Table 4. DPPH scavenging activity of plant extracts; Vales are the mean ± SD of three replicates.

DPPH SCAVENGING ACTIVITY (%)						
***Annona squamosa* L. (Leaves)**						
Plant Extract (µg/mL)	**100**	**250**	**500**	**750**	**1000**	**IC50 Mean SD (µg/mL)**
CLM	85.82±0.18	89.42±1.17	93.34±0.67	94.4±0.06	94.4±0.06	11.74±0.22
CLH	58.55±0.54	61.88±0.60	76.10±0.68	86.29±0.67	93.88±0.40	84.67±0.47
CLW	47.94±0.23	60.94±0.37	65.29±0.17	68.94±0.29	72.66±0.29	112.35±1.76
***Annona squamosa* L. (Seeds)**						
CSM	49±0.30	83.74±0.89	96.86±0.68	98.08±0.64	98.35±0.47	110±0.264
CSH	46.92±0.17	50.68±0.17	57.93±0.23	63.14±0.29	63.45±0.20	115.45±1.12
CSW	55.58±0.30	73.52±0.35	80.88±0.29	87.38±0.24	91.34±0.27	75.57±0.67
Standard						
Name (µg/mL)	**10**	**20**	**30**	**40**	**50**	**IC50 Mean SD (µg/mL)**
Ascorbic acid	52.74±0.34	71.22±0.22	86.86±0.48	94.34±0.32	98.79±0.18	7.1±0.42

Phenolic compounds and Flavonoid were found in higher concentrations in the methanol extract of leaves sample and were absent in the hexane and aqueous extracts of the seeds sample. Alkaloid, Oil, Phytosterol and Coumarin were found among all the six extracts (leaves and seeds) of *Annona squamosa* L. Tannin was detected in higher concentration in the aqueous extract of leaves sample and was missing in the hexane extract of the seeds sample. It was found in moderate concentration in the remaining other extracts. Saponins were detected in higher concentration in the aqueous extract of seeds sample and were absent in the hexane extract of the leaves sample. It was found in moderate concentration in the remaining other extracts. Carbohydrates were detected in higher concentrations in the methanol and aqueous leaves sample and were missing in the hexane extract of seeds sample. Protein was detected in higher concentration in the methanol, aqueous extracts of seeds sample and aqueous extracts of leaves sample, whereas it was found absent in the hexane, aqueous extracts of the leaves sample and hexane extract of seeds sample.

Antioxidant Activity by DPPH

The antioxidant potential of *Annona squamosa* L. samples (leaves and seeds) was studied against DPPH compared to ascorbic acid. Removing one electron from the analyte transforms stable free radical DPPH into a non-radical form (yellow colour).

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The DPPH activity increased with increasing concentrations of plant extract prepared in various extraction solvents. The antioxidant activity of plant extracts is shown in Table 4, along with the IC50 value for each plant extract.

The antioxidant activity of *Annona squamosa* L. leaves and seeds samples was graphically depicted in form of a dose-response curve and IC50 values, as shown in Figure 1–4.

The antioxidant potentials of all plant samples were dose dependent; as their concentration (100 - 1000 g/mL) grew, so did their antioxidant potential. Experiments were carried out three times and antioxidant activity of each plant sample extract and the standard (ascorbic acid) were determined.

Maximum radical scavenging activity was observed as 94.4±0.06 and 98.35±0.47 for a sample of *Annona squamosa* L.'s leaves and seeds extracted with methanol with concentration are 1000 µg/mL. Methanol extract of *Annona squamosa* L. leaves sample yielded the lowest IC50 value of 11.74 µg/mL.

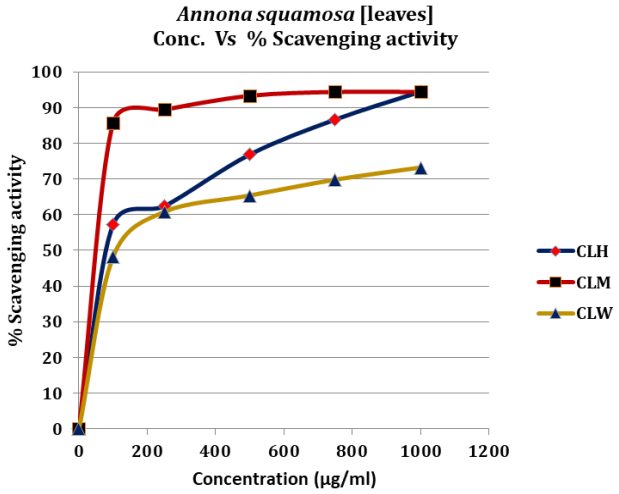

Figure 2. DPPH Scavenging Activity of *Annona squamosa* L. (leaves sample) extracts

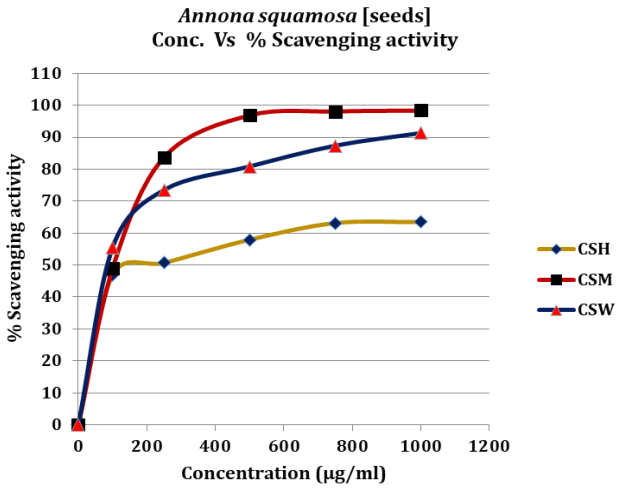

Figure 3. DPPH Scavenging Activity of *Annona squamosa* L. (seeds sample) extracts

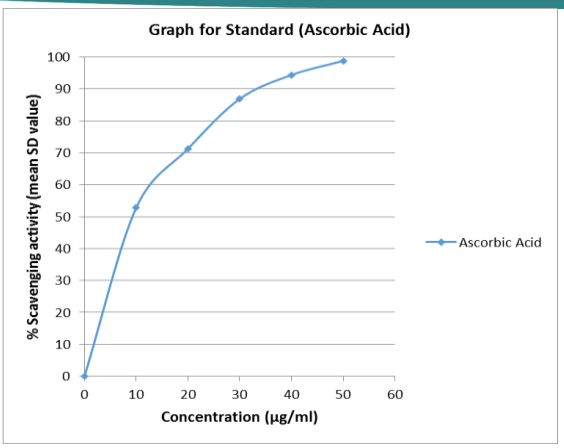

Figure 4. Graph for the standard (Ascorbic acid)

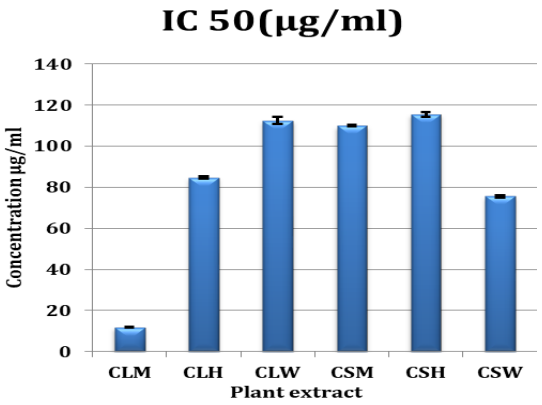

Figure 5. IC50 vs. Concentration of crude extracts of plant samples

Anticancer activity

Different concentrations of *Annona squamosa* L. extracts were tested for their capability to restrain the growth of breast cancer cell lines (MCF-7) using MTT assay. Extract and fractions had dose-dependent consequence on preventing cell proliferation in MCF-7 cell lines.

Number of viable cells in a breast cancer cell line was clearly influenced by treatment with *Annona squamosa* L. seed extracts (Table 5). At varied concentrations of 5, 10, 20, 40 and 80 µg/mL, Cell viability (%) of MCF-7 cell line was decreased from 96.45±3.25 to 35.69±1.07, from 88.88±9.93 to 37.12±2.29, from 78.89±3.46to 36.55±1.06 for hexane, methanol and water extracts respectively in 24 hours using MTT assay (Figure 6). The MCF-7 cell viability was appreciably reduced by the methanolic extract at 10, 20, 40, and 80 µg/mL. Nevertheless, it was determined that at an 80 µg/mL concentration, all three *Annona squamosa* L. (seeds) extracts significantly reduced the viability of MCF-7 cells, with a 62–64% inhibition of cell proliferation (Figure 7). Number of cells in MCF-7 cell line decreased with an increase in the extract concentration applied. The IC50 (µg/mL) values of CSH, CSM and CSW were obtained as 27.74±0.71, 16.12±0.77 and 20.29±2.92 respectively.

DOI: https://doi.org/10.52756/ijerr.2023.v30.024

Table 5. Anticancer effect of *Annona squamosa* L. (seeds) extracts against MCF-7 cell line

Sample	Concentration (µg/mL)	% Cell Viability	% Cell inhibition	IC50(µg/mL)
CSH	0	100±00^{ns}	0.0±00^{ns}	27.74±0.71
	5	96.45±3.25^{ns}	3.55±3.25^{ns}	
	10	85.33±7.77^{ns}	14.66±7.77^{ns}	
	20	60.99±7.09 * *	39.01±7.09 * *	
	40	36.16±1.41 * * *	63.83±1.41 * * *	
	80	35.69±1.07 * * * *	64.30±1.07 * * * *	
CSM	0	100±00ns	0.0±00ns	16.12±0.77
	5	88.88±9.93^{ns}	11.11±9.93^{ns}	
	10	61.22±2.16 * * *	38.57±2.43 * * *	
	20	42.55±0.71 * * * *	57.45±0.71 * * * *	
	40	39±0.71 * * * *	61±0.71 * * * *	
	80	37.12±2.29 * * * *	62.87±2.29 * * * *	
CSW	0	100±00^{ns}	0.0±00^{ns}	20.29±2.92
	5	78.89±3.46 * *	21.10±3.46 * *	
	10	61.74±1.41 * * *	38.25±1.41 * * *	
	20	52.40±1.61 * * *	47.59±1.61 * * *	
	40	47.00± 0.28 * * * *	52.99±0.28 * * * *	
	80	36.55±1.06 * * * *	63.45±1.06 * * * *	
Each value represents mean±SD of three experiments. CSH: Hexane extract of *Annona squamosa* L. (seeds), CSM: Methanol extract of *Annona squamosa* L. (seeds), CSW: Water extract of *Annona squamosa* L. (seeds),ns: p > 0.05, * : p < 0.05, * *:p< 0.01, * * *:p < 0.001 , * * * *:p < 0.0001				

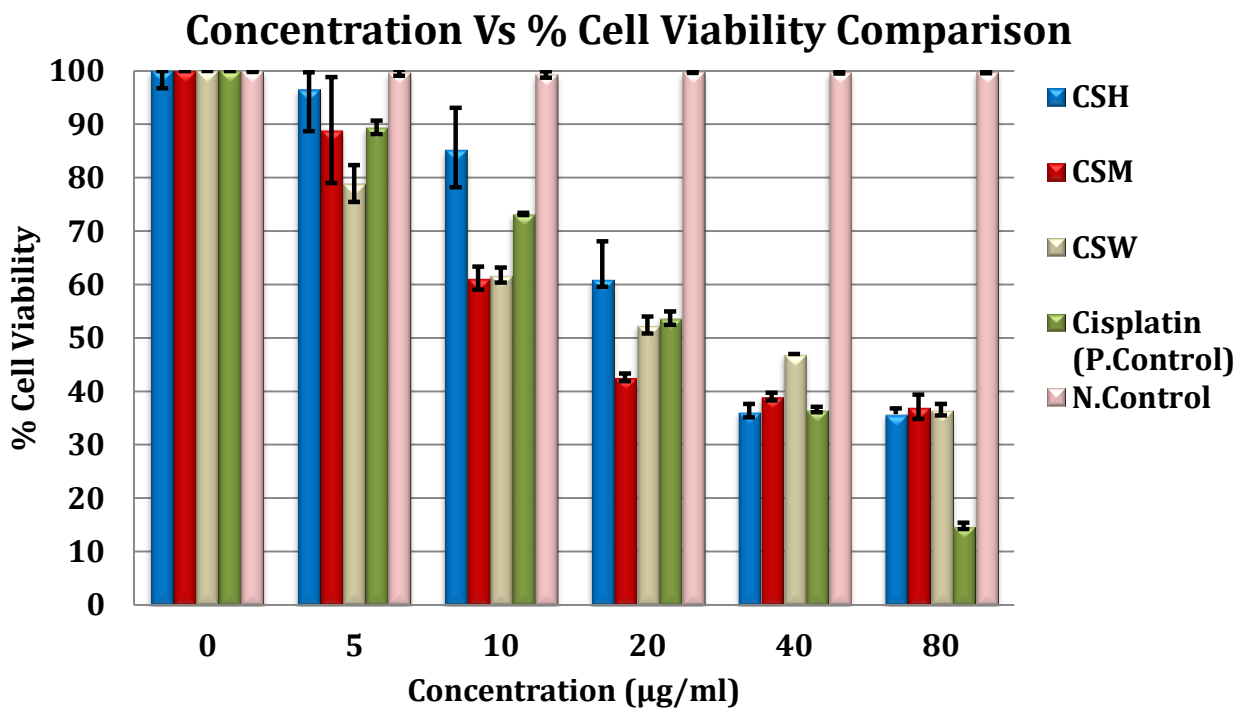

Figure 6. Effect of *Annona squamosa* L. (seeds) extracts on the viability of MCF-7 cells subjected to oxidative stress as determined by the MTT assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this. CSH: Hexane extract of *Annona squamosa* L. (seeds), CSM: Methanol extract of *Annona squamosa* L. (seeds), CSW: Water extract of *Annona squamosa* L. (seeds)

DOI: https://doi.org/10.52756/ijerr.2023.v30.024

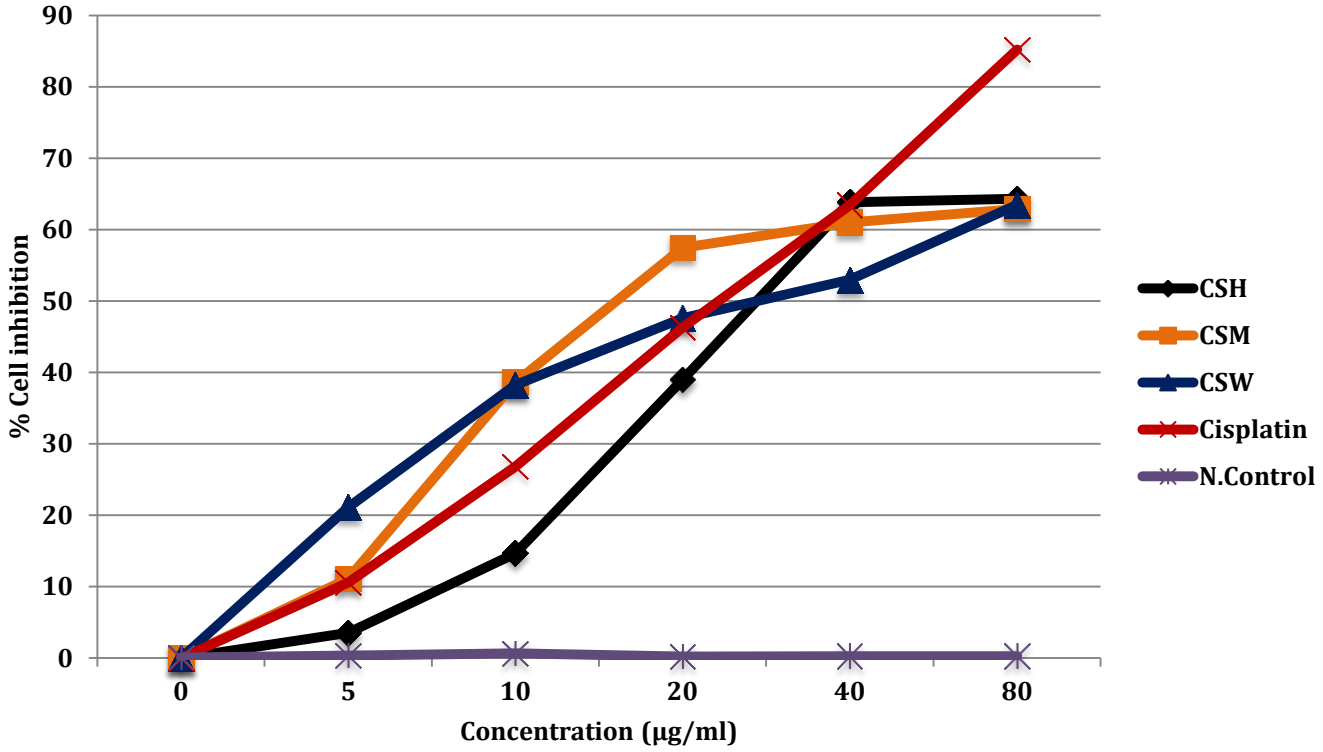

Figure 7. Effect of *Annona squamosa* L. (seeds) extracts on the inhibition (%) of MCF-7 cells. The values of the negative (untreated) control group were fixed as 0% inhibition and the % inhibition in the other groups were calculated relative to this. CSH: Hexane extract of *Annona squamosa* L. (seeds), CSM: Methanol extract of *Annona squamosa* L. (seeds), CSW: Water extract of *Annona squamosa* L. (seeds)

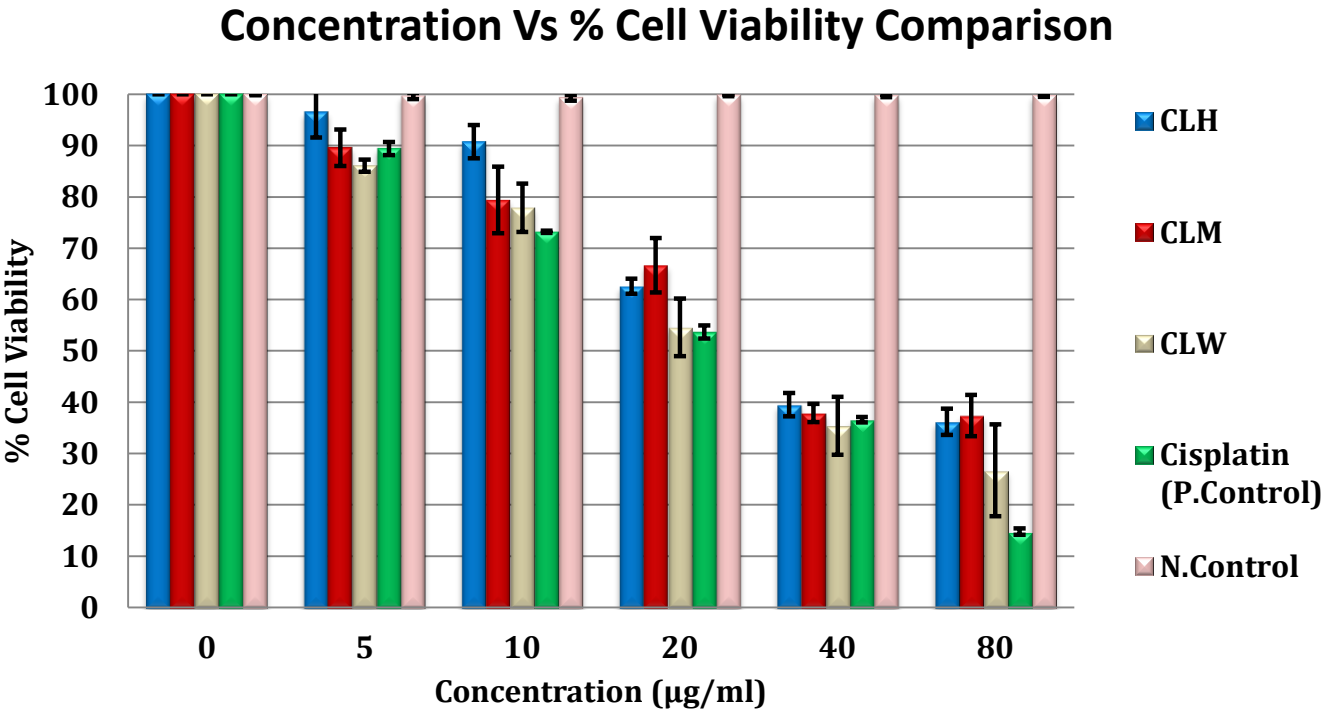

Figure 8. Effect of *Annona squamosa* L. (leaves) extracts on the viability of MCF-7 cells subjected to oxidative stress as determined by the MTT assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this. CLH: Hexane extract of *Annona squamosa* L. (leaves), CLM: Methanol extract of *Annona squamosa* L. (leaves), CLW: Water extract of *Annona squamosa* L. (leaves)

DOI: https://doi.org/10.52756/ijerr.2023.v30.024

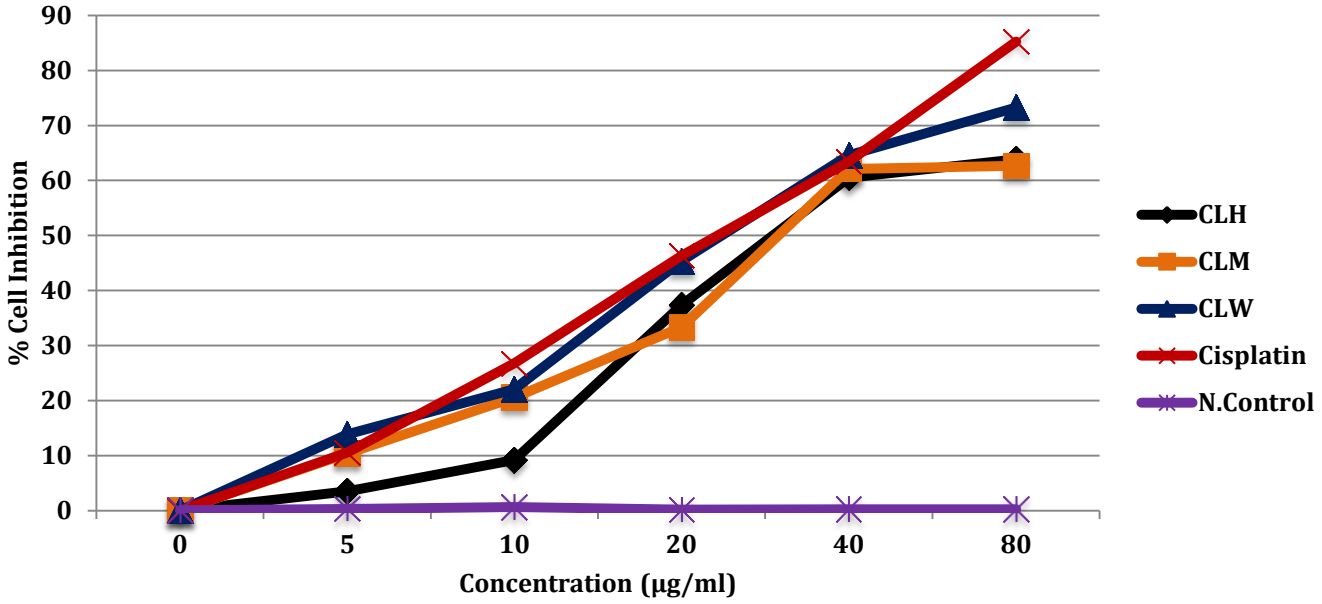

Figure 9. Effect of *Annona squamosa* L. (leaves) extracts on the inhibition (%) of MCF-7 cells. The values of the negative (untreated) control group were fixed as 0 % inhibition and the % inhibition in the other groups was calculated relative to this. CLH: Hexane extract of *Annona squamosa* L. (leaves), CLM: Methanol extract of *Annona squamosa* L. (leaves), CLW: Water extract of *Annona squamosa* L. (leaves)

Table 6: Anticancer effect of *Annona squamosa* L. (leaves) extracts against MCF-7 cell line

Sample	Concentration (µg/mL)	% Cell Viability	% Cell inhibition	IC50 (µg/mL)
CLH	0	100 ± 00^{ns}	0.0 ± 00^{ns}	29.73±1.27
	5	96.47 ± 4.93^{ns}	3.52 ± 4.93^{ns}	
	10	90.78±3.25 *	9.21±3.25 *	
	20	62.64±1.47 * * *	37.35±1.47 * * *	
	40	39.47±2.28 * * *	60.52±2.28 * * *	
	80	36.16±2.55 * * *	63.83±2.55 * * *	
CLM	0	100 ± 00^{ns}	0.0 ± 00^{ns}	28.60±3.68
	5	89.59±3.56 *	10.40±3.56 *	
	10	79.42±6.5 *	20.57±6.5 *	
	20	66.66±5.29 * *	33.33±5.29 * *	
	40	37.92±1.77 * * *	62.08±1.77 * * *	
	80	37.35±4.03 * * *	62.65±4.03 * * *	
CLW	0	100 ± 00^{ns}	0.0 ± 00^{ns}	24.35±1.19
	5	86.12±1.2 *	13.87±1.2 *	
	10	77.94±4.69 * *	22.05±4.69 * *	
	20	54.60±5.62 * *	45.39±5.62 * *	
	40	35.38±5.67* * *	64.62±5.67* * *	
	80	26.72±8.95 * * * *	73.28±8.95 * * * *	
	Each value represents mean ±SD of three experiments. CLH: Hexane extract of *Annona squamosa* L. (leaves), CLM: Methanol extract of *Annona squamosa* L. (leaves), CLW: Water extract of *Annona squamosa* L. (leaves), ns:p > 0.05, *:p < 0.05, * *:p < 0.01, * * *:p <0.001 , * * * * :p < 0.0001]			

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Annona squamosa L. (leaves) antiproliferative activity was obtained using different concentrations from 5µg/mL to 80 µg/mL (Table 6). At varied concentrations of 5µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL, the cell viability (%) of MCF-7 cell line was decreased from 96.47±4.93 to 36.16±2.55, from 89.59±3.56 to 37.35±4.03, from 86.12±1.2 to 26.72±8.95 for hexane, methanol and water extracts respectively in 24 hours using MTT assay (Figure 8). The IC50 (µg/mL) values of CLH, CLM and CLW were obtained as 29.73±1.27, 28.60±3.68 and 24.35±1.19 respectively.

Discussion and Conclusion

Antioxidants are tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. The antioxidant potential of *Annona squamosa* L.extracts was investigated in the search for new bioactive compounds from natural resources. The findings of the antioxidant assay indicate that the methanol extract of *Annona squamosa* L. leaves exhibited a minimum IC50 value (11.74 ± 0.22 µg/mL) and maximum activity (94.4 ± 0.06 %) at 1000 µg/mL in the DPPH assays. It became clear that *Annona squamosa* L. (leaves) and (seeds) samples present the medicinally important phytochemicals and significant antioxidant activity compared with reference antioxidant ascorbic acid for DPPH scavenging activity.

Cancer is a major health issue that remains the leading cause of mortality globally. Because of advances in understanding the molecular process of cancer growth, a large number of anticancer medicines have recently been identified and produced. Unfortunately, the introduction of synthetic chemotherapeutic drugs has not resulted in a considerable increase in overall survival during the last few decades. New techniques and newer chemoprevention medications are required to improve the efficacy of existing cancer treatments (Choudhari et al., 2020). Plants have been utilised to heal ailments by people since ancient times. They are employed as a complement to medication or in synthesising chemical substances. Traditional alternative medicine based on natural sources is used by over 80% of the world's population, according to the WHO (Contant et al., 2021). *Annona squamosa* L. is one of those plants with significant traditional usage; yet, it is less well characterised biologically. Several researches have been conducted to demonstrate the anticancer efficacy of *Annona squamosa* L. (leaves) extracts. Therefore, this study sought to investigate whether extracts from the leaves and seeds of *Annona squamosa* L. had any anticancer effects on breast cancer cells.

Extracts of *Annona squamosa* L. (leaves) and (seeds) were tested for their anticancer effects on breast cancer cell lines (MCF-7) in this in vitro investigation. Maximum activity was observed in methanol extract of *Annona squamosa* L. (seeds) with IC50 16.12±0.77 µg/mL. Our data showed that the dose-dependent inhibition of MCF-7 cell growth by *Annona squamosa* L. (leaves) and (seeds) extracts suggests that they may have anticancer potential.

Conflict of Interest

None

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How to cite this Article:

Nehal Rami, Bhushan Kulkarni, Sandesh Chibber, Devendrasinh Jhala, Nilam Parmar and Krupali Trivedi (2023). *In-vitro* antioxidant and anticancer potential of *Annona squamosa* L. Extracts against breast cancer. *International Journal of Experimental Research and Review*, *30*, 264-275.

DOI : https://doi.org/10.52756/ijerr.2023.v30.024