IJAAR PUBLISHING®

American Research Journal of Contemporary Issues | ISSN: 3027-0189
Vol. 1, Issue 3 (October-December, 2023) | www.ijaar.org

ANTIOXIDANT AND CYTOTOXIC INVESTIGATION OF *Annona squamosa* Linn (SUGAR APPLE) ON HUMAN BREAST CANCER CELLS

[1]Binta M. A; [*2]Musa A. A and [1]Idris S. I

[1]Department of Science Laboratory Technology, Kano State Polytechnic, Nigeria
[2]Department of Pharmaceutical Technology, Kano State Polytechnic, Nigeria

**Corresponding author's e-mail:aamusakanopoly@gmail.com,+2348061619400*

ABSTRACT

Background and Objectives: *Cancer is a common cause of death worldwide in human populations. Surgery, chemotherapy and radiotherapy still remain the corner stone of treatment. However, herbal medicines are gaining popularity on account of their lesser harmful side effects on non-targeted human cells and biological environment. Annona squamosa L. is a common delicious edible fruit with different parts of the plant having various therapeutic potentials; however, little is known about the anticancer property of the plant. Thus, antioxidant and cytotoxic properties of its leaf-extracts were evaluated to support its traditional claim for preventive health care.*
Methods: *Qualitative phytochemical screening was carried out to detect the presence of bioactive components of A. squamosa l using standard procedure. The cytotoxic effect of the extracts on Human breast cancer cells (MCF-7) was determined by MTT assay. For the antioxidant activity, 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used. Total phenolic content (TPC) was evaluated using Folin-Ciocalteu techniques.*
Results: *The ethanolic extract of A. squamosa exhibited higher percentage yield of 27.4 % compared to 22.5 % of aqueous during the solvent extraction procedure. The cytotoxic activity of the extracts showed IC_{50} values of 0.27 and 3.24 mg/ mL for ethanolic and aqueous extracts, respectively indicating stronger cytotoxic effects on MCF-7 cell lines in ethanolic extract. The TPC was also higher in ethanolic extract compared to aqueous which was 0.131 and 0.105 mg of GA/ g of extracts, respectively. For the antioxidant activity, both ethanolic and aqueous extracts showed IC_{50} values of > 8 mg/mL in comparison of 0.03 mg/mL of the standard ascorbic acid, indicating low antioxidant activity. Both ethanolic and aqueous extracts of A. squamosa l exhibited a weak, negative correlation between TPC and percentage inhibition of DPPH. The phytochemical screening of the extracts reveals the presence of several phytochemical compounds namely alkaloid, saponin, flavonoid and fixed oil and fats with maximum presence of phytochemicals in ethanolic compared to aqueous extract of A. squamosa.*
Conclusion: *the present result shows that the ethanolic extract of A. squamosa which exhibited higher percentage yield, antiproliferative activity, TPC content and maximum presence of phytochemicals can be used as a potential source of anticancer agent but not a good source of natural antioxidants. Thus, A. squamosa leaf-extracts may be developed as a potential novel drug for the treatment of breast cancer in the future.*

Keywords: *Annona squamosa* L.; leaf, cancer cell lines; antioxidant property; cytotoxicity.

11/10/2023
Binta M. A, Musa A. A & Idris S. I. (2023). Antioxidant and Cytotoxic Investigation of *Annona Squamosa* Linn (Sugar Apple) on Human Breast Cancer Cells. *American Research Journal of Contemporary Issues*, 1(3), 68-83.

INTRODUCTION

Cancer is the common cause of death in human worldwide. Currently, surgery, chemotherapy and radiotherapy, which are the main and the major therapies, are still unavailable to the populations of many third world and underdeveloped countries. However, herbal medicines are gaining popularity on account of their lesser harmful side effects even on non-targeted biological environment (Maya *et al.,* 2013). Breast cancer is the second most common cancer and the most common cause of death in women worldwide (Ferlay *et al.,* 2015). Nevertheless, surgery, chemotherapy, and radiotherapy remain the cornerstone of breast cancer treatment (Wang *et al.,* 2014). Chemotherapeutic agents have several disadvantages and significant side effects, such as the inability to differentiate between cancer and normal cells and drug resistance associated with repeated treatment. Hence, there is an urgent need for new drugs. It is necessary to identify natural products that target multiple signaling pathways and cause growth inhibitory effects on cancer cells with fewer harmful effects on healthy cells and the biological environment (Maya *et al.,* 2013). Therefore, in the field of phytoscience, researchers are working to elucidate the side effects, calculate appropriate dosages, and find the best method to extract and identify bioactive components. The bioactive compounds are secondary plant metabolites that elicit pharmacological or toxicological effects in humans and animals; however, one of the most critical challenges that researchers have faced is that a single plant may contain many bioactive compounds (Aḥmad *et al.,* 2006).

Annona squamosa L. is a small group of an edible plant of the genus Annona and the family Annonaceae. It is commonly known as a sugar apple or custard apple (Lim, 2012). It is mainly grown in gardens for its delicious fruit and its great ornamentals value. It is a small (about 5-6 m in height) deciduous tree with irregular branches. Different parts of *A. squamosa L.,* such as the bark, root, seed, fruits, flowers, and leaves have been used in traditional medicine to treat various diseases (Kalidindi *et al.,* 2015).

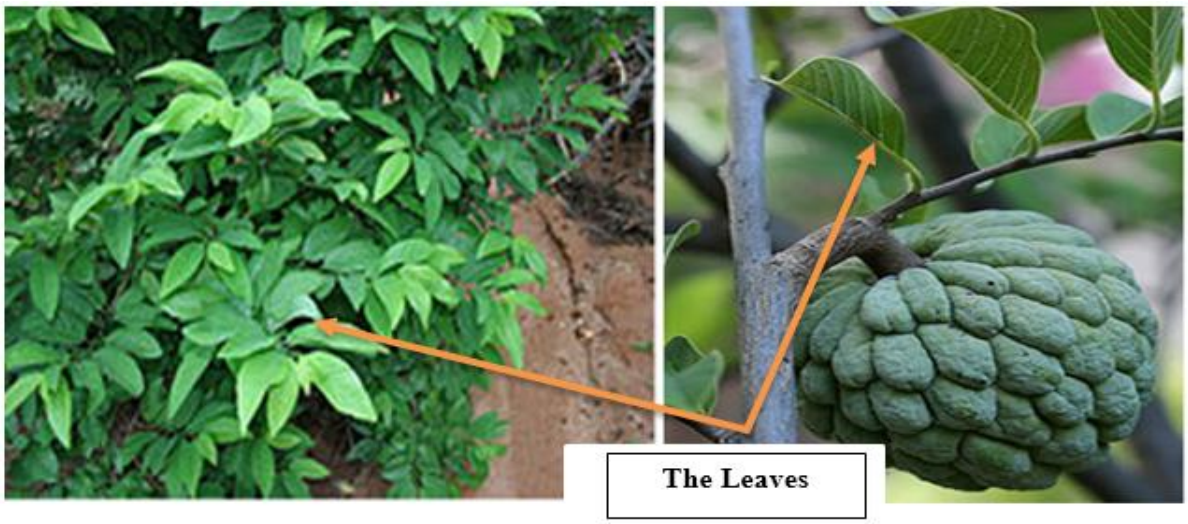

Figure 1.0: Annona squamosa (Sugar apple tree)

The plant has been used in traditional medicine of several tropical countries to treat epilepsy, dysentery, cardiac problems, worm infection, constipation, hemorrhage, antibacterial, dysurea, fever and ulcer. It also has anti-fertility and abortifacient properties (Soni *et al.,* 2012). It is effective remedy for various types of inflammatory diseases as well as tumorous growths. All parts of this plant i.e. leaf; bark, shoot and roots have various compounds of medicinal importance and hence were used in different kinds of health problems. Ripe fruits

of plant are applied to malignant tumors to hasten suppuration while vermin are destroyed by the used of its dried unripe fruit powder. The seeds are acrid and poisonous and their powder served as fish poison and as insecticidal agent. A seed paste has been used in eradication of head lice, for destroying worm inthe wound of cattle's and also has shown antifertility activity or being used as valuable contraceptive. The crushed leaves are sniffed to overcome hysteria and fainting spells, they are also applied on ulcer and wounds (Parvin *et al.,* 2003; Sobiya *et al.,* 2009). The plant aerial parts are used as antibacterial, anti-diabetic, anti-hyperlipidemic, anti-microbial, antioxidant, anti-head lice effect, antitumour, cytotoxic, hepatoprotective, insecticidal, chemopreventive & anti-lipid peroxidative, mosquitocidal, pesticidal, molluscicidal, anti-plasmodial, vasorelaxant, anti-thyroidic, anti-fertility, antiviral and anthelmintic activity. Several bioactive compounds have been isolated from *A. squamosa L.* leaves, including alkaloids, steroids, annonaceous acetogenins, terpenoids, glycosides, saponins, flavonoids, and phenolics and these compounds were found to be responsible for various biological activities (Neha and Dushyant, 2011).

A. squamosa Linn plant parts are known to have various biological activities, although there are fewer studies of the leaf-extracts. Hence, to provide a clear understanding of the mechanism action of *A. squamosa* leaf-extracts, evaluation for the anticancer property should confirm its use as an alternative to chemotherapy for breast cancer. Thus, the objective of the study is to investigate the in vitro anti-breast cancer property of *A. squamosa* leaf extracts. Thus, the total phenolic contents (TPC), antioxidant and cytotoxic effects of aqueous and ethanolic leaf-extracts of *A. squamosa* will be evaluated to support its traditional claim for preventive health care.

Breast cancer is a disease in which cells in the breast grow out of control. There are different kinds of breast cancer. Breast cancer is the most common cancer overall among women in both developed and less developed regions of the world, representing 25% of new cancer cases among women, and the second cause of death in developed regions after lung cancer (Anubhav *et al.,* 2021; Ferlay *et al.,* 2015). Breast cancer is cancer formed in the breast cells. Breast cancer is the most common malignancy in women, but it can occur less frequently in men. The kind of breast cancer depends on which cells in the breast turn into cancer. Breast cancer can begin in different parts of the breast. A breast is made up of three main parts: *lobules, ducts, and connective tissue.* The lobules are the glands that produce milk. The ducts are tubes that carry milk to the nipple. The connective tissue (which consists of fibrous and fatty tissue) surrounds and holds everything together. Most breast cancers begin in the ducts or lobules. Breast cancer can spread outside the breast through blood vessels and lymph vessels. When breast cancer spreads to other parts of the body, it is said to have metastasized (Ferlay *et al.,* 2015). In the United States, breast cancer is the second-leading cause of cancer death in women, after lung cancer. It's also the leading cause of cancer death among women ages 35 to 54.

The current medical treatment for breast cancer includes surgery, chemotherapy and radiation therapy (American Cancer Society, 2014). However, some of these methods cause undesired side effects by the non-specific targeting of both normal and cancer cells (Chueahongthong *et al.,* 2011). Thus, research for cancer treatment using natural products has been increasing rapidly (Valiyari, 2012). Numerous studies have identified medicinal plant extracts which not only displayed antioxidant effect, but also cytotoxicity to many forms of cancer (Valiyari, 2012; Chueahongthong *et al.,* 2011). Therefore**,** research for cancer treatment using natural products is of great concern. Therefore, *Annona squamosa* L. is an important medicinal plant used in traditional medicine for the treatment of various diseases. Different parts of *A. squamosa* have various therapeutic potentials; however, to date, little is known about the

anticancer property of the leaves. Thus, the total phenolic contents (TPC), antioxidant and cytotoxic properties of leaf-extracts of *A. squamosa* need to be evaluated to support its traditional claim for preventive health care.

3.0 MATERIALS AND METHODS

3.1 Chemicals and reagents

All the reagents used in this research will be purchased from Sigma Chemicals Co. (St. Louis, MO, United States), Fisher, QrecTM and Merck (Germany). For cell culturing purposes, MCF-7 breast cancer cells will be cultured in Dulbecco's modified eagle medium (DMEM, Sigma, USA), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratory GmbH, Austria), 1% penicillin G (PAA Laboratory GmbH, Austria). Ethanol (95%), sodium nitrite (NaNO2), aluminum chloride (AlCl3), sodium hydroxide, ascorbic acid, gallic acid, quercetin, Phosphate buffered saline (PBS) and Trypsin solution were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). All the chemicals were of analytical grade.Folin-Ciocalteu Reagent (FCR), gallic acid, sodium carbonate, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), and L- ascorbic acid, were purchased from Sigma-Aldrich Chemical Co. (St. Louis). For the phytochemical analysis, the chemical used including ammonium hydroxide, sulphuric acid, chloroform, ferric chloride, sodium chloride, lead acetate, potassium iodide were all purchased from Merck, Germany.

3.2 Experimental Design

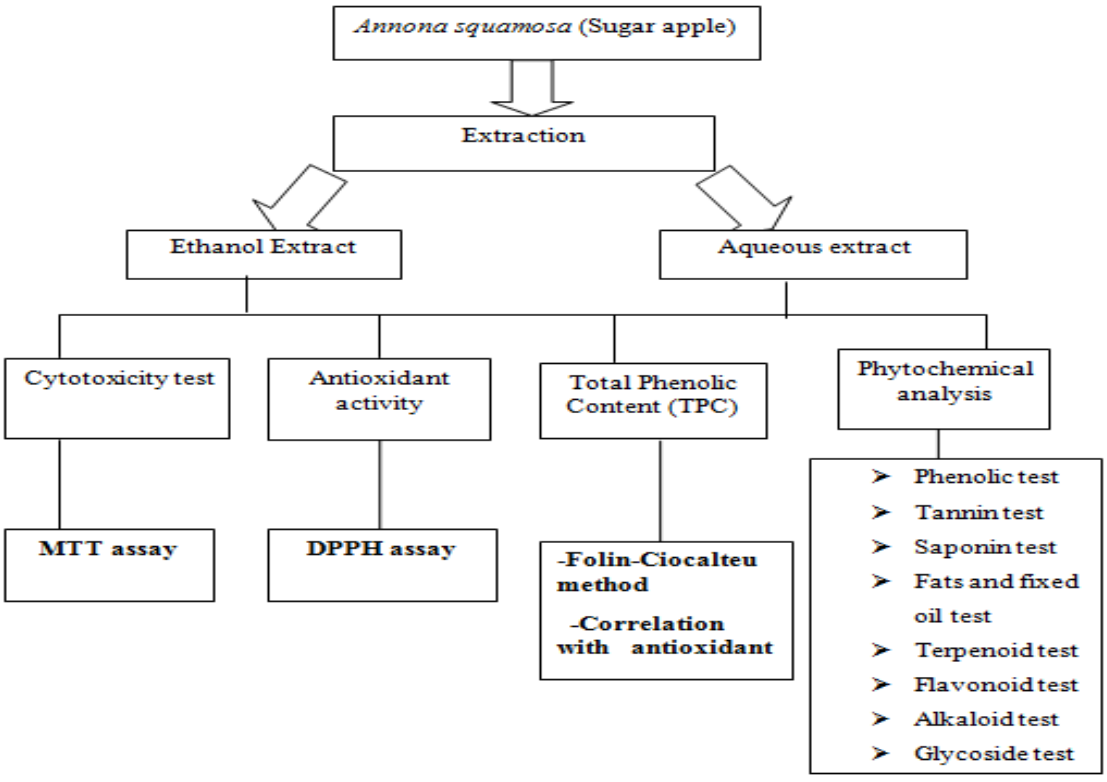

Figure 2.0: Experimental design of the overall scope of the study

3.3 Culture of Human Breast cancer cells (MCF-7)

MCF-7 breast cancer cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin- streptomycin antibiotic. The MCF-7 cells were maintained in a humidified incubator (Contherm Scientific Ltd, New

Zealand) with an atmosphere of 95% air and 5% CO_2 at 37 °C. At 50- 70% confluence, cells were rinsed with phosphate buffer saline (PBS) and harvested from 25cm^2 flask using 0.25 % trypsin solution. Then, cells were sub-cultured into 25 cm^2 flask or 96 -well plate according to the experiments.

3.4 Collection, Preparation and Extraction of Plant Material

The dried leaves of *A. squamosa* were collected from farm-land, Kano state, Nigeria. The leaves were blended into uniform powder form with an electrical blender. Following that, the blended leaves were subjected to extraction by using two different solvents namely ethanol and aqueous. The powdered plant samples were extracted by maceration with ethanol in the ratio of 1: 20 (50 g/ 1000 mL) and left for 72 hr at room temperature. The solvent were filtered first through a Whatmann filter paper and then through cotton wool. The filtrates were concentrated using Eyela™ rotary evaporator (Sigma- Aldrich, USA) with the water bath set at 45 ºC. The obtained crude extracts were stored at –20°C. For aqueous leaf extract, air-dried sample was extracted using sterile distilled water in the ratio of 1:20. The mixture was heated on a hotplate and continuously stirred with magnetic stirrer for 3 hr at 65°C. The mixture was then cooled and filtered using Whatmann filter paper. The filtrate was evaporated using Eyela™ rotary evaporator with the water bath set at 45ºC. The obtained crude extracts was stored at – 20°C. After the pure extracts were collected successfully, the yields of each kind of extracts were calculated as following formula:

$$\text{Yields (\%)} = \frac{\text{Weight of extracts obtained (g)}}{\text{Weight of dried } A.\ squamosa \text{ leaves (g)}} \times 100$$ (Stanković, 2011)

3.5 DPPH Radical Scavenging Activity Assay

The antioxidant activity of the plant extracts was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Tepe *et al.,* 2005). Stock solution of DPPH was prepared by adding 10 mg of DPPH powder to 25 ml of 95% ethanol. Ascorbic acid was used as the standard. It was prepared by adding 16 mg of ascorbic acid to 2 ml of 95% ethanol to get a final concentration of 8 mg/ml. The extract solution was transferred to a 96-well plate and serially diluted at ten-fold dilution using 95% ethanol with a range of concentration from 8 mg/ml to 0.01875 mg/ml. 100 µl of DPPH reagent was added to each well except blank. Ethanol solution was used as blanks. The control only contained DPPH solution instead of sample. Following incubation at room temperature for 30 min, absorbance was measured at 517 nm. The change in the absorbance produced at 517nm was used as a measure for antioxidant activity. All samples and readings were prepared and measured in triplicates. DPPH radical scavenging activities of the extracts was expressed as IC_{50} values. IC_{50}, effective concentration of the extract required for 50% scavenging of DPPH radicals was calculated from the plotted graph of scavenging activity against sample concentration.

3.6 Total Phenolic Content (TPC)

Total phenolic content was measured by Folin-Ciocalteu method with a slight modification (Singleton *et al.,* 1999; Magalhães *et al.,* 2010). 30 µl of samples was diluted up to 2370 µl deionized distilled water and followed by the addition of 150 µl of Folin-Ciocalteu reagent. The mixture was vortexed for 15s in dark. Following 1 min incubation, 450 µl of 20% (w/v) sodium carbonate was added to the mixture and incubated at 40°C for 30 min in the dark before measuring the absorbance (A) in 96-well plate at 750 nm. The samples was performed in quadruplicate. The results were expressed as gallic acid equivalents (GAE) by mean of a calibration curve.

3.7 Cytotoxicity assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

The HT29 colon cancer cell lines (1×10^5 cells/ well) was seeded in a 96 - well plate, and incubated at 37°C under 5% CO_2 in a humidified atmosphere for a period of 24 hr before the addition of plant extract. Crude extract was dissolved in 100% dimethyl sulfoxide (DMSO) and prepared fresh prior to assay. The diluted solution of *A. squamosa* extract ranging from 0.06, 0.13, 0.25, 0.50, 1.00, 2.00, 4.00 and 8.00 mg/ mL was added to the culture plate in triplicates, in order to examine a wide range of plant extract for potential toxicity. The control well consist of untreated cells (cell + medium). The plate was then incubated for 24 hr under the same conditions. Following the treatment, 20 µL of MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) solution was added to each of the 96 -well and incubated for 3 hr at 37°C. The plate was read with a microplate reader with the absorbance at 570 nm. High optical density readings corresponded to a high intensity of dye colour, that is, to a high number of viable cells able to metabolize MTT salts. IC_{50} value was determined from the plotted graph of percentage inhibition against sample concentration which is defined as the concentration needed to reduce cell growth by 50%. All samples and readings were prepared and measured in triplicates.

3.8 Qualitative Phytochemical Screening

The ethanolic and aqueous extracts of *A. squamosa* were subjected to phytochemical analysis to detect the presence of bioactive compounds. Various compounds such as ferric chloride, tannin, saponin, Fats and Fixed oils, terpenoids, flavonoids and alkaloids were screened qualitatively using standard methods (Ayoola *et al*., 2008; Aziman *et al*., 2012; Prasad *et al.*,. 2014).

3.9 Statistical Analysis

Statistical analysis of data was conducted by analysis of variance (ANOVA one way) using Statistical Package for the Social Sciences (SPSS) software version 18.0 and a probability value of $p \leq 0.05$ represent a significant difference between the means.

4.0 RESULTS AND DISCUSSION

The air-dried leaves of *A. squamosa* were extracted using two different solvents; aqueous and ethanol. Water and ethanol are widely used for plant extraction due to their low toxicity and high extraction yield. The extraction yield of plants depends highly on the solvent polarity (Amita and Shalini, 2014). As shown in Table 4.1, the percentage yield of ethanol was higher compared to that of aqueous. This is likely in light of the fact that, the type of solvent used in the extraction procedure influenced the solubility of the active component of the leaves (Saad *et al.*, 2014).

Table 4.1: The percentage yield of the plant using aqueous and ethanolic extraction

Solvent	Yield (%)
Aqueous	22.5
Ethanol	27.4

4.1 Cytotoxicity assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

The cytotoxic effects of ethanolic and aqueous crude extract of *A. squamosa*on the MCF-7 breast cancer cells were determined using the MTT assay. In this assay, a tetrazolium salt (3-

[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide, MTT) is converted to a purple formazan product by enzymes active only in living cells (Fotakis and Timbrell, 2006). The concentration of the plant extract was ranged from 0.0625 mg/mL to 8.0 mg/ mL. From the result stated in Table 2, the inhibition of MCF-7 breast cancer cells treated with ethanolic extract of *A. squamosa* was much higher compared to aqueous extract. This is represented by lower IC_{50} value which was 0.27 and 3.24 mg/mL for ethanol and aqueous extract, respectively (Figure 1). Statistical analysis shows that there is significant difference ($p< 0.05$) between the percentage inhibition of MCF-7 and extract concentration for both ethanolic and aqueous leaf extracts.

***Table 4.2**: Percentage viability and inhibition of MCF-7 breast cancer cells tested against different concentrations of ethanolic and aqueous Leaf-extracts of A. squamosa (0.0625 mg/mL – 8.0 mg/mL)*

Concentration (mg/ mL)	Viability (%)		Inhibition (%)	
	Ethanolic	Aqueous	Ethanolic	Aqueous
0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	0 ± 0.00
0.06	55.03 ±1.08	98.57 ± 1.78	41.87 ± 1.21	4.33 ± 2.02
0.13	24.55 ± 0.60	90.15 ± 3.58	75.45 ± 0.67	8.71 ± 4.11
0.25	17.22 ± 0.34	82.14 ± 2.21	81.25 ± 0.38	16.15 ± 2.45
0.50	15.23 ± 0.25	66.15 ± 4.41	78.55 ± 0.28	28.55 ± 4.92
1.00	13.11 ±0.14	60.17 ± 0.81	88.59 ± 0.17	34.88 ± 5.19
2.00	11.55± 0.17	55.84 ± 0.42	90.14 ± 0.21	43.16 ± 2.49
4.00	8.68± 0.05	41.66 ± 9.85	92.33 ± 0.044	51.20 ± 4.79
8.00	4.88± 0.09	22.15 ± 0.53	96.70 ± 0.084	68.99 ± 0.45

Note; each value represents the mean± SD for three replicates

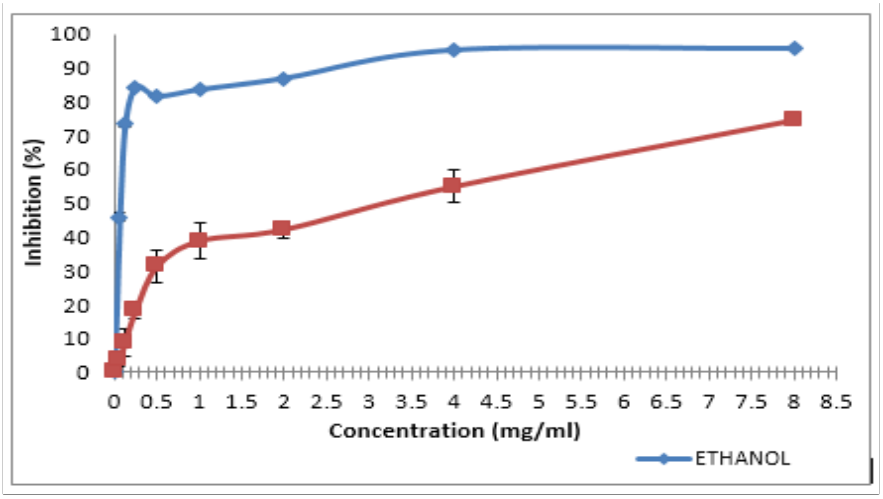

Figure 4.1:The graph shows percentage inhibition of MCF-7 breast cancer cells treated with different concentrations of ethanolic and aqueous extract of A. squamosa leaves. The IC_{50} value was determined from the graph

Table 4.3: IC_{50} values of ethanolic and aqueous extract of A. squamosa leaves

Extract	IC_{50} (mg/mL)
Ethanolic	0.27
Aqueous	3.24

Note; the lower the IC_{50} value the higher the inhibition of MCF-7 breast cancer cells and thus, indicates high cytotoxic activity of the pant (*A. squamosa*).

4.2 Phytochemical Screening

The ethanolic and aqueous extract of the *A. squamosa* were qualitatively screened for the presence of phytochemicals. The chemical compounds considered in this study include phenols, flavonoids, tannins, terpenoids, alkaloids, saponin and fixed oils as shown in Table 4.4.

Table 4.4: Qualitative analysis of phytochemical constituents of A. squamosa in ethanolic and aqueous crude extracts

No	Phytochemical Test	Reagent used (test performed)	Indication for the presence of phytochemicals	Result	
				Ethanol	Aqueous
1.	Phenolic test	Ferric Chloride Test	Appearance of bluish black precipitate	++	++
2.	Tannin test	Gelatin Test	Formation of white precipitate	++	-
3.	Saponin test	Foam Test	Produce foam than lasts for more than 10 minutes	++	+
4.	Fats and Fixed oil test	Filter Paper press Test	Oily stain was obtained	++	+
5.	Terpenoid test	Salkowski Test	Formation of reddish brown color at interface	-	-
6.	Flavonoid test	Lead Acetate Test	Yellow precipitate obtained	++	+
7.	Alkaloid test	Wagner's Reagent	Reddish brown precipitate obtained	+	-

Key: (++) indicates highly presence, **(+)** indicates moderately presence **(-)** indicates absence

Phytochemical screening of ethanolic and aqueous leaf-extracts of *A. squamosa*revealed the presence of phytochemicals such as saponin, fats and fixed oil, flavonoid and alkaloid by the positive reaction with the respective test reagents. However, more of the phytochemical compounds were detected in ethanolic compared to aqueous extracts. The variation in the expression of the phytochemicals could be due to the difference in polarity of the solvents used (Prasad *et al*., 2014). Therefore, the presence of these metabolites explains the various uses of this plant in traditional medicine.

4.3 Total Phenolic Content (TPC)

The intensity of blue colour observed during the experimental procedure indicates the quantity of phenolic compounds which can be measured by spectrophotometer (Conforti *et al*., 2008). The total phenolic content in the ethanolic and aqueous extracts were expressed in

terms of gallic acid equivalent to the equation of y = 1.048x + 0.043 (R^2= 0.992), whereby y = absorbance at 750 nm and x = concentration of total phenolic compounds in mg per mL of the extract (Figure 4.1). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract as shown in Table 2. These values indicated that 1g of plant extract contains an amount of phenolic compounds equivalent to about 1 mg of pure gallic acid (Suhailah *et al*., 2011).

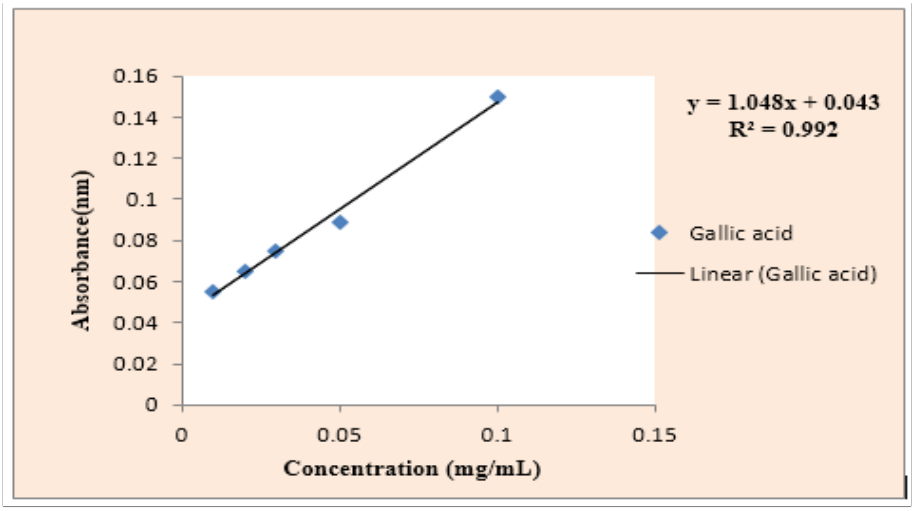

Figure 4.2: Concentration of gallic acid (mg/mL) against absorption at 750 nm

Table 4.5: Total phenolic contents in the ethanolic and aqueous extracts of A. squamosa leaves expressed in terms of gallic acid equivalent (mg of GA/g of extract). Each value is the average of three replicates ± standard deviation.

Extract	mg of GA/g of extract
Ethanolic	0.131 ± 0.00082
Aqueous	0.105 ± 0.0015

3.3 Evaluation of Antioxidant property

DPPH free radical scavenging activity of aqueous and ethanol extracts were evaluated to determine their antioxidant properties. The results are presented in Table 3.

Table 4.6: Percentage inhibition of DPPH tested against different concentrations of ethanolic and aqueous extracts of A. squamosa leaves (8.0 mg/mL – 0.008 mg/mL). Each value represents the mean± SD for three replicates.

Concentration (mg/ mL)	Inhibition (%)		
	Ethanolic	Aqueous	Standard (Ascorbic acid)
8.0	20.8 ± 0.18	24.4 ± 0.006	96.8 ± 0.02
4.0	14.9 ± 0.12	15.8 ± 0.005	96.8 ± 0.02
2.0	12.8 ± 0.09	9.9 ± 0.035	96.6 ± 0.02
1.0	9.3 ± 0.097	5.8 ± 0.018	96.7 ± 0.02
0.5	8.9 ± 0.153	5.1 ± 0.034	96.8 ± 0.02
0.25	8.4 ± 0.106	3.7 ± 0.045	96.7 ± 0.02
0.13	7.5 ± 0.113	2.5 ± 0.012	96.7 ± 0.02
0.06	5.5 ± 0.101	2.4 ± 0.005	96.6 ± 0.03
0.03	3.8 ± 0.088	2.7 ± 0.009	64.6 ± 0.40
0.016	1.9 ± 0.055	2.7 ± 0.004	33.8 ± 0.90
0.008	0.6 ± 0.021	0.8 ± 0.002	19.5 ± 1.10

Both ethanolic and aqueous leaf-extracts of *A. squamosa* showed low free radical scavenging activity on DPPH as the IC_{50} values were > 8 mg/mL as compared to the standard ascorbic acid which was 0.03 mg/mL (Figure 2). The concentration of the extract at which the percentage inhibition reaches 50% is defined as the IC_{50} value which is inversely proportional to the antioxidant activity. Therefore, the lower the IC_{50} value the higher the antioxidant activity of the extracts (Akrout *et al*., 2012). Statistical analysis shows that there was significant difference ($p< 0.05$) between the percentage inhibition of DPPH and the extract concentration for both ethanolic and aqueous extracts. These findings were likely in agreement with Almey *et al*. (2010) who found that both methanolic and aqueous extracts of *A. squamosa*had the highest IC_{50} value which indicates low antioxidant activity. Thus, the antioxidant property of the plant extracts depends highly on the extraction solvents (Aksoy *et al.*, 2013).

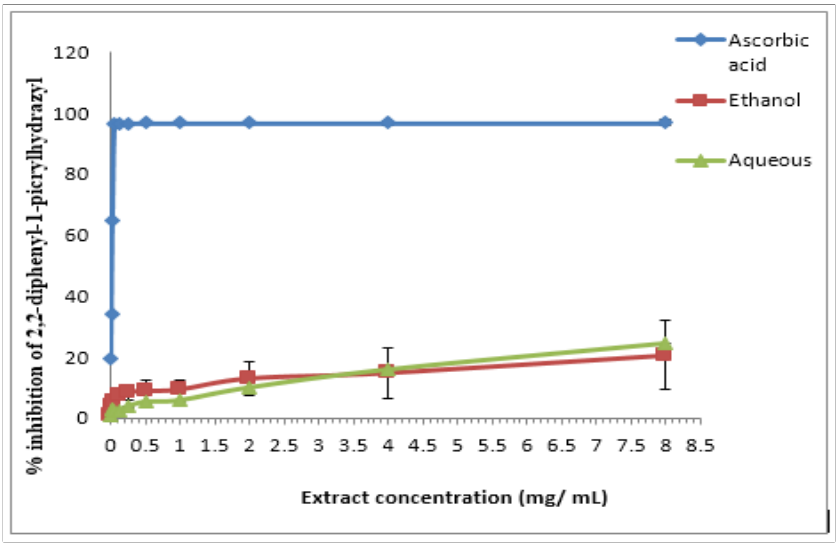

Figure 4.3: Percentage inhibition of the ethanolic and aqueous extracts of A. squamosa leaves and standard on DPPH. The values were expressed as mean ± standard deviation (n=3). Ascorbic acid was used as the standard.

Table 4.7: IC_{50} values of ethanolic and aqueous extract of C. hystrix leaves

Extract	IC_{50} (mg/mL)
Ethanolic	>8
Aqueous	>8
Ascorbic acid	0.03

DISCUSSION

Based on the results of extraction process, it can be explained that *A. squamosa* contains compounds that dissolve better in ethanol than in aqueous or absolute water. Previous studies conducted by Suhailah *et al*., (2011) reported that percentage yield of ethanolic extract of five traditional Malaysian plants were higher compared to aqueous extract. In addition, Amita and Shalini (2014) stated that the ethanolic extract was an efficient extract and provides the highest extraction yield compared to other polar solvents including water. However, this finding seems to be in contrast with Nurain *et al*. (2013) who reported that higher percentage yield of some plantswas found in aqueous compared to ethanolic and other organic solvents. This variation could be due to factors such as cultivar origin, geographical origin as well the stage of harvest. From ancient decades, plants have been utilized for the treatment of various human diseases. They are used as complementary medicine and/ or to synthesize chemical compounds.

According to the World Health Organization (WHO), more than 75% of the world's population depends on nature-derived traditional alternative medicine for their primary healthcare needs (Bailon-Moscoso *et al.,* 2016, Semlali *et al.,* 2021, Contant *et al.,* 2021). One of these plants with extensive traditional utilization is *Annona squamosa*. However, biologically the plant is less well characterized up to date. Few studies have illustrated the anticancer property of *A. squamosa* leaf extracts. Therefore, this study was aimed to investigate the anticancer properties of *A. squamosa*leaf-extracts on Human breast cancer cells. For the current study, the anticancer activity of *A. squamosa* leaf extracts was investigated in vitro against breast cancer cell lines; MCF-7. Our results revealed that the different *A. squamosa* leaf-extracts were found to induce cytotoxicity and inhibit the proliferation of MCF-7 cells in a dose-dependent manner (Table 2).

It is well known that the leading cause of death among patients with cancer is the ability of cancer cells to metastasize and invade (Seyfried and Huysentruyt, 2013), and our results also present the demonstration that different *A. squamosa* leaf extracts strongly inhibited breast cancer migration in MCF-7 cells (Table 2). These results agreed with those of Pardhasaradhi *et al.* (2005), whichshowed that treatment of MCF-7 with *A. squamosa* seed extracts resulted in nuclear condensation, DNA fragmentation, ROS generation, and apoptosis induction via down regulation. The remarkable anticancer property of the extracts may be due to flavonoids and phenolic compounds and to some extent due to the high level of *germacrene-D* on the leaves which exerted anticancer activity against different cell lines, or to the presence ofother bioactive compounds which have also been known to havean anticancer activity such as humulene, phytol and/or a combinationof these bioactive compounds (Essien *et al.,* 2016; Kim *et al.,* 2015; Pejin *et al.,* 2014). Collectively, ethanolic extracts havethe highest total phenolic content, thus agrees with the findings documented by Rawan *et al.*,(2022) that, the methanolic extracts f *A. squamosa* extracts have the highest total phenolic contents, while acetonic extracts have the highest total flavonoid content, these differenceson the ratio of the bioactive compounds explain the potentialdifferences between the extracts.Also, according to

research conducted by Chen *et al.,* (2012b), *A. squamosa* extracts had strong cytotoxic effects on H22 hematoma cells in which *A. squamosa* seed-extracts inhibited the growth of H22 hepatoma cells by 69.55% in mice without any side effects.

The variation in the expression of the phytochemicals could be due to the difference in the polarity of the solvents or due to different in geography, climate soil condition and age of *A. squamosa* plant (Prasad *et al*., 2014). Thus, ethanol may possibly aid in extraction of novel bioactive compounds in plant materials more than aqueous solvent. Ethanol is mainly used as an extraction solvent because it is relatively safe and cheap for herbal medicine preparation compared with the other toxic organic solvents. Therefore, the yields of extract and consequential cytotoxic activity of plant materials may be largely dependent on the nature of extraction solvent, due to the presence of different bioactive compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. The present findings were also supported by Prasad *et al*. (2014); Aziman *et al*. (2012), who reported that the ethanolic extract of *A. squamosa*leaves shows negative result for the ferric chloride and gelatin tests. Flavonoids and tannins are phenolic compounds that act as primary antioxidants or free radical scavengers in plants (Ayoola *et al*., 2008).

For the antioxidant properties observed in *A. squamosa*, the DPPH free radical scavenging assay might not be a good choice to determine the antioxidant activity of *A. squamosa*. This is because, less antioxidant activity was observed in this study using DPPH. Thus, the results were in contrast with the findings of Biba Vikas *et al.,* (2017) in which they reported that, the free radical scavenging potential of *A. squamosa* leaves using different antioxidant models of screening was shown to possess strong antioxidants. Therefore, different assay works according to different principles and detects different compounds (Gursoy *et al*., 2009). Moreover, in a comparative study of aqueous and ethanolic aromatic herbs using four antioxidant activity assays, Nurain *et al*. (2013) reported that both the ethanolic and aqueous extracts of *A. squamosa*were among the plants that exhibited low antioxidant activity on DPPH free radical scavenging assay. In contrast, highest antioxidant activity was observed using Oxygen Radical Absorbance Capacity (ORAC) assay, followed by Ferric Reducing Antioxidant Power Assay (FRAP). The presence of other reducing agents not reactive towards DPPH radical could be one of the possible reasons for high IC_{50} values of the plant (Nurain *et al*., 2013). Thus, method of extraction could also be another factor that influences the antioxidant activity.

The TPC contents were also observed to be low forboth ethanolic and aqueous extracts of *A. squamosa*. However, the antioxidant activity of ethanolic extract was found to be higher compared to that of aqueous. This study was supported by Nurain *et al*. (2013), who reported that higher TPC was found in ethanolic extract compared to aqueous extracts of various plants studied. Various studies on total phenolic content of some plants had been published in several papers. Almey *et al.,* (2010) and Nurain *et al.,* (2013), reported that both ethanolic and aqueous leaf-extracts of *C. hystrix* had low TPC values. In addition, Jayshree (2008) also reported that, in the antioxidant activities studied, *A. squamosa* aqueous extract supplementation is useful in controlling the blood glucose level, improves the plasma insulin, lipid metabolism and is beneficial in preventing diabetic complications from lipid peroxidation antioxidant systems in experimental diabetic rats. Therefore, variation in the results; whether strong or low, may be due to the different procedures and standards that were used during the experimental study. Extrinsic factors such as agronomic, environmental and handling and storage might give different levels of TPC contents as well (Huda-Faujan *et al*., 2007).

CONCLUSION

Based on the results obtained and previous studies analyzed, it can be concluded that the ethanolic extract of *A. squamosa* leaves exhibited higher percentage yield, cytotoxicity and antioxidantproperty compared to that of aqueous. Phytochemical screening reveals the presence of several phytochemical compounds.

In summary, *A. squamosa* leaf-extracts demonstrated strong potential anticancer property against breast cancer cells within-vitro experiment using MCF-7 cell lines. The studied plant also exhibited varying degrees of total phenolic contents and antioxidant properties. The present study revealed fewer amounts of phenolic compounds, Hence, *A. squamosa* leaves have low potential power to be used as a good source of natural antioxidants. Therefore, further investigation may contributeto the development of a potential new anticancer therapy for different types of Human breast cancers. Further research is needed fully to understand the mechanism of action of *A. squamosa* leaf-extracts and its role in cell autophagy, in order to identify the pathways targeted by these extracts. The present results therefore, demonstrated that by exhibiting good cytotoxic effect on the MCF-7 cells, *A. squamosa* leaves may have the potential to be used in cancer treatments.

DECLARATION OF COMPETING INTEREST

The authors declare no known competing interests or personal relationships that could have appeared to influence the work reported in this research paper.

ACKNOWLEDGEMENTS

The authors extend their appreciation to the Researcher's financial Supporting Organization; Tertiary Education Trust Fund (TETFUND) Nigeria, and Kano State Polytechnic for its support and encouragement throughout the period of this research.

REFERENCES

Aḥmad, I., Aqil, F., Owais, M. (2006). Modern phytomedicine : turning medical plants into drugs. Wiley-VCH, Weinheim.

Akrout, A., Mighri, H., Krid, M., Thabet, F., Turki, H., El-Jani, H. and Neffati, M. (2012). Chemical Composition and Antioxidant Activity of Aqueous Extracts of Some Wild Medicinal Plants in Southern Tunisia. *International Journal of Life Science and Medical Science.* 2(1): 1-4.

Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z. and Kargıoğlu, M. (2013). Free Radical Scavenging Activity, Total Phenolic Content, Total Antioxidant Status, and Total Oxidant Status of *Endemic ThermopsisTurcica*. *Saudi Journal of Biological Sciences.* 20(3): 235-239.

Almey, A. A., Ahmed Jalal Khan, C., Syed Zahir, I., Mustapha Suleiman, K., Aisyah, M. R. and Kamarul Rahim, K. (2010). Total Phenolic Content and Primary Antioxidant Activity of Methanolic and Ethanolic Extracts of Aromatic Plants Leaves. *International Food Research Journal*. 17(4): 1077-1084.

American Cancer Society (2014). Colorectal Cancer Facts and Figures. Retrieved January15,2015,from:http://www.cancer.org/research/cancerfactsstatistics/colorectal-cancer-facts-figures.

Amita P., Shalini T. (2014). Concept of standardization, extraction andpre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry 2014; 2 (5): 115-119.

Anubhav, Dubey, Deepanshi Tiwari, Yatendra Singh, Om Prakash, Pankaj Singh (2021). Drug repurposing in Oncology: Opportunities and challenges. Int J of Allied Med Sci and Clin Res. 9(1):68-87.

Ayoola, G. A., Coker, H. A., Adesegun, S. A., Adepoju-Bello, A. A., Obaweya, K., Ezennia, E. C. and Atangbayila, T. O. (2008). Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research*.7(3): 1019-1024.

Aziman, N., Abdullah, N., Noor, Z. M., Zulkifli, K. S. and Kamarudin, W. S. S. W. (2012*).* Phytochemical Constituents and In Vitro Bioactivity of Ethanolic Aromatic Herb Extracts. *SainsMalaysiana*. *41*(11): 1437-1444.

Bailon-Moscoso, N., Romero Benavides, J.C., Ramirez Orellana, M.I., Ojeda, K.,Granda, G., Ratoviski, E.A., Ostrosky-Wegman, P., 2016. Cytotoxic and genotoxiceffects of extracts from Annona montana M. fruit. Food Agric. Immunol. 27 (4),559–569.

Chen, Y., Xu, S.S., Chen, J.W., Wang, Y., Xu, H.Q., Fan, N.B., Li, X., 2012b. Anti-tumoractivity of Annona squamosa seeds extract containing annonaceousacetogenincompounds. J. Ethnopharmacol. 142, 462–466. https://doi.org/10.1016/j.jep.2012.05.019.

Chueahongthong, F., Ampasavate, C., Okonogi, S., Tima, S. andAnuchapreeda, S. (2011). Cytotoxic Effects of Crude Kaffir Lime (Citrus Hystrix, DC.) Leaf Fractional Extracts on Leukemic Cell Lines. Journal of Medicinal Plant Research. 5(14): 3097-3105.

Conforti, F., Sosa, S., Marrelli, M., Menichini, F., Statti, G. A., Uzunov, D., Tubaro, A.T., Menichini, F. and Loggia, R. D. (2008). In Vivo Anti-Inflammatory and In Vitro Antioxidant Activities of Mediterranean Dietary Plants. *Journal of Ethnopharmacology*. 116(1):144-151.

Contant, C., Rouabhia, M., Loubaki, L., Chandad, F., Semlali, A., 2021. Anetholeinduces anti oral cancer activity by triggering apoptosis, autophagy and oxidative stress and by modulation of multiple signaling pathways. Sci Rep 11 (1).

Essien, E.E., Newby, J.M., Ogunwande, I.A., Setzer, W.N., Ekundayo, O., 2016. Essential oil constituents, anticancer and antimicrobial activity of Ficusmucoso and Casuarinaequisetifolia leaves. Am. J. Essent. Oil. Nat. Prod. 4, 1–6.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer: 136, 359–386. https://doi.org/10.1002/ijc.29210.

Fotakis, G. and Timbrell, J. A. (2006). In Vitro Cytotoxicity Assays: Comparison of LDH, Neutral Red, MTT and Protein Assay in Hepatoma Cell Lines following Exposure to Cadmium Chloride. *Toxicology letters*. 160(2): 171-177.

Huda-Faujan, N., Noriham, A., Norrakiah, A.S. and Babji, A.S. (2009). Antioxidant activity of plants methanolic extracts containing phenolic compounds. African Journal of Biotechnology 8(3): 484-489.

Jayshree (2008). Annona squamosa phytochemical analysis and antimicrobial screening. Journal of Pharmacy Research. 1(1).

Kalidindi, N., Thimmaiah, N.V., Jagadeesh, N.V., Nandeep, R., Swetha, S., Kalidindi, B. (2015). Antifungal and antioxidant activities of organic and aqueous extracts of Annona squamosa Linn. leaves. J. Food Drug Anal. 23 (4), 795–802.

Lim, T.K., (2012). Annona squamosal. In: Edible Medicinal and Non-Medicinal Plants: Volume 1, Fruits. Springer Netherlands, Dordrecht, pp. 207–220. https://doi.org/ 10.1007/978-90-481-8661-7_30.

Lim, T.K., 2012. Annona squamosal. In: Edible Medicinal and Non-Medicinal Plants: Volume 1, Fruits. Springer Netherlands, Dordrecht, pp. 207–220. https://doi.org/ 10.1007/978-90-481-8661-7_30.

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S. and Lima, J. L. (2010). Rapid Microplate High-Throughput Methodology for Assessment of Folin-Ciocalteu Reducing Capacity. Talanta. 83: 441-447.

Maya, S., Sabitha, M., Nair, S.V., Jayakumar, R. (2013). Phytomedicine-loaded polymeric nanomedicines: potential cancer therapeutics. In: Dutta, P.K., Dutta, J. (Eds.), Multifaceted Development and Application of Biopolymers for Biology, Biomedicine and Nanotechnology. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp. 203–239.

Neha, Pandey and Dushyant, Barve (2011). Phytochemical and Pharmacological Review on *Annona squamosa* Linn. International Journal of Research in Pharmaceutical and Biomedical Sciences. 2(4); 1404-1412.

Nurain, A., Noriham, M.N. Zainon, W.S.K. Wan Saidatul and S.Z. Khairusy, (2013). Comparative Study of Aqueous and Ethanolic Aromatic Malaysian Herbs Extracts Using Four Antioxidant Activity Assays. *International Journal of Agricultural Research.* 8: 55-66.

Pardhasaradhi, B.V.V., Reddy, M., Ali, A.M., Kumari, A.L., Khar, A., 2005. Differential cytotoxic effects of Annona squamosa seed extracts on human tumour cell lines: role of reactive oxygen species and glutathione. J Biosci 30 (2), 237–244.

Parvin, ShahnajMst, Islam Ekramul M, Rahman MotiurMd and Haque, Ekramul, Md (2003). Toxicological evaluation of Annotemoyin-1 isolated from Annona squamosa from long evans Rats. Pakistan Journal of Biological Sciences, 6(18): 1593-1596.

Pejin, B., Kojic, V., Bogdanovic, G., 2014. An insight into the cytotoxic activity of phytol at in vitro conditions. Nat. Prod. Res. 28 (22), 2053–2056.

Prasad, M.P., Balamurugan, P. and Rajkumar, A.R. (2014). Comparative Phytochemical Analysis of Rutaceae Family (Citrus Species) Extract. International. *Journal of Scientific Research*. 3(4): 2277-8179.

Rawan, Al-Nemari, Abir, Ben, Bacha, Abdulrahman Al-Senaidy, Mikhlid, H., Almutairi, MahaArafah, Hadel Al-Saran, Nael, Abutaha and Abdelhabib, Semlali (2022). Cytotoxic effects of Annona squamosa leaves against breast cancer cells via apoptotic signaling proteins. *Journal of King Saud University* – Science 34; 102013.

RibaVikas, Akhil B.S, Remani P., and K., Sujathan (2017). Free Radical Scavenging Properties of Annna squamosa. Asian Pac. J. Cancer Prev. 18(10): 2725-2732.

Saad, R., Khan, J., Krishnanmurthi, V., Asmani, F., & Yusuf, E. (2014). Effect of Different Extraction Techniques of Persicariaodorata Extracts Utilizing Anti-bacterial Bioassay. *British Journal of Pharmaceutical Research*, *4*(18).

Semlali, A., Chakir, J., Rouabhia, M., 2011. Effects of whole cigarette smoke onhuman gingival fibroblast adhesion, growth, and migration. J. Toxicol. Env. Heal.A 74 (13), 848–862.

Seyfried, T.N., Huysentruyt, L.C., 2013. On the origin of cancer metastasis. Crit. Rev. Oncog. 18 (1 - 2), 43–73.

Singleton, VL, Orthofer, R., Lamuela-Raventos, RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299:152-78.

Sobiya R, Jannet DV, Aiyavu J., and Panneerselvam, C. (2009). The heapatoprotective effect of alcoholic extract of Annona squanmosa leaf on experimentally induced liver injury in Swiss albino mice. International Journal of Integrative Biology, 5(3): 182-186.

Soni H, Malik SS and Singhai AK (2012). Evaluation of leaf of aqueous extract of MeOH extract of Aannon squamosa on cell viability American. J. Pharm. Tech. Res., 2(4): 936-944.

Suhailah W. Q., Mahmood A., Abdulla, Lee Suan Chua , Nigar N., Mazatulikhma Mat Zain and Salehhuddin H. (2011). Antioxidant, Total Phenolic Content and Cytotoxicity Evaluation of Selected Malaysian Plants. Molecules; 16, 3433-3443; doi:10.3390/molecules16043433.

Tepe, BD, Daferera D., Sokmen, A., Sokmen, M., and Polissiou, M. (2005). Antimicrobial and antioxidant activities of the essential oil and various extracts of Salvia tomentosa Miller (Laiaceae). J Food Chem ;90:333-40.

Valiyari, S. (2012). Dichloromethane and Methanol Extracts of Scrophulariaoxysepala Induces Apoptosis in MCF-7 Human Breast Cancer Cells. Advanced Pharmaceutical Bulletin. 2(2): 223.

Wang, D.S., Rizwani, G.H., Guo, H., Ahmed, M., Ahmed, M., Hassan, S.Z., Hassan, A., Chen, Z.S., Xu, R.H. (2014). Annona squamosa Linn: cytotoxic activity found in leaf extract against human tumor cell lines. Pak. J. Pharm. Sci. 27, 1559–1563.