

## INVESTIGATION THE CYTOTOXICITY AND APOPTOSIS OF ANNONACEAE NANOPARTICLES AND 5-FU ENCAPSULATED CHITOSAN NANOPARTICLES AGAINST TONGUE SQUAMOUS CELL CARCINOMA CELL LINES (SSC-25)

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### ABSTRACT

**Objective:** cancer result from abnormal genetic makeup of a cell resulting in continuous cell division with often production of metastasis throughout the body. Apoptosis is very effective in killing cancer cells. We performed this study to investigate the effect of annonaceae and 5FU drug nanoparticles against scc-25 cell lines human tongue squamous cell carcinoma by studying their effect on cell cycle progression and apoptosis

**Methods:** Cell Line cells were obtained from American Type Culture Collection , cells were cultured using DMEM (Invitrogen/Life Technologies). Annonaceae nanoparticles and 5FU drug were used. The effect of the tested materials were investigated by MTT assay, cell cycle analysis & flow cytometry.

**Results:** the cancer cells treated by annonaceae nanoparticles revealed obvious cytotoxicity at  $iC50$   $7 \mu\text{g/ml}$ , in addition it stimulated the upregulation of annexin-v leading to apoptosis of cancer cells and growth arrest at G1 phase with prominent late apoptosis analyzed by cell cycle analysis & flow cytometry.

**Conclusion:** Our findings demonstrate that annonaceae nanoparticle might be capable of inducing cancer cell death against SCC-25 cell lines by cell cycle arrest and induction of apoptosis.

### INTRODUCTION

Cancer is group of cells developing from abnormal genetic makeup of a cell resulting in uncontrolled cell division. The cancer cells reveal aberrant cell growth, not responding to inhibitory growth signals with often production of metastasis

throughout the body <sup>[1]</sup>

One of the effective strategies to kill cancer cells is induction of apoptosis, a programmed cell death mechanism for removing unwanted cells in tissue <sup>[2]</sup>. This procedure is mainly associated with morphological change, heterochromatin

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condensation, cell shrinkage and budding in addition to loss of organelles in the cytoplasm, and formation of apoptotic bodies<sup>[3]</sup>.

Therefore, continual research of natural compounds having the power of induction of apoptosis pathways in cancer cells is extremely important and becoming more of interest in the field of oncology<sup>[4]</sup>.

Many medicinal plants have been subjected to scientific studies where their secondary metabolites/bioactive compounds are discovered to have the anticancer effect. *Annona muricata* which belongs to the Annonaceae family, native to some central and south America. It is an oval fruit with green skin and it contains black seeds involved in a white pulp. This plant has been used in traditional medicine to treat different conditions like fever, rheumatism, cancer, and also as sedative and immunosuppressive activity.<sup>[5]</sup>

Intensive research on the chemical composition of the annonaceae leaves<sup>[6]</sup> and seeds<sup>[7]</sup> lead to the finding of acetogenin compounds which explains its therapeutic effects. Acetogenin (ACG) is characterized by its unbranched C32 or C34 fatty acid with a  $\gamma$ -lactone at the end of the cytoskeleton<sup>[8]</sup>. This molecular structure is a very potent compound against cancer as it deprives the highly energy demanding cancer cells from adenosine triphosphate (ATP) supply via the disruption of mitochondrial electron transport system, hence resulting in apoptosis<sup>[9]</sup>.

Our aim in this study is to investigate the cytotoxic effect of annonaceae nano-particles and 5-fluorouracil nanoparticles against tongue cancer cell lines (scc-25) and evaluate their ability to induce cell cycle arrest and apoptosis in cancer cells.

## MATERIALS AND METHODS

Cell Line Scc-25 (human tongue squamous cell carcinoma). cells were obtained from American

Type Culture Collection, cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone), 10  $\mu$ g/ml of insulin (Sigma), and 1% penicillin-streptomycin. All of the other chemicals and reagents were from Sigma, or Invitrogen.

Cell culture protocol was done according to standard procedures at *confirmatory diagnostic unit, VACSERA-Egypt*. . Plate cells (cells density 1.2 – 1.8  $\times$  10,000 cells/well) in a volume of 100 $\mu$ l complete growth medium + 100 ul of the tested compound per well in a 96-well plate for 24 hours before the MTT assay .

The cultures were incubated s at 37°C for 24 hrs. After treatment of SCC-25 cells with the serial concentrations of the nano- annonaceae and 5FU drug (100, 25, 6.25, 1.56 and 0.39 ug/ml ) . Incubation is carried out for 48 h at 37°C, then the plates are to be examined under the inverted microscope and proceed for the MTT assay.

## Preparation of Nanoparticles Containing Annona Muricata Leaves Extract Using Gelation Ionic Method

### Plant materials

The voucher specimen was leaves powder of *Annona muricata* code E536, Origin Peru.

### Chemical materials

2,2-diphenyl-1-picrylhydrazil (DPPH) (Sigma Aldrich), poly (acrylic acid) (PAA) (Sigma-Aldrich), calcium chloride, ascorbic acid, dimethyl sulfoxide (DMSO), propylenglycol.

### Nanoparticles preparation

*Annona Muricata* and 5FU nanoparticles were prepared at *National research center, Egypt*. The plant extract was used to develop nano-suspension with poly acrylic acid (PAA) and calcium chloride. PAA solution was used at a concentration of 0.05% (in water) with NaOH to pH 8 and 0.1% calcium chloride solution in water.<sup>[10]</sup>.

The stability of nanoparticles was observed for 5 days in term of color, turbidity and sediment. Evaluation of nanoparticle included particles size using a Malvern

Particle sizer and examination of the zeta potential using a Malvern zeta potential measuring device. Furthermore, the nanoparticles plant suspension was dried using spray drier with inlet temperature of 190 0C and outlet temperature of 90°C. The evaluation of the dry powder of nanoparticles *soursop* (*A. muricata*) included organoleptic, moisture content and examination of dried nanoparticle morphology using Scanning Electron Microscopes (SEM).<sup>[11]</sup>

Evaluation	Nanoparticle Suspension
Yellow	Color
Transparent	Opacity
none	Sediment
nm 558.4	Particle size
mv 27.07	Potential zeta

### Fluorouracil Encapsulated Chitosan Nanoparticles preparation

Chitosan, derived from crab shell, in the form of flakes was purchased from Sigma-Aldrich (Medium Molecular Weight, Catalogue no. 448877).

The degree of deacetylation and molecular weight for the medium-molecular-weight chitosan (MWM chitosan) is 75–85% and 190–310 kDa based on viscosity, respectively.<sup>[12,13]</sup>

Sodium tripolyphosphate (TPP) (purity: 85%), 5-fluorouracil (5-FU) (purity: 99%), and phosphate-buffered saline (PBS) tablets (pH 7.4), were maintain chitosan concentration at 0.75 (mg/mL). Prepared chitosan solutions were mixed with 5-FU solutions (5-FU dissolved in water), and 1.0mg/mL 5-FU containing chitosan solutions were maintained. Tween 80 (Sigma,Germany) (0.5% (v/v)) was added to chitosan solutions, and pH was arranged as 4.6–4.8. Prepared 5-FU-containing chitosan solutions were flushed mixed with 0.5mg/mL TPP solutions with a ratio of volume ratio of

(2 : 1) (v/v) (chitosan : TPP). The nanoparticle suspension was gently stirred for 20 min at room temperature to allow excess 5-FU adsorption on the nanoparticles to reach isothermal equilibrium. 5-FU encapsulated chitosan nanoparticles were centrifuged at 12000 g for 30 min, resuspended in water and freeze-dried used for further analyses. Prepared 5-FU encapsulated chitosan nanoparticles were analyzed by Zetasizer Nano S (Malvern,UK) in order to determine mean average particle size distributions.<sup>[14]</sup>

All nanoparticle solutions used for the present study were freshly prepared and sonicated before treatment at *confirmatory diagnostic unit, VACSERA-Egypt*. Nanoparticles were sterilized with UV exposure prior to the treatment

### Cytotoxicity assay protocol (MTT)

The MTT method of monitoring in vitro cytotoxicity is a mean to measure the activity of living cells by mitochondrial dehydrogenases. The key component is (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions<sup>[15]</sup>. The crystals are dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured.<sup>[16]</sup> An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material using BIOLINE ELIZA READER. *IN VITRO TOXICOLOGY ASSAY KIT MTT BASED Stock No. TOX-1. 7H258*, Sigma-Aldrich, Inc. the MTT assay was done according to the standard procedure at *confirmatory diagnostic unit, VACSERA-Egypt*. Spectrophotometrically measure absorbance at a wavelength of 570 nm for spectrophotometric measurement using BIOLINE ELIZA READER.

**Cell cycle analysis:**

Cell cycle analysis was done by BD FACS Calibur (Becton Dickinson, USA). The Kit used for enzyme-assay: ab139418\_Propidium Iodide Flow Cytometry Kit/BD using Solvent DMSO following the standard procedure at *confirmatory diagnostic unit, VACSERA-Egypt*.

**Statistical analysis**

Statistical analysis was performed using statistical analysis program (SPSS, 16.0, International Business Machines, USA). Comparisons between groups (controls and treatments) were performed by student t-test and one-way ANOVA test. Statistical significance was accepted at P value lower than 0.05.

**RESULT**

**MTT assay**

The MTT cytotoxicity assay revealed that annonaceae nanoparticles have highly significant cytotoxicity on SSC-25 cancer cell lines at IC50 7.64  $\mu\text{g/ml}$ , as well as nano-5FU drug revealed highly significant cytotoxicity on SSC-25 cancer cell lines at IC50 2.005  $\mu\text{g/ml}$  as shown in (table1) fig. (1,4).

TABLE (1) Showing the IC50 values of annonaceae nanoparticles and 5fu drug against tongue carcinoma cell line (scc-25). Level of significance set at  $p < 0.005$ . student t-test

Ser	Sample code	IC50 $\mu\text{g/ml}$
		SCC25
1	<i>annonaceae</i>	7.64 $\pm$ 1.94
2	5FU drug	2.005 $\pm$ 0.11
	p-value	P < 0.005

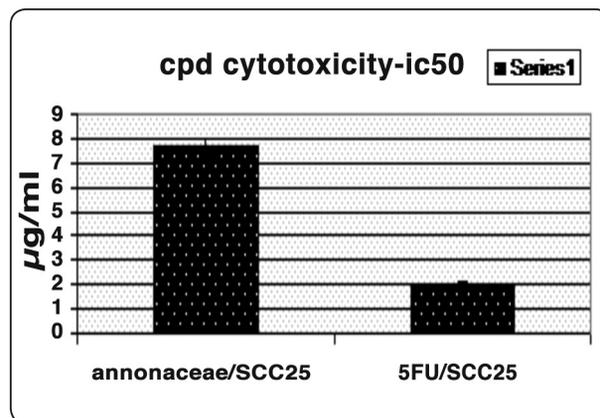


Fig.(1): Bar chart showing comparison of cytotoxicity-ic50 value of annonaceae nanoparticles and 5fu drug against tongue carcinoma cell line (scc-25).

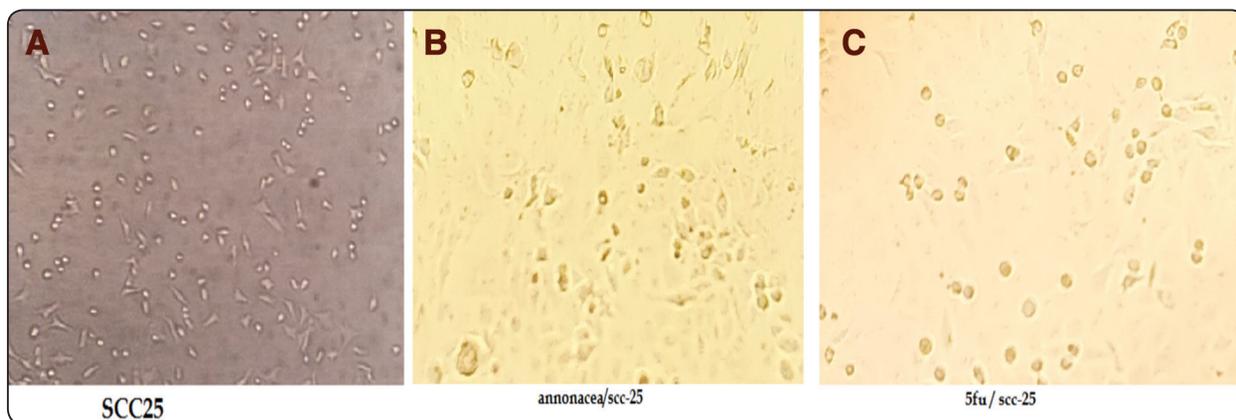


Fig. (2) cell culture images by inverted microscope. A. SCC-25 cell line before treatment. B. SCC-25 when treatment with **annonacea** nanoparticles after 48hs incubation. C. SCC-25 when treated by 5FU nanoparticles after incubation period 48hs.

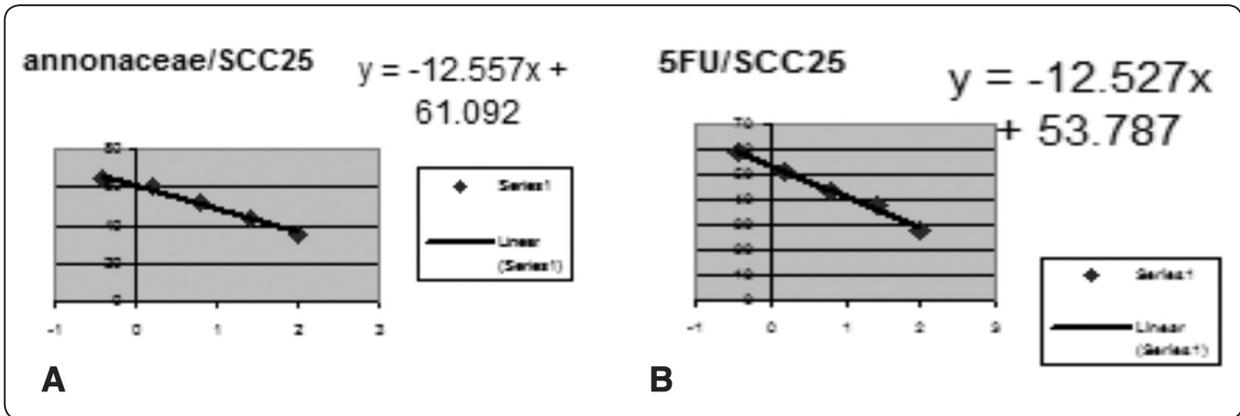


Fig. (3) A- graph demonstrate an inverse linear relation between the serial nano-annonaceae concentrations and cell viability of cancer cells (SCC-25). B- graph demonstrate an inverse relation between the serial concentrations of nano-5FU and cell viability of cancer cells (SCC-25).

In the current study an inverse relation between the serial nano-annonaceae concentrations and nano-5FU drug on cell viability of tongue cancer cells (SCC-25 as shown in fig (3) revealed by BIOLINE ELIZA READER.

TABLE (2) Showing highly significant difference between control untreated cancer cells and the treated cancer cells with either nano-annonaceae or nano-5FU drug ANOVA test.

Groups	Control	annonaceae nano	5FU	p-value (ANOVA)
mean± SD	0.557 ±1.866	0.288± 1.60	0.282± 1.46	0.005

**CELL CYCLE analysis**

The cell cycle analysis revealed accumulation of large number of cancer cells at G1 phase showing growth arrest by 54.15 % when treated by annonaceae nanoparticles at IC50 7.64 µg/ml. In addition apoptosis of 17% of cancer cells at pre G1 Phase as shown in table (3) fig. (5).

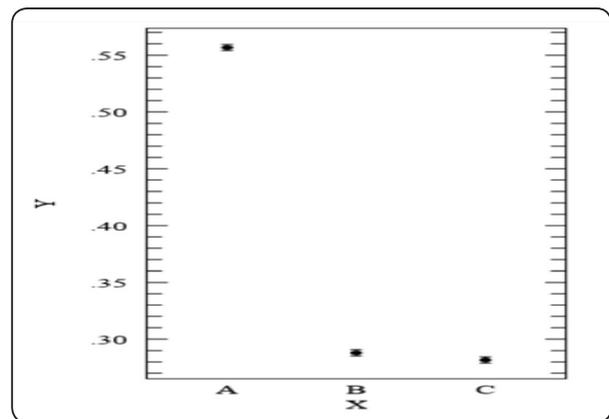


Fig. (4) plot graph showing the cytotoxic effect of nano-annonaceae (B) and nano- 5FU drug (C) on cancer cell viability in comparison to control group (A). ANOVA test with plot output variance.

The 5FU drug nanoparticles revealed growth arrest of 68 % of cancel cells at G1 phase when treated at IC50 2 µg/ml. In addition apoptosis of 24.41% of cancer cells at pre G1 Phase as shown in table (3) fig. (5).

On the contrary, the control untreated cancer cells revealed growth arrest of 41.33 % of cancel cells at G1 phase and apoptosis of 1.86% of cancer cells at pre G1 Phase as shown in table (3) fig. (5).

TABLE (3) Showing cell cycle arrest of treated cancer cells (scc-25) by the tested materials

Sample data			Results				
Ser	Sample code	Used conc. $\mu\text{g/ml}$	%G0-G1	%S	%G2/M	%Pre-G1	Comment
1	Annonaceae	7.64 $\mu\text{g/ml}$	54.15	39.46	6.39	17.11	Pre G1apoptosis & Cell growth arrest at G1
2	5FU	2.005 $\mu\text{g/ml}$	68.26	28.62	3.12	24.41	Pre G1apoptosis & Cell growth arrest at G1
3	Control		41.33	46.58	12.09	1.86	Growth arrest at G1

TABLE (4) Showing highly significant difference between control untreated cancer cells and the treated cancer cells with either Annonaceae nano-particle or 5FU in reference to cell cycle arrest at G1 phase ANOVA test.

Groups	Control	Annonaceae nano	5FU	p-value (ANOVA)
mean $\pm$ SD	41.33 $\pm$ 1.80	54.15 $\pm$ 2.40	68.26 $\pm$ 2.20	0.000

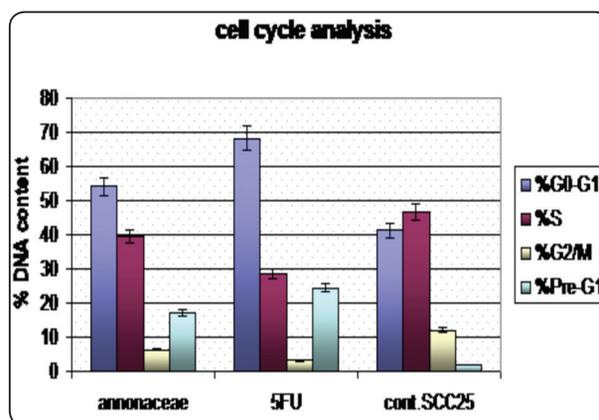


Fig. (5) Bar chart showing more growth arrest of cancer cells at G0-G1 phase of cell cycle 68.26 .

### Apoptosis analysis

The annonaceae nanoparticles at 7  $\mu\text{g/ml}$  induced apoptosis of 17% of cancer cells showing more late apoptosis of 9 % of cancer cells and less necrosis of cancer cells table (5) (fig7,8 ).

The 5FU drug at IC50 2  $\mu\text{g/ml}$  induced apoptosis of 24.4 % of cancer cells showing more late apoptosis of 14.6 % of cancer cells and more necrosis of cancer cells when compared to anonaceae noanoparticles table (5) (fig 7,8 ). While, the non-treated tongue cancer cells Scc-25 revealed minimal apoptosis of 1.89 % of cancer cells table (5) (fig 7,8 ).

In the current study, an important observation was found, the nano-5FU drug was significantly cytotoxic at low value IC50 2 $\mu\text{g/ml}$  against scc-25

with prominent apoptotic effect as shown in table (5) fig (6,7) more than annonaceae nanoparticles which revealed prominent significant cytotoxic and apoptotic effect at value IC50 7 table (5) fig. (6,7) while, annonaceae nanoparticles showed significantly less necrosis of scc-25 in comparison to5FU drug table (6) fig.(6, 8)

### Flow cytometry analysis:

The images revealed that when the cancer cells treated by annonaceae nanoparticles at concentration 7  $\mu\text{g/ml}$  and nano-5FU drug at 2 $\mu\text{g/ml}$  stimulated apoptosis through upregulation of annexin-v resulting in apoptosis of cancer cells and growth arrest at G1 phase with prominent late apoptosis analyzed by cell cycle analysis & flow cytometry. (fig.8).

TABLE (5) showing the apoptotic effect values of annonaceae nanoparticle and 5FU drug against SCC-25 tongue cancer cell lines.

		conc. μg/ml	Apoptosis			Necrosis
			Total	Early	Late	
1	<i>annonaceae</i>	<b>7μg/ml</b>	17.11	5.81	9.16	2.14
2	5FU	<b>2μg/ml</b>	24.41	6.29	14.65	3.47
3	cont		1.86	1.04	0.29	0.53

TABLE (6) Showing highly significant difference between Annonaceae nano-particle and nano-5FU in reference to degree of inducing necrosis against the treated cancer cells. Student t-test.

Groups	Necrosis of cancer cells	annonaceae nano	5FU	p-value (t-test)
mean±SD			2.14±1.41	3.47±4.00

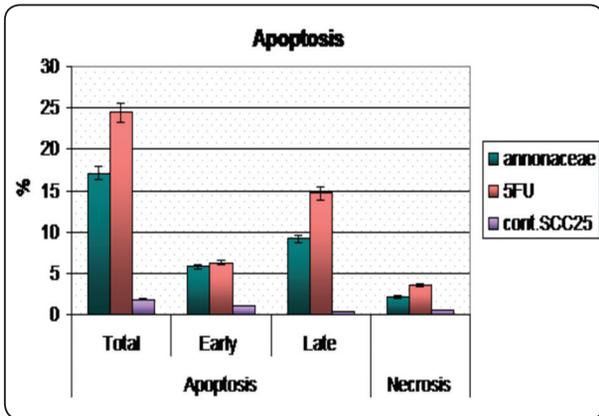


Fig. (6) bar chart showing the prominent apoptotic effect of annonaceae nanoparticles and 5FU drug against SCC-25 tongue cancer cell lines.

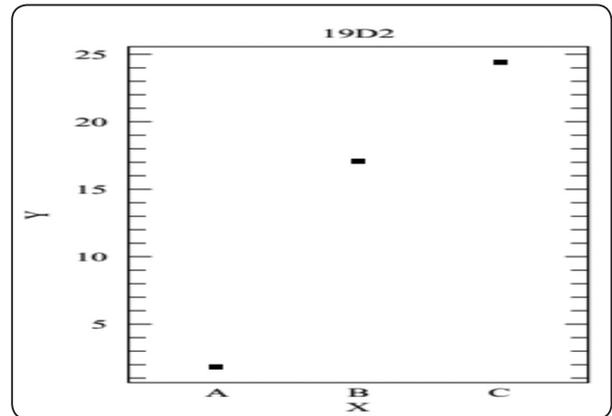


Fig.(7) plot graph showing the significant apoptotic effect of annonaceae nanoparticles (B) and nano-5FU drug (C) on cancer cells in comparison to control group (A), ANOVA test with plot output variance.

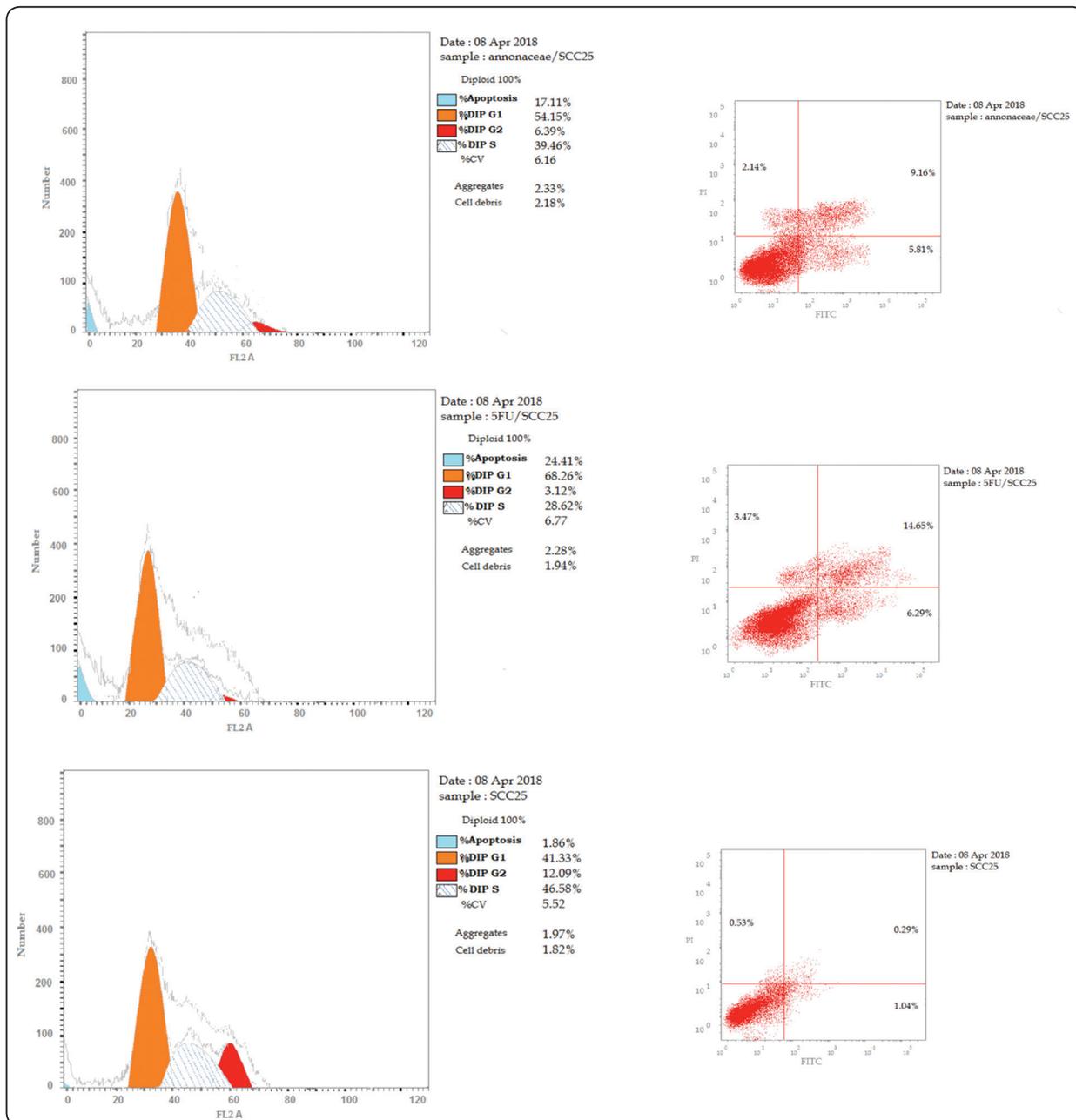


Fig (8) flow cytometry plot and cell cycle analysis of Annonaceae NPs and 5fu NPs against scc-25 cell line. A- The annonaceae NPs at 7  $\mu\text{g/ml}$  induced apoptosis of 17% of cancer cells showing more late apoptosis of 9 % of cancer cells and less necrosis of cancer cell. B-5FU NPs at IC50 2  $\mu\text{g/ml}$  induced apoptosis of 24.4 % of cancer cells showing more late apoptosis of 14.6 % of cancer cells. C- the non-treated tongue cancer cells Scc-25 revealed minimal apoptosis of 1.89 % of cancer cells

## DISCUSSION

Studies of compounds from herbal plants to discover new treatment regimen for cancer with minimal harmful side effect are of great interest. According to a WHO report (2002), 60% of medicinal drugs are isolated from natural sources, including anti-cancer drugs<sup>[17]</sup>

The main finding of this investigation is that annonaceae nanoparticles have significant ( $p < 0.005$ ) cytotoxicity on SSC-25 cancer cell lines at IC<sub>50</sub> 7.64  $\mu\text{g/ml}$  when compared to the control untreated cancer cells at incubation period 48 hours. This could be considered a very promising finding since the US Food and Drug Administration (FDA) and national cancer institute USA, considered that plant extract with IC<sub>50</sub> values  $\leq 20 \mu\text{g/ml}$  following incubation between 48 and 72 h, are considered pharmaceutically active can be used as bench mark for suitable screening cancer drugs from plants and herbs<sup>[18]</sup>.

Moreover, the cell cycle analysis revealed accumulation of large number of cancer cells at G1 phase showing growth arrest by 54.15% when treated by annonaceae nanoparticles at IC<sub>50</sub> 7.64  $\mu\text{g/ml}$ . In addition apoptosis of 17% of cancer cells at pre G1 Phase, showing more late apoptosis of 9% of cancer cells and less necrosis of cancer cells by 2.14 %.

The previous observation in accordance with different studies who revealed that Annonaceae family contain Certain types of alkaloids, such as jerantinine B<sup>[19]</sup>, liriiodenine<sup>[20]</sup>, and vinoreline<sup>[22]</sup>, exhibit the ability to induce apoptosis and block the cell cycle in the G1 phase and inducing apoptosis by mitochondria membrane potential disruption.

The same result was obtained by constant Anatole pieme et al., 2014, They revealed that *Annona muricata* (annonaceae) extracts induced G0/G1 cell cycle arrest in HL-60 cancer cell lines at different concentrations with IC<sub>50</sub> 6-12  $\mu\text{g/ml}$  after

24 hours of treatment. They noticed marked G0/G1 cell cycle arrest however low effect on G2/M phase. They concluded that *A. Murita* might have strong anti-proliferative effect through G0/G1 cell cycle arrest and induction of apoptosis through disruption of membrane mitochondrial potential causing release of apoptotic factors result in cell cycle arrest at G0/G1 phase and inhibiting cell proliferation<sup>[23]</sup>.

Fluorouracil (also referred to as 5-fluorouracil, 5fu), which is a medication for cancer chemotherapy therapy, is a structural analog of thymine that blocks the enzyme thymidylate synthetase to depress the formation of DNA<sup>[24]</sup>. Fluorouracil has been one of the most widely used chemotherapy drugs in treating solid organ tumors, including tumors of the colon, rectum, breast, pancreas, and stomach; however, its common side effects are gastrointestinal symptoms, alopecia, cardiotoxicity, neutropenia, dermatitis, cardiac toxicity, and damage of the central nervous system limit its applications<sup>[25][27]</sup>.

In the present study, the MTT assessment revealed that 5fu nanoparticles showed significant ( $p < 0.005$ ) cytotoxicity on SSC-25 cancer cell lines at IC<sub>50</sub> 2.005  $\mu\text{g/ml}$  when compared to the control non-treated cancer cells. In addition to cell cycle analysis by flow cytometry, revealed growth arrest of 68 % of cancel cells at G1 phase when treated by 5FU nanoparticles, moreover, apoptosis of 24.41% of cancer cells at pre G1 Phase, showing more late apoptosis of 14 % of cancer cells and necrosis of cancer cells by 3.47 % compared to non-treated cancer cells Scc-25.

A previous study conducted by shaimaa M. Masloub et al, 2016. showed the same previous observation, they revealed that 5FU nanoparticles showed a prominent cytotoxic effect on Hep-2 cell lines compared to non- treated cancer cells utilizing MTT assay, in addition to the quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assessment of caspase-3 expression, revealed a significant increase in caspase-3 expression in

Hep-2 cell line when treated by 5FU nanoparticles compared to non-treated cancer cells <sup>[27]</sup>

In the current study, an important observation was found, the 5FU nanoparticles were significantly cytotoxic at low value IC<sub>50</sub> 2 µg/ml against scc-25 with prominent apoptotic effect more than annonaceae nanoparticles which revealed prominent significant cytotoxic and apoptotic effect at value IC<sub>50</sub> 7 compared to non-treated tongue cancer cells Scc-25. While, annonaceae nanoparticles showed significantly less necrosis of scc-25 cells in comparison to 5FU nanoparticles.

The previous observation may produce new insights for replacing chemotherapeutic drugs like 5fu drug with herbal extracts as promising as Annonaceae Murita nanoparticles for treatment certain types of cancer as oral tongue cancer. This might reduce the unwanted side effects of chemotherapeutic drugs and make the treatment procedure less suffering to the patient with cancer.

## CONCLUSION

The annonaceae nanoparticle might be capable of inducing cancer cell death against SCC-25 cell lines by cell cycle arrest and induction of apoptosis.

## RECOMMENDATION

Further studies should be performed to analyze the major bioactive compounds of annonacea extracts as well as investigation their effect on molecular level of cell pathways and it should be screened against more cancer cell lines for the potential use of this plant for chemotherapy in the future.

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## Compliance with ethical standards

Conflicts of interest. The authors declare that they have no conflict of interest.

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