Original Article

# Investigation of the Apoptotic Effect of Pumpkin seed oil (Cucurbita Pepo l.) loaded Chitosan nanoparticles on tongue squamous cell carcinoma cell line (scc-25): In vitro study.

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## Abstract

**Background**: Researchers are continuously working on formulating anticancer drugs of plant origin for treatment of oral cancer. Pumpkin seed oil is one of them. The use of nanoparticles as a drug delivery system has shown great success as it provides more selectivity and better drug efficiency.
**Aim:** The following study was performed to determine whether the pumpkin seed oil (Cucurbita pepo L.) loaded Chitosan nanoparticle (NPSO_CH) has a cytotoxic and apoptotic effect on tongue squamous cell carcinoma cell line or not.
**Methodology:** After obtaining the SCC-25 (tongue squamous carcinoma) cells, cell culture protocol was done according to standard procedures. The cultures were treated with the serial concentrations of the Nano chitosan loaded pumpkin seed oil and Taxol drug, after that incubation is carried out for 48 h at 37ºC.
Chitosan/pumpkin seed oil nanoparticles were prepared using ionotropic gelation at room temperature. Caspase 9 enzyme essay was carried out using ROBONIK P2000 ELISA READER wl 450 nm.
**Results:** Results of MTT cell cytotoxicity assay showed that there is an inverse relation between the serial NPSO_CH concentrations and Taxol drug on cell viability of tongue cancer cells (SCC-25). IC50 of NPSO_CH/SCC25 was 22.0±0.86 µg/ml while that of Taxol/SCC25 was 6.28±0.29 µg/ml. The caspase 9 enzyme assay revealed a significant increase of caspase 9 expression values after treatment of cancer cell lines SCC-25 with NPSO_CH and Taxol (18.63, 25.94 pg/ml) respectively, in comparison to the untreated control cancer cells line SCC-25(2.49 pg/ml) .
**Keywords:** : Pumpkin seed oil loaded chitosan nanoparticles (NPSO_CH), Taxol, SCC-25

### Introduction

According to a study made by Omar Kujan, Camile S Farah and Newell W Johnson in 2017 on the incidence and mortality of oral and oropharyngeal cancer in the middle east and north African region. They reported that OCC-OPCs is one of the top 20 cancers in the MENA area. Moreover, a relatively high number of oral cancer cases in the MENA region are discovered when they become symptomatic (a single lump or ulcer or as a red or white lesion), at a late stage and thus, poor prognosis and survival outcomes (Kujan, Farah and Johnson, 2017). The World Health Organization (WHO) has assumed that the Eastern Mediterranean

area (a larger definition of MENA that includes Afghanistan and Pakistan) will have the highest elevation in cancer occurrence in all WHO areas in the coming 15 years. It has been estimated that more than 270,000 people lose their life of cancer each year in the middle eastern territory (Kujan, Farah and Johnson, 2017).

The most commonly used treatments for oral cancer are surgery, chemotherapy and radiotherapy. The problem with these treatment modalities is their lack of selectivity, they destroy malignant cells as well as normal cells, leading to undesirable side effects and complications. There are other treatment modalities that are being used in intention to improve the patient's quality of life which include: Monoclonal antibodies (Adept Antibody Directed Enzyme Pro-drug Therapy), photodynamic therapy, Iressa, Thalidomide or the use of natural plant extract components, like green tea tomatoes and others (Murnal, no date).

Researchers have been determined to develop inventive drug formulas based on bioactive natural products as a functional substitute to the commonly used chemotherapy. Pumpkins (Cucurbit sp.) belonging to the Cucurbitaceae family are commonly cultivated all over the world as a vegetable (Duse et al., 2018).

There is an increasing interest in vegetable oils of particular constitution and nutritional values and since pumpkin seed oil is a good candidate in this regard, it is extracted by cold press or steam distillation (Patel, 2013). Pumpkin seed oil is believed to have many benefits including: antioxidant effect, hypolipidemic effect, antimicrobial effect, heart protective effect and anticarcinogenic effect (Syed, Akram and Shukat, 2019). Due to the presence of carotenoids, tocopherols, and sterols, many studies were made to discover the anticarcinogenic action of pumpkin seed oil on various forms of cancers as prostate cancer and breast cancer (Amin et al., 2019).

Nanoparticles can be defined as ultra-dispersed solid supramolecular structures with a sub micrometer size ranging from 10 to 1,000 µm." " Nanotechnology depend on the potential to characterize, control, and arrange materials on a nanoscale, which acquires the products features and behaviors different to those present at the larger scale"(Wijayadi and Rusli, 2019).

NPs can improve the steadiness of drugs and control their targeted delivery, allowing for a fixed and consistent concentration at the site of a lesion and easing drug extravasation into the tumor system, leading to reduction of side effects (Duse et al., 2018). Chitosan nanoparticles are drug carriers that have the privilege of slow & controlled drug effect, which makes drug solubility better, enhancing efficacy, and reducing its toxic effect.

Since nanomaterials might behave diversely when compared to bulk material, the objective of this research was conducted to examine the anticancer effect of pumpkin seed oil in nanoform utilizing Chitosan nanoparticles against oral tongue squamous cell carcinoma cell line (SCC-25). Especially as far to our knowledge, few studies have been made to test pumpkin seed oil (PSO) against head & neck cancer in the available English literature. This study would be considered the first to assess NPSO_CH against oral tongue squamous cell carcinoma in vitro.

## Materials and methods

### Study Design

Cell Line SCC-25 (human tongue squamous cell carcinoma) cells were acquired through American Type Culture Collection. Cells were sub-cultured to obtain 3 study groups, which were subjected to NPSO_CH/SCC-25 group, Taxol/SCC-25(as positive control) and negative control group (untreated SCC-25) not subjected to any compounds. First the chitosan/pumpkin seed oil NPs were prepared, then the NPs were assessed by TEM then the tested compounds were investigated by MTT viability assay followed by IC50 calculation.

The apoptosis analysis was measured by active caspase-9 expression using ROBONIK P2000 ELISA READER wave length 450 nm.

**Preparation of materials:**

**1.Preparation of plant material:**

Pumpkin seeds (Cucurbita Moschata, L. Family Cucurbitaceae) were acquired through the local market, Cairo, Egypt. pumpkin seeds were dried in an oven at 40 °C and transformed into powder. For construction of PSO, a measured weight of the dried Pumpkin seeds powder was deposited in an extraction apparatus (Soxhlet) and exposed to extraction using petroleum ether (40-60ºC) to develop the oil. The solvent was totally withdrawn by evaporation under reduced pressure at a temperature not passing 40°C utilizing motor driven evaporator. The whole procedure was done at National Research Center (NRC), Cairo Egypt (Sahar et al., 2016).

**2.Preparation of Taxol**

The commonly used chemotherapeutic Taxol was supplied in vials 100 mg (16.7 mL) obtained from Bristol-Myers Squibb Company Princeton, NJ 08543 USA. Taxol was used in serial dose concentration of 100, 10, 1, 0.1 and 0.01 µg/ml.

**3.Preparation of Chitosan/ pumpkin seed oil NPs using ionotropic gelation method:**

The NPSO_CH was prepared at Advance materials and Nanotechnology Department in the National Research Center (NRC), Cairo Egypt. The methodology to formulate the NPSO_CH in the current study was developed by ionotropic gelation method which revealed very good efficacy for targeted drug-delivery applications (Desai 2016).

Chitosan nanoparticles were produced utilizing the ionotropic gelation method Chitosan mixture (1% (w/v)) was developed by mixing chitosan powder in distilled water (DW) containing 0.05M NaCl with 1% acetic acid (w/v), then stirred overnight using magnetic stirrer (300 rpm for 24 hrs) at room temperature (25 °C). CaCl2 solution (0.25% (w/v)) was prepared by dissolving CaCl2 in DW (Thakur and Thakur 2015).The pH of chitosan mixture was modified to 5.5 using 2 M NaOH solution. Afterwards, it was purified by 0.45-µm cellulose acetate filter to extract any unsolved chitosan. TPP (0.25 wt%) was liquefied in double-distilled water at a concentration of 0.25% by weight and purified through a 0.25-µm cellulose acetate filter. 60 mL Chitosan solution and pumpkin seed oil solution (10.0 mg) that were previously dissolved in (DCM) were mixed forming emulsion dispersion. The final loaded NPs were then prepared at room temperature using chitosan /CaCl2 mixture 15mL of chitosan -CaCl2 mixture solutions were added dropwise to the 60 mL chitosan /pumpkin seed oil emulsion under 550 rpm stirring for 30 min. The prepared NPs were rapidly rotated at 10000 rpm for 15 min, followed by washing several times by distilled water. Finally, the collected wet pellets were dispersed in DW and freeze dried at (-65 °C) for 72 hrs. The NPs were stored in a desiccator until further analysis.

**Cell line culturing**

Cell culture protocol was done at confirmatory diagnostic unit of the research and development (R&D) sector, VACSERA, Egypt. according to manufacturer's manual of continuous cell line cell culture ATCC protocol (http://www.atcc.org). cells in plate (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100µl growth medium + 100 ul of the studied agent per well in a 96-well plate for 24 hours before the MTT assay. Cells were cultured using DMEM (Invitrogen/Life Technologies) provided with 10% FBS (Hyclone,), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. Compounds were from Sigma, or Invitrogen.

Cultures were incubated at 37°C for 24 hrs. Afterwards, SCC-25 cells were exposed to the Nano chitosan loaded pumpkin seed oil and Taxol drug in serial concentrations of (100, 25, 6.25, 1.56 and 0.39 ug/ml). Incubation was done for 48 h at 37ºC, then the plates were observed under the inverted microscope and moved for the MTT assay and caspase-9 assay.

**Cell Culture Protocol**

All steps were performed in the laminar flow hood under complete aseptic conditions as follows:

1. Culture medium was removed to a centrifuge tube.

2. The cell layer was washed with 0.25% (w/v) Trypsin 0.53 mM EDTA mix to get rid of traces of serum that contains Trypsin inhibitor.

3. 2.0 to 3.0 ml of Trypsin EDTA mix was poured to the flask and cells were noticed under an inverted microscope until cell layer was distributed (within 5 to 15 minutes).

4. 6.0 to 8.0 mL of complete growth medium was added and mixed cells by gently pipetting.

5. The suspension was transferred to the centrifuge tube from step 1, and centrifuged at 125 xg for 5 to 10 minutes. The supernatant was shed.

6. The cell prill was suspended in fresh growth medium. Proper aliquots of the cell suspension were provided to new culture vessels.

7. Cells were sub cultured in plastic-96 well plates. Cultures were stored at 37°C for 24 hrs. ready for MTT assay and caspase enzyme assay.

**Cytotoxicity assay protocol (MTT)**

The MTT is a colorimetric assay in which mitochondria nicotinamide adenine dinucleotide phosphate- dependent cellular oxide-reductase enzymes reveal the number of active cells present. The reduction of MTT can happen in metabolically active cells, so the level of activity is a true measure of the viability of cells (Stockert et al., 2018) The mitochondrial reductase enzymes are able of changing the yellow soluble MTT stain to insoluble purple colored formazan. MTT solubilization mix is used to disintegrate the insoluble purple formazan by-product into colored mix. The extent of light absorption of this colored mix can be measured at certain wave length by a spectrophotometer (Wang, Yu and Wickliffe, 2011).

**Steps**

The cell viability test was performed in triplicates at cell culture unit- VACSERA, Giza. Egypt. According to the manufacturer's instruction manual of MTTA Assay with ELISA Reader Sigma-Aldrich protocol. All the steps were performed in the laminar flow hood under complete aseptic condition. The assay was incubated for 48 hours as upcoming:

Every test includes blank wells, containing medium without cells, their absorbance is to be subtracted from other measurements. Then treated test wells: NPSO_CH and Taxol with serial dilution (100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml & 0.01 µg/ml).

1.Cultures were taken from incubator into laminar flow hood.

2. Reconstitution of every vial of MTT [M-5655] was utilized with 3 ml of medium without phenol red and serum. Reconstituted MTT was applied in an amount equal to 10% of the culture medium volume.

3.Cultures were returned to incubator for 2-4 hours according to cell type and maximum cell density. (An incubation period of 2 hours is commonly adequate) Incubation times was consistent for making comparisons.

4.Afterwards, cultures were taken from incubator and the produced formazan crystals were diffused by adding an amount of MTT

Solubilization Solution [M-8910] equal to the original culture medium volume.

5. Gentle mixing was carried out in a gyratory shaker to promote dissolution. pipetting up and down was done totally liquefy the MTT formazan crystals.

6. Absorbance was detected utilizing spectrophotometrically at a wave length of 570 nm. The background absorbance was measured at 690 nm and subtracted from the 450 nm measurement.

**IC50 Value Calculation**

The half-maximal hindering concentration (IC50) represents concentration of a specific pharmacological agent which required to inhibit a given biological activity by 50% (Aykul and Martinez-Hackert, 2016). In the present study the results were interpreted and the IC50% value was calculated as follow:

1.the mean OD of each column of the 96-well was calculated by dividing the sum of OD of the column wells by the number of wells.

2.The mean OD of every column with its concentration treatment was divided by the mean of the control untreated cells to get the percent of viability (viability %) and cytotoxicity of each concentration treatment. The IC50 values for the current study are presented in table (1) & fig.(2 & 3).

**Caspase 9 assay**

Cells were provided from American Type Culture Collection, cells were expanded in RPMI 1640 with 10% fetal bovine serum at 37°C, subjected to the agents to be tested for caspase 9, and lysed with Cell Extraction Buffer. This preparation was adulterated according to Standard Diluent Buffer and detected for human active caspase-9 content. (Cells are Plated in a density of 1.2 – 1.8 × 10,000 cells/well in a volume of 100µl complete growth medium + 100 ul of the examined agent per well in a 96-well plate for 24 hours before the enzyme assay. Utilizing ROBONIK P2000 ELISA READER wave length 450 nm.

**Statistical analysis**

Data were statistically provided in terms of mean + standard deviation (+ SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of casp9 between the study groups was done utilizing one way analysis of variance (ANOVA) test with Tukey post hoc multiple 2-group comparisons. For comparing MTT between the 2 agents Student t test for independent samples. Two-sided p values less than 0.05 was considered statistically significant. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Window .

**Results**

1.Cell viability and MTT cytotoxicity measurements

The measurements of the two studied groups revealed that both NPSO_CH and Taxol treated groups demonstrated dose dependent reduction in cell viability when treated with increasing doses (from 0.01 up to 100 µg/ml). Comparing the two groups of the current study, the lowest value of % viability was shown by nan-Taxol followed by NPSO_CH at 48h were 30.28% and 36.09% respectively.

In the current study there is an inverse relation between the serial NPSO concentrations and Taxol drug on cell viability of tongue cancer cells (SCC-25) as shown in (fig. 1) revealed by BIOLINE ELIZA READER.

2.IC 50 results

The MTT cytotoxicity assay revealed that the lowest dose of nan- chitosan loaded pumpkin seed oil (NPSO_CH) to inhibit 50% of the cancer cells (SSC-25) was 22 µg/ml at 48h incubation period, meanwhile the least dose needed to do the same inhibitory reaction

on cancer cells was offered by Taxol ( 6.28 µg/ml ) at same incubation period 48 h. when comparing between the two intervention agents of the current study at the same duration (48 h), Taxol drug revealed highly significant lower dose in comparison to NPSO as shown in (table 1) &( fig.3 ,4 & 5 ).

3.Caspase 9 expression

The caspase enzyme assay using ROBONIK P2000 ELISA READER revealed prominent increase of caspase 9 expression values after treatment of cancer cell lines SCC-25 with NPSO and Taxol (18.63, 25.94 pg/ml) respectively when compared to the untreated cancer cells (SCC-25) which revealed caspase 9 expression value at (2.49 pg/ml)(fig.5&6). Taxol treated group revealed the highest value expression of caspase 9 over the NPSO_CH treated group denoting the superior potential of Taxol for apoptotic stimulation. All expression values of the different groups were measured after 48 hours incubation period.

Regarding comparison of the studied groups, ANOVA test revealed significant differences

between them and showing significant statistical increase of caspase 9 expression in Taxol and NPSO_CH groups (table 2). Tukey's post hoc test revealed that Taxol was highly significant from the other groups (table 3). In addition, there was statistically significant difference between NPSO_CH and the untreated group.

**Table (1)** Showing the IC50 values of NPSO and Taxol drug against tongue carcinoma cell line (scc-25). Level of significance set at $p<0.005$. Student t-test.

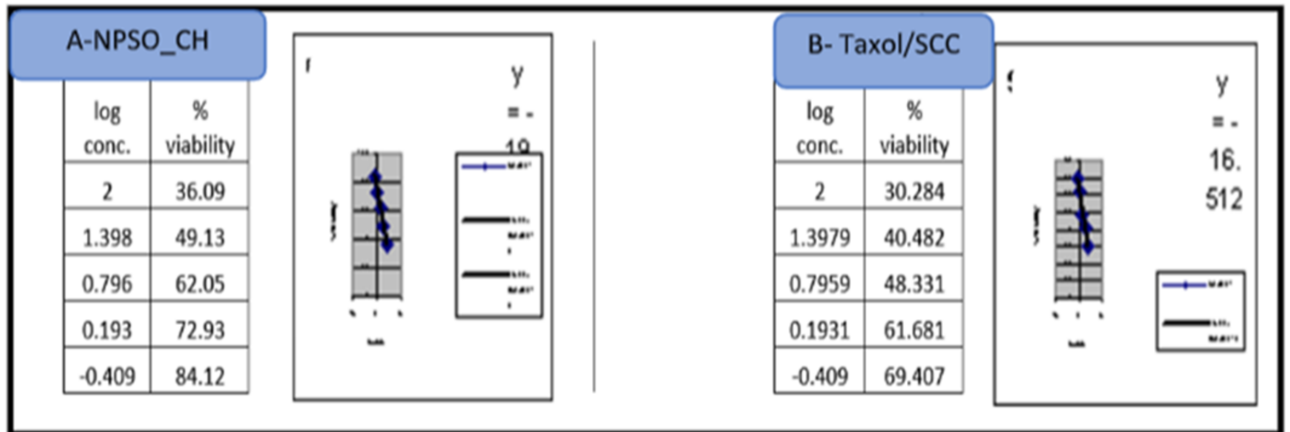
| **cytotoxicity**<br>**IC50 (µg/ml) results** | |
|---|---|
| **Intervention (48 h)** | **values** |
| NPSO_CH/SCC25 | 22.0±0.86 |
| Taxol/SCC25 | 6.28±0.29 |
| **P value** | **0.002** |

**Table (2)** Showing highly significant difference between caspase 9 expression in control untreated cancer cells and the treated cancer cells with either NPSO or Taxol using ANOVA test.

| | Sum of Squares | df | Mean Square | F | p value |
|---|---|---|---|---|---|
| Between Groups | 667.973 | 2 | 333.986 | 918.895 | 0.000 |
| Within Groups | 1.817 | 5 | 0.363 | | |
| Total | 669.790 | 7 | | | |

**Table (3)** Tukey HSD post hoc multiple 2-group comparisons

| (I) Group | (J) Group | Mean Difference (I-J) | Std. Error | p value | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Upper Bound | Lower Bound |
| Control | NSPO_CH/SCC25 | -16.1438(*) | 0.5504 | 0.000 | -17.935 | -14.353 |
| | Taxol/SCC25 | -23.4505(*) | 0.5504 | 0.000 | -25.241 | -21.660 |
| NSPO_CH/SCC25 | Control | 16.1438(*) | 0.5504 | 0.000 | 14.353 | 17.935 |
| | Taxol/SCC25 | -7.3067(*) | 0.4923 | 0.000 | -8.908 | -5.705 |
| Taxol/SCC25 | Control | 23.4505(*) | 0.5504 | 0.000 | 21.660 | 25.241 |
| | NSPO_CH/SCC25 | 7.3067(*) | 0.4923 | 0.000 | 5.705 | 8.908 |




| log conc. | % viability |
|---|---|
| 2 | 36.09 |
| 1.398 | 49.13 |
| 0.796 | 62.05 |
| 0.193 | 72.93 |
| -0.409 | 84.12 |

| log conc. | % viability |
|---|---|
| 2 | 30.284 |
| 1.3979 | 40.482 |
| 0.7959 | 48.331 |
| 0.1931 | 61.681 |
| -0.409 | 69.407 |

**Figure (1)** Concentrations of Taxol and cell viability of cancer cells (SCC-25) by BIOLINE ELIZA READER A- Graph demonstrating an inverse relation between the serial NPSO concentrations and cell viability of cancer cells (SCC-25). B-Graph demonstrating an inverse relation between the serial Taxol and cell viability of cancer cells (SCC-25).

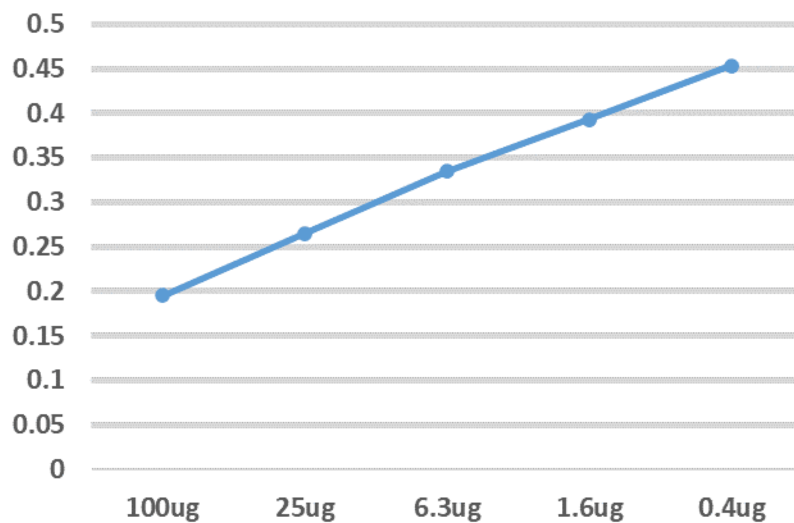



**Figure (2)** Line chart showing the MTT assay (cell viability) using NSPO_CH/SCC-25 with different concentrations at 48 h incubation period.

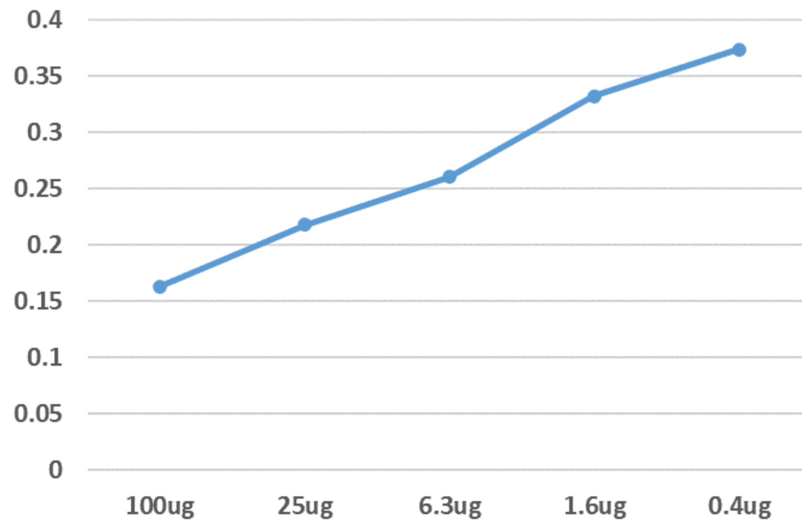



**Figure (3)** Line chart showing the MTT assay (cell viability) using nano-Taxol/SCC25 with different concentrations at 48 h incubation period.

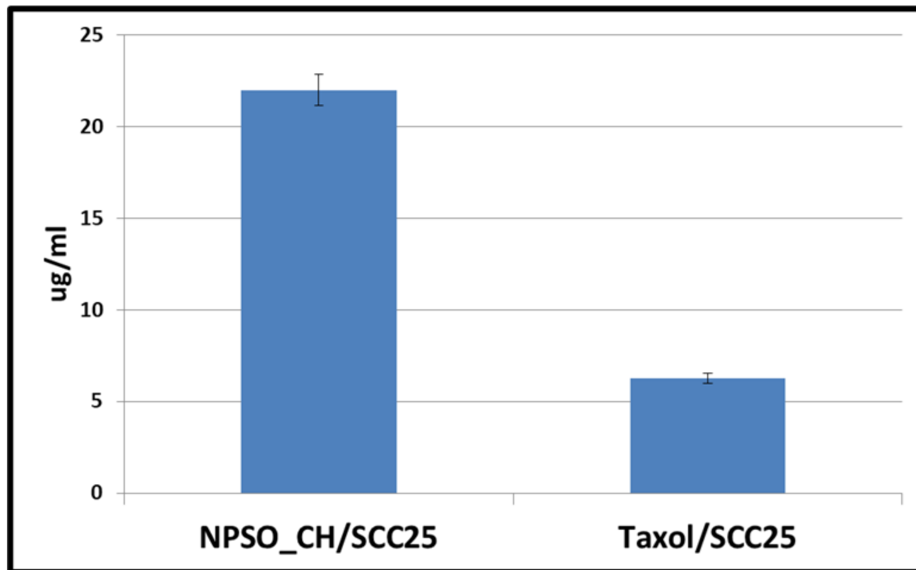



**Figure (4)** Bar chart showing comparison of cytotoxicity-IC50 (ug/ml) of NPSO and Taxol drug.

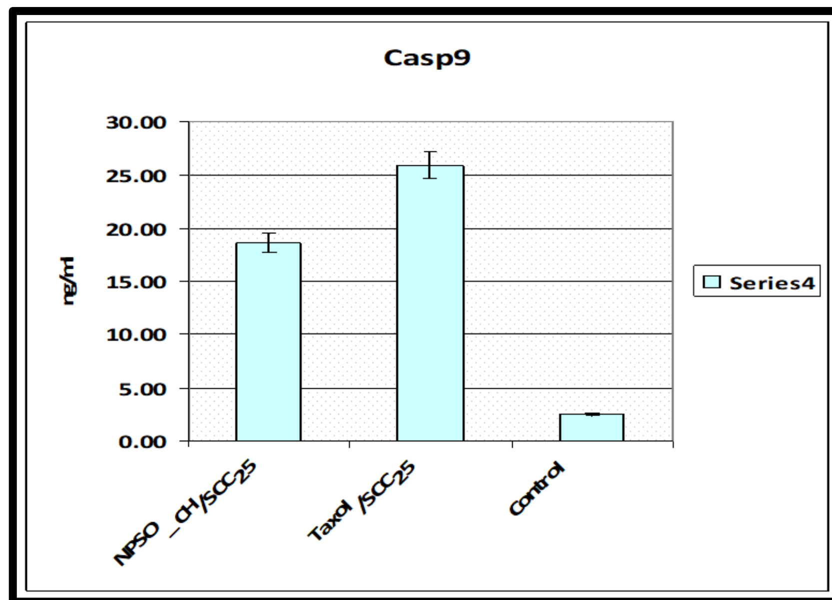



Figure (5) Bar chart showing comparison of caspase 9 expression values of NPSO, Taxol drug and control tongue carcinoma cell line (SCC-25). Notice the significant higher expression of caspase 9 in Taxol/SCC25 & NPSO/SCC25 in comparison to the untreated control SCC-25.

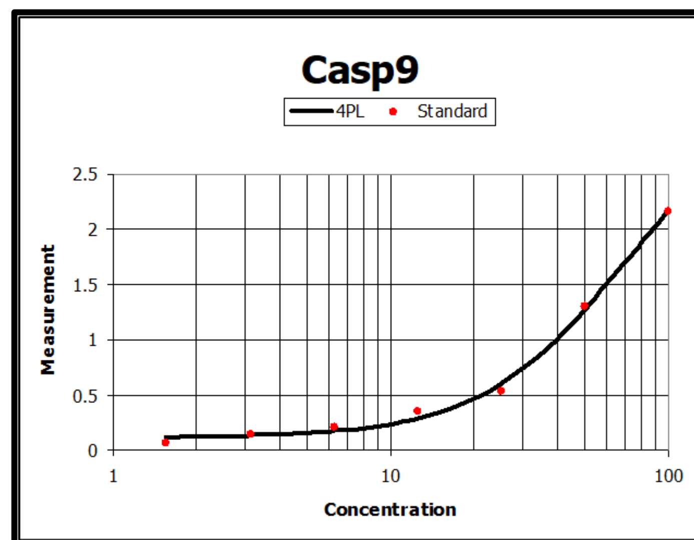



Figure (6) The graph demonstrates a positive linear relation between the serial NPSO concentrations and Caspase 9 expression of cancer cells (SCC-25).

**Discussion**

Cancer is regarded as a consequence of a series of genetic alterations that affect normal cell converting it into a malignant cell, one of these essential alterations is evasion of cell death. Therefore, change in events of apoptosis or cells becoming resistant to this process could result in carcinogenesis by either distorting the balance between pro-apoptotic and anti-apoptotic proteins or decrease in caspase function or even blocking in death receptor signaling (Shokrzadeh et al., 2010).

Studies have shown that the mechanisms of chemo-prevention might be attributed to the

action of anti-oxidants, stimulation of apoptosis, immune promoting effect, anti-inflammatory, anti-hormonal effect, arrest of cell cycle and repression of cell growth and angiogenesis. Many potent anti-cancer medications have been derived from natural products, as well as combination of natural products with conventional agents for improving the anticancer effect and reducing resistance of malignant cells to therapy (Rathinavelu et al., 2013)

Taxol (generic name paclitaxel) is an antineoplastic agent isolated from the Pacific yew. It is a microtubule-stabilizing chemotherapeutic drug which is credibly used in therapy of various types of cancer and it was authorized by the United States (US) Food and Drug Administration (FDA) for therapy of advanced non-small cell lung carcinoma, ovarian carcinoma, breast carcinoma, and acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma. It has been used commonly as a single agent and in coalescence chemotherapy (Rowinsky and Donehower, 1995). On the other hand, Taxol is not well tolerated as it may produce major hurtful drug reactions (ADRs), involving hypersensitivity reactions, , neuropathy, hematological toxicity and myalgia (Markman, 2003).

Nanomedicine represents the implementation of nanotechnology and drug formulation for better drug efficacy. Chitosan nanoparticle delivery system was selected for loading hydrophobic pumpkin seed oil by ionotropic gelation method to improve NPSO stability, concentration and adsorption when application to cancer cells (M. Wang and Thanou 2010).In the current study, the cytotoxicity and apoptotic effect of pumpkin seed oil loaded chitosan nanoparticle (NPSO_CH) were tested against tongue squamous cell carcinoma cell line (SCC-25)

To verify that the prepared NPSO_CH in the current study has attained the criteria of NPs, it was characterized by using TEM. It was confirmed that NPs were spherical, monodispersed, un-agglomerated and uniformly distributed within the nano-size, which ensure that NPSO_CH could be used for drug delivery. (Ruman et al. 2021).

In the present study, NPSO showed cytotoxicity on SCC-25 cell line at IC50 22 ug/ml compared to Taxol drug which showed cytotoxicity at a significantly lower value of IC50 6.28 ug/ml ($p<0.005$) when the cells were incubated for 48hrs. Similarly, in 2020, a study was made by Azari et al. to measure the cytotoxic effect of hull-less seed Pumpkin extract on human papillary thyroid cancer cell line. They found out that, as the concentration of the extract was elevated the number of dead malignant cells increased, and the viability of cells decreased, i.e. at value of 800 µg/mL, the viability of cells declined up to 30%, at value 1600 µg/mL the percentage of dead malignant cells increased to more than 50% where the cell viability inclined significantly(Azari et al. 2020).

The previous observation agrees with a study conducted by Bahadori et al in 2021. They showed that both Hull less seed pumpkin (HLPS) hydro-alcoholic extract and Paclitaxel (PTX) induced cell death in papillary thyroid carcinoma (PTC) cell lines. The IC50 value of PTX was significantly lower than the IC50 HLPS ($P<0.05$). The IC50 values of HLPS was 1.379 ug/ml, while, the IC50 value of PTX was 0.683 ug/ml when the cells were incubated for 48hrs (Bahadori et al. 2021).

Interestingly in the current study, the IC50 cytotoxicity value of NPSO on SCC-25 cell line was 22 µg/ml significantly higher than the traditional chemotherapeutic drug Taxol. Many studies showed numerous values of IC50 of pumpkin extract that varied according to the cancer cell lines used in each study.

The previous observation in accordance with an interesting study conducted by Sahar Y Al-Okbi1 et al., 2016. They studied the anticancer activity of Egyptian and European pumpkin

seed oil (PSO). The cytotoxicity of the oils was assessed in different human cancer cell lines involving HepG-2 (liver cancer cells), MCF-7 (breast cancer cells) and Caco-2 (colon cancer cells). They revealed antitumor effect as they were capable of hindering the multiplication of colon cancer cell line (Caco-2) with the same IC50 value of 0.483mg. Egyptian oil revealed increased cytotoxic activity than the European towards liver cancer cell line (HepG2), at IC50 0.483 mg, while the European oil was 0.517mg. On the other hand, Egyptian oil showed lower cytotoxic performance against cancer cell line (MCF-7), with IC50 0.517 mg compared to the European oil of IC50 0.483 mg (Sahar et al. 2016).

The same observation was revealed by Shokrzadeh et al. in 2010. In their study, cytotoxicity of hydro-alcoholic extracts of pumpkin Cucurbita pepo and Solanum nigrum was measured on normal [Chinese hamster ovarian cells (CHO) and rat fibroblast], cancer HepG2 (human hepatocarcinoma) and CT26 (human colon carcinoma) cell lines. The results revealed that IC50 of S. nigrum extract was remarkably lesser than that of the C. pepo extract performed on all cell lines ($P < 0.05$). The IC50 of S. nigrum extract showed higher cytotoxicity than the extract of Taxus baccata (Taxol) and Cisplatin. IC50 of the agents performed on the cell lines raised in the coming sequence: Cisplatin < T. baccata < S. nigrum < C. pepo. The value of the cytotoxicity of C. pepo extract was ($IC50 = 132.6 \pm 4.3$ μg/ml) on HepG2 cell line and ($IC50 = 293.2 \pm 10.3$ μg/ml) against fibroblast cell line, this might reflect the less cytotoxic effect of pumpkin extract on normal tissue (Shokrzadeh et al. 2010).

Interesting biochemical molecules known as ribosome-inactivating proteins (RIPs) were isolated from mature seeds of pumpkin (Cucurbita Moschata). These molecules have been reported to have anticancer effect and cytotoxic performance when passed into cytoplasm according to a study made by Heng et al. in 2003. They presented a selective cell - killing agent based on the RIP action known as immunotoxin Moschatin fabricated from seeds of Cucurbita Moschata pumpkin specie, this agent successfully hindered the growth of melanoma cells M21 with an IC50 of 0.04 μg/ml as it demonstrated powerful rRNA N-glycosidase activity and high suppressing action on protein synthesis in malignant cells. This might indicate the potential chemo preventive action of pumpkin seeds extracts (Heng et al. 2003).

Another study was conducted by Richter et al. in 2013 to evaluate the effect of pumpkin seed oil on human breast cancer cells (MCF7). Their study came up with the conclusion that phytoestrogen found in the pumpkin seeds had a cytotoxic effect on breast cancer cells. In fact, their study came up with a very interesting finding, which is that PSO produced a notable downregulation of the ER-α (Estrogen Receptor -α) in the MCF7 breast cancer cell line, while expression of ER-β was unchanged. This might indicate possible protection against breast cancer cell growth. (Richter et al. 2013).

In the current study, the caspase enzyme essay revealed a remarkable increase in the expression of caspase 9 after treatment of the SCC-25 cell line with NPSO and Taxol 18.63 pg/ml, 25.94 pg/ml respectively, in comparison to its expression in the untreated SCC-25 cell line, which was as low as 2.48 pg/ml. These finding show that NPSO might have an apoptotic effect on tongue SCC-25 cell line.

The same observation was also reported by Shen et al. in 2017. It was reported that polysaccharides, which were obtained from pumpkins, were capable of inducing the apoptotic process in HepG2 cells which might be mediated by blocking JAK2/STAT3 pathways. That is why, this pathway might be appreciated as one of therapeutic selections for hepatocellular carcinoma (Shen et al. 2017).

Cucurbitacins extracted from the pumpkin seeds play a role in inducing apoptosis by modifying genes and stimulation and hindrance

of pro- or antiapoptotic proteins. Mainly, Cucurbitacins cause suppression of JAK/STAT pathways and other procedures in relation to the antiapoptotic action like PARP cleavage, JAK3, and lowered pSTAT3 levels (Chari, Polu, and Shenoy 2018).

Ren et al. in 2012 showed that the multiplication of prostate cancer (PCa) cell lines was blocked by dose dependent measurements of cucurbitacin. They used DU145, PC3, and LNCaP which are considered to be the standard prostate cancer cell lines used in therapeutic research. Their study indicated that cucurbitacin has the capability to promote cell cycle block and amplify levels of apoptosis (Ren et al. 2012).

Rathinavelu, et al. (2013) also performed a study on the cytotoxic effect of pumpkin seeds on prostate cancer in vitro. Their study revealed that the viability of LNCaP cells was remarkably ($p \leq 0.05$) reduced in a dose dependent manner by both the PS-AQ and PS-ET extracts when compared to vehicle controls. The IC50 values were 49 µg/ml and 55µg/ml for PS-AQ and PS-ET extracts sequentially. These findings prove that the extracts of pumpkin have potent cytotoxic activity. They also measured the effect of PS extracts on mitochondrial membrane and their effect on ROS generation in LNCaP Cells. Their results proved that the anticancer effects is due to oxidative stress, mitochondrial depolarization and change in apoptosis mechanisms (Rathinavelu et al. 2013).

It has also been discovered that pumpkins contain important phytochemicals like carotenoids which include α- and β-carotene, lycopene, and derivatives, such as retinoic acid, that have been examined for their outstanding anti-proliferative activity on cancer. Moccia et al. in 2020 reported that a carotenoid-enriched extract (CE) produced from pumpkins revealed an anti-proliferative action on HG3 cell line, human B Chronic lymphocytic leukemia (CLL) cells. CE extract caused a 40% inhibition in cell multiplication in comparison with untreated cells. In HG3 cells exposed to CE, they found a 30% rise of autophagosome intra-cellular (Moccia et al. 2020).

Carotenoids involving β-carotenes have the capability of protecting protein against oxidative damage. It was reported that fruits and vegetables with high β-carotene concentration possess antioxidant characteristics. It was revealed that β-carotenes in pumpkin seed oil possess powerful cellular antioxidant effect (Mohamed and Ahmed 2016).

The clinical trial performed by Hong et al. in 2009 on benign prostatic hyperplasia (BPH) patients treated with pumpkin seed oil, revealed that the group treated with pumpkin seed oil showed a reduction in their international prostate symptom score after 3-months, quality of life score was also improved after 3 months and maximal urinary flow rate were gradually improved after 6 months (Hong, Kim, and Maeng 2009). These results might indicate that pumpkin seed oil could be a prospective treatment option in dealing with (BPH) and thus preventing early stage of prostatic cancer.

In the present study the IC50 cytotoxicity value of NPSO on SCC-25 cell line differs from IC50 values of pumpkin extract on other different cell lines which reflect the variable response of diverse types of cancer cells to herbal extracts. Worth noting that the remarkable increase in the expression of caspase 9 after treatment of the SCC-25 cell line with NPSO could encourage further studies to prove whether NPSO can be incorporated in the cancer drug industry since it revealed significant apoptotic effect against oral squamous cell carcinoma (SCC-25).

**Conflict of Interest:**

The authors declare no conflict of interest.

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**Ethics:**

This study protocol was approved by the ethical committee of the faculty of dentistry- Cairo university on:18-2-2020, approval number: 20232

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