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EFFECTIVENESS OF PUMPKIN SEED AQUEOUS EXTRACT AS AN ANTICANCER AGENT

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ABSTRACT: One of the primary causes of human mortality is cancer. Herbal plants may offer new and enhanced anticancer compounds to combat cancer. The current study prepared, characterized, and evaluated a pumpkin seed aqueous extract (PAE) for its antioxidant and anticancer activities. The extraction yield from PAE was recorded 13 g/ 100g Defalted flour. The total phenol (TPCs) content of the PAE was 144.99 mg GAE g^{-1} dry extract, while the total flavonoid (TFCs) content was 11.84 mg QE g^{-1} dry extract. Twelve phenolic compounds (Gallic acid, Caffeic acid, Rutin, Coumaric acid, Vanillin, Naringenin, Querectin, Ellagic acid, 3.4-Dihydroxybenzoic acid, Keampferol, Ferulic acid, and Syringic acid) dominated in the chromatogram from the separation of PAE. It can be noted that the antioxidant activity of PAE increased gradually with increasing concentrations. When the concentration of extract was increased from 100 to 2000 µg/mL, the DPPH radical scavenging activity increased from 8.39% to 29.86%. The MTT assay revealed that the PAE inhibited the proliferation of the human cancer cell lines (HCT 116 and A-549) in a concentration-dependent manner. PAE had an IC_{50} against HCT116 and A-549 (213.59, and 208.72 µg/mL, respectively). Therefore, PAE can be used as a source to produce pharmaceutical and medicinal products.

Key words: Pumpkin seed, Bioactive compounds, MTT-assay, DPPH, A459, HCT 1116.

INTRODUCTION

Cancer ranks among the foremost causes of mortality, accounting for one in six death (about 9.6 million individuals) in 2018; nonetheless, 70% of cancer-related fatalities transpire in middle- and low-income nations (**Bray *et al.*, 2018**). Surgery, chemotherapy, radiotherapy, and hormonal therapy are the most common ways to treat cancer. In recent years, natural product-based therapeutic and diagnostic approaches have shown the potential to improve cancer treatment (**Vickers, 2004**). Using herbal medications is one of the most ancient and secure approaches to treating various diseases **(Jamshidi-Kia *et al.*, 2017**). Prooxidants and antioxidants are crucial in regulating the equilibrium between free radicals and the body's antioxidant system **(Rahal *et al.*, 2014**). Antioxidants are those molecules that prevent, reduce or completely eliminate the action of free radicals and protect the body from oxidative damage (**Lobo *et al.*, 2010**). Thus, effective antioxidants could delay oxidation reaction or obstruct the development of free radicals or break the generation of the autoxidation chain reaction that generates free radicals/oxidants. They also act as reducing agents and metal chelators which convert hydroperoxides into stable compounds. Some antioxidants act as metal chelators that transform metal prooxidants into stable form. Oxidative/nitrosative stress results from disequilibrium in oxidant-antioxidant balance in the favor of reactive species with the increase of reactive oxygen and/or RNS production, respectively (**Kurutas, 2015**). Studies have linked oxidative stress with the pathogenesis of inflammation-related cancers, and agents with the capacity to protect cells against ROS attack by quenching free radicals, are thought to be potent chemo preventive candidates (**Chi *et al.*, 2015**). In fact, the correlation of antioxidative

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function with anticancer property has traditionally been reported in a range of biological and chemical agents, including dietary kelp and plant phenolics (**Dia and de Mejia, 2010**). Medicinal plants contain several active principles with specific therapeutic effects. They represent a source of chemical compounds such as tannins, flavonoids, saponins, resins and alkaloids with curative properties, often not provided by synthetic chemical compounds (**Fabricant and Farnsworth, 2001**). Pumpkin seeds, typically thrown during processing, are highly nutritious and yield superior grade oil, serving as a good source of protein. Furthermore, they serve as an excellent source of minerals, dietary fiber, beneficial vitamins, and monounsaturated fatty acids, which promote cardiovascular health. The distinctive flavor of pumpkin seeds and pumpkin seed oil is widely recognized and appreciated globally, enhancing the aromatic profile during the roasting process. Pumpkin seed has garnered significant attention because of its high nutritional and health-protective properties, as well as its pharmacological activity, including antidiabetic, antifungal, antibacterial, anti-inflammatory, and antioxidant benefits (**Lestari and Meiyanto, 2018**). Therefore, the current study prepared, characterized, and evaluated a pumpkin seed aqueous extract (PAE) for its antioxidant and anticancer activities.

MATERIALS AND METHODS

Preparation of Pumpkin Seeds Aqueous Extract (PAE)

The pumpkin seeds were isolated manually from the pulp of the fruits, and then the collected seeds were dried and ground into a powder using a grinder (Moulinex Type LM201, Mayenne, France). Seeds were grounded and defatted with hexane to remove lipids and lipophilic molecules. Ten grams of ground seeds were extracted with 100 ml of distilled water (10% W/V) using a magnetic stirrer for 2 h, followed by filtration by filter paper Whatman No.1 The remains were re-extracted twice under the same conditions and the remaining water was lyophilized (Thermo- electron Corporation–Heto power dry LL 300 Freeze dryer). The extract was preserved in a refrigerator for further analysis (**Benariba *et al.*, 2013**).

Total Phenolic Compounds (TPCs) Estimation

The TPCs have been determined using the Foline-Ciocalteu reagent, outlined by **Singleton *et al.* (1999)**. One mL of sample (1000 µg/mL) was mixed with 5 mL of Folin-Ciocalteu reagent (previously diluted with water 1:10, *V/V*) and 4 mL of sodium carbonate (75 g/L). The tubes were vortexed for 15 seconds before being left to stand at 40°C for 30 min to allow for color progression. The absorbance was determined at 765 nm. Gallic acid was used to create a standard curve (20-200 µg/mL), and the lowering of Folin-Ciocalteu reagent by samples was quantified as mg of gallic acid equivalents (GAE) per gram of extract. The calibration equation for gallic acid was y = 0.001x + 0.0563 (R2 = 0.9792), where y is absorbance and x are concentration of gallic acid in µg/mL.

Total Flavonoids (TFs) Determination

Total flavonoids (TFs) were estimated according to the protocol of **Ordonez *et al.* (2006)**. Two mL aliquot of 20 g/L $AlCl_3$ ethanol solution was blended with 1 mL of the extract (1000 µg/mL solvent). After 60 min, the absorbance at 420 nm was estimated. Quercetin was applied to gain the standard curve (20–200 µg/mL), Total flavonoids contents expressed as quercetin equivalent (QE), which was calculated based on the calibration curve. The calibration equation for quercetin was y = 0.0012x + 0.008 (R_2 = 0.944), where y is absorbance and x are concentration of quercetin in µg/mL

Polyphenolic Compounds Identification

To quantify phenolic compounds in PAE, HPLC was utilized as described (**Abd Elhamid *et al.*, 2022**). Phenolics were identified by HPLC-Agilent 1100 apparatus with a UV/Vis detector and absorbance was recorded at 254 nm. C18 column (125 mm × 4.60 mm, 5 µm particle size) was used. Phenolic components were extracted utilizing gradient mobile phase consisting of two solvents: solvent A (methyl alcohol) and solvent B (acetic acid) in water at a ratio of 1:25. The flow rate was regulated to 1.0 mL/min, and column temperature was consistently kept at 37°C for whole test duration.

Antioxidant Activity Estimation (DPPH-assay)

The antioxidant activity of PAE (100-2000 µg/mL) was measured using the DPPH assay (**Güllüce *et al.*, 2003**). A 50 µL sample solution was added to 5 mL of 0.004% methanolic DPPH and incubated for 30 minutes at room temperature in dark. We measured absorbance at 517 nm. We calculated antioxidant capacity of DPPH radicals (%) utilizing following formula:

$$\text{Radicals scavening activity (Inhibition)}(\%) = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs}}\text{control}\right] \text{x } 100$$

DPPH assay (IC_{50}) values showed concentration of compounds to scavenge 50% of DPPH.

Anticancer Activity

MTT-assay

Cell viability was estimated utilizing 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate efficacy of PAE (50-1000 µg/mL) against cancer (A549 and HCT-116) cell lines, following the methods outlined in **Mosmann (1983) and Dawoud *et al.* (2022)**. The cells were grown in DEME media with heat-inactivated fetal bovine serum (10%), penicillin (10 U/mL, Sigma-Aldrich), and streptomycin (10 µg/mL, Sigma-Aldrich). Cells (1.0×10^4) were incubated in sterile 96-well microplates. After incubation, the monolayer sheet of cells was separated, and the growth media was decanted. The cells were exposed to BAME at various concentrations in volume of 150 µl per well. The control was treated with an equal volume of saline. All plates were placed in a 5% CO_2 incubator and incubated at 37°C for 48 h. After removing media, cells were washed with phosphate-buffered saline (PBS). Following this, 50 µl/well of MTT solution (Sigma -Aldrich, 0.5 mg/mL) was added to the plates and incubated for 4-5 h. Subsequently, 50 µl/well of DMSO solution was added. Absorbance of each well was measured at 590 nm utilizing an ELIZA reader. The test was conducted in triplicate for all cells. The viability percentage was calculated as follows:

$$\textbf{Cell viability}\ (\%) = \left(\textbf{Ab}\frac{\textbf{sample}}{\textbf{Ab}}\textbf{control}\right)\textbf{x 100}$$

The anti-cancer activity of PAE was assessed by determining the IC_{50} values 48 hours after treatment.

Morphological Analysis

The impact of PAE on cell morphology was examined using HCT 116 and A549 cells as a model among the tested cell lines. The cells were initially seeded in 12-well plates with DMEM supplemented with 10% fetal bovine serum at density of 5× 105 cells/well, and then they were cultured for 48 h. After that, the media were aspirated, and cells were treated with varying concentrations (50-1000 µg/mL) of PAE. Changes in cell morphology were documented using a normal inverted microscope (Nikon) at 200 x magnification compared to untreated cells.

Statistical Analysis

IBM Inc. in Armonk, NY, developed the SPSS software for Windows version 22, which we use to conduct data analysis. Unless otherwise specified, threshold of significance was $P \leq 0.05$.

RESULTS AND DISCUSSION

Yield and Identification of Phenolics and Flavonoids Constituents

In an experimental study of PAE, the total phenol content, total flavonoid, and extraction yield are given in Table 1. The extraction yield from PAE was recorded 13 g/100g seed. The total phenol (TPCs) content of the PAE was 144.99 mg GAE g^{-1} dry extract, while the total flavonoid (TFCs) content was 11.84 mg QE g^{-1} dry extract. **Hagos *et al.* (2023**) recorded that the total phenolic content of pumpkin peel, flesh, and seed samples ranged from 354±1.4 to 385±2.1 mg GAE/100 g, 288±0.8 to 369±1.8 mg GAE/100 g, and 80.6±0.4 to 102±1.2 mg GAE/ 100 g, respectively. These variations might be related to geographic and environmental factors as well as different parts of the pumpkin (**Akomolafe, 2021**).

A representative chromatogram of the HPLC phenolic compounds analysis of PAE was shown in Fig. 1. Twelve peaks (Gallic acid, Caffeic acid, Rutin, Coumaric acid, Vanillin, Naringenin, Querectin, Ellagic acid, 3.4-Dihydroxybenzoic acid, Keampferol, Ferulic acid, and Syringic acid) dominated in the chromatograms from the separation of PAE. Table 2 summarizes the contents

Table 1. Yield of extracted substances (g), TPCs (GAE g^{-1} dry extract), and TFs (QE g^{-1} dry extract)

Parameters	Concentration
Extraction yield	13 g/ 100g Defalted flour
TPCs	144.99 mg GAE g^{-1} dry extract
TFs	11.84 mg QE g^{-1} dry extract

TPCs: total phenolic compounds; TFs: total flavonoids

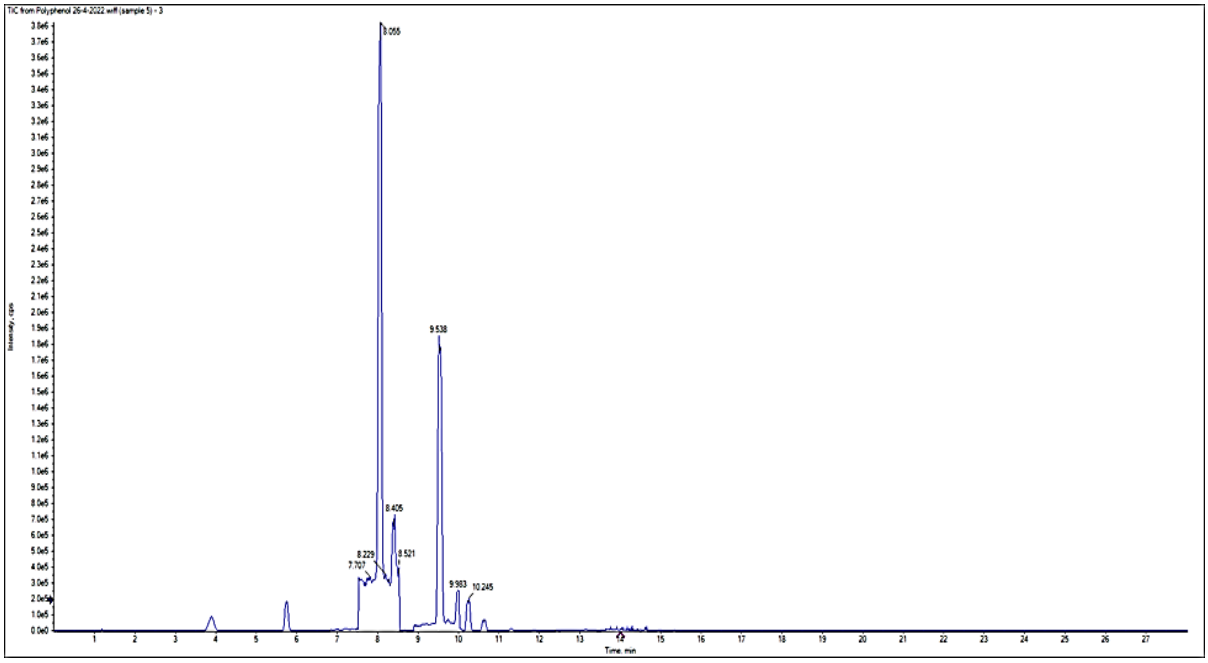

Fig. 1. HPLC-chromatogram of major phenolic compounds in pumpkin seed aqueous extract (PAE)

Table 2. Major phenolic compounds concentration (µg/g)) in pumpkin seed aqueous extract (PAE)

Component	Concentration (µg/g extract)
Gallic acid	2.49
Caffeic acid	14.03
Rutin	0.03
Coumaric acid	4.18
Vanillin	36.58
Naringenin	0.62
Querectin	0.01
Ellagic acid	0.07
3.4-Dihydroxybenzoic acid	7.96
Keampferol	0.02
Ferulic acid	6.01
Syringic acid	37.00

of phenolic compounds 1–12 in the PAE. It was shown that Syringic acid and Vanillin are the main phenolic compounds in the PAE. Syringic acid and Vanillin have the highest concentrations (37 and 36.58 µg/g dry extract, respectively) of all the components.

Antioxidant Activity

Antioxidants activity (% inhibition) for PAE using DPPH assay are presented in Fig. 2. PAE has antioxidant activity. These results are compatible with our results recorded in total phenolic compounds and total flavonoids content. It can be noted that the antioxidant activity of PAE increased gradually with increasing concentrations. When the concentration of extract was increased from 100 to 2000 µg/mL, the DPPH radical scavenging activity increased from 8.39% to 29.86%. The antioxidant activity of pumpkin seed is due to the presence of phenolic compounds (**Peiretti *et al.*, 2017**). Recently, research on polyphenolic compounds has become subject of interest because of their numerous health benefits. Several reports have linked the antioxidant activities of many plant foods/extracts to the polyphenolic compounds, and its believed to be due to the redox properties of their polyhydroxy molecule, which is known for their ROS adsorbing and neutralizing potentials, chelating of transitional metal catalysts and activation antioxidant enzymes activities (**Akomolafe *et al.*, 2016**).

Anticancer Activity

PAE at different concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/ mL) were tested *in vitro* for their anticancer activity on the HCT 116, and A-549 human cancer cell lines using the MTT assay. Figs. 3 and 4 show estimates of the cell viability percentages as well as their cytotoxic activity. There was a linear relationship between cell viability (%) and extract concentration, and overall, cell viability (%) decrease with increasing concentration. The MTT assay revealed that the PAE inhibited the proliferation of the human cancer cell lines (HCT 116 and A-549) in a concentration-dependent manner. PAE had an IC_{50} against HCT 116 and A-549 (213.59, and 208.72 µg/mL, respectively). (**Rathinavelu *et al.*, 2013**) demonstrated the cytotoxic effects of aqueous and alcoholic pumpkin seed extracts by induced DNA fragmentation and poly ADP ribose polymerase cleavage. The oestrogen-like molecule is also reported to promote the proliferation of cancerous cells. It has been shown that PS extracts are cytotoxic to breast cancer cell lines (MCF-7) and human chorionic carcinoma cell lines (JEG-3 and BeWo cell lines), and stimulate the production of oestradiol (**Richter *et al.*, 2013**).

Figs. 5 and 6 demonstrates the impact of PAE on the morphological structures of A549 and HCT116. The control groups showed no significant changes. However, in the treated groups, a reduction in cell numbers was observed, which was concentration dependent.

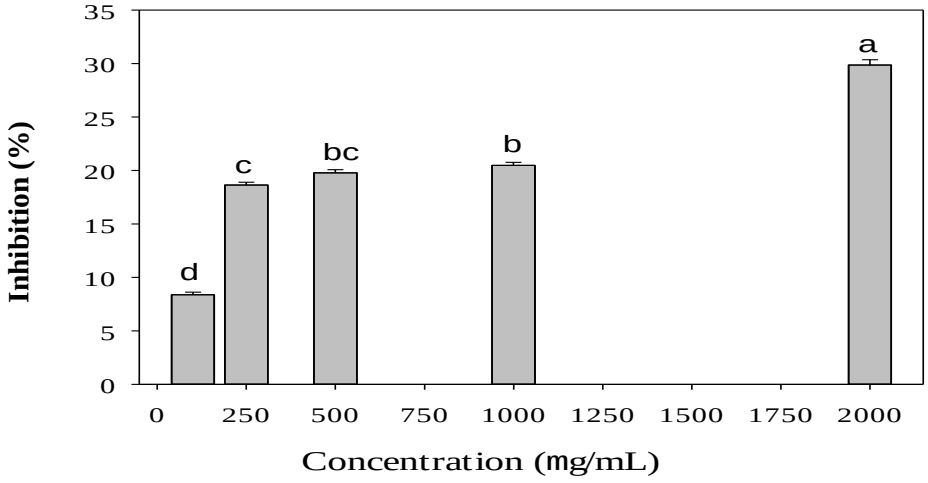

Fig.2. Radicals scavenging activity (Inhibition%) of pumpkin seed aqueous extract (PAE) at different concentration (100, 250, 500, 1000, 1500, and 2000 µg/mL)

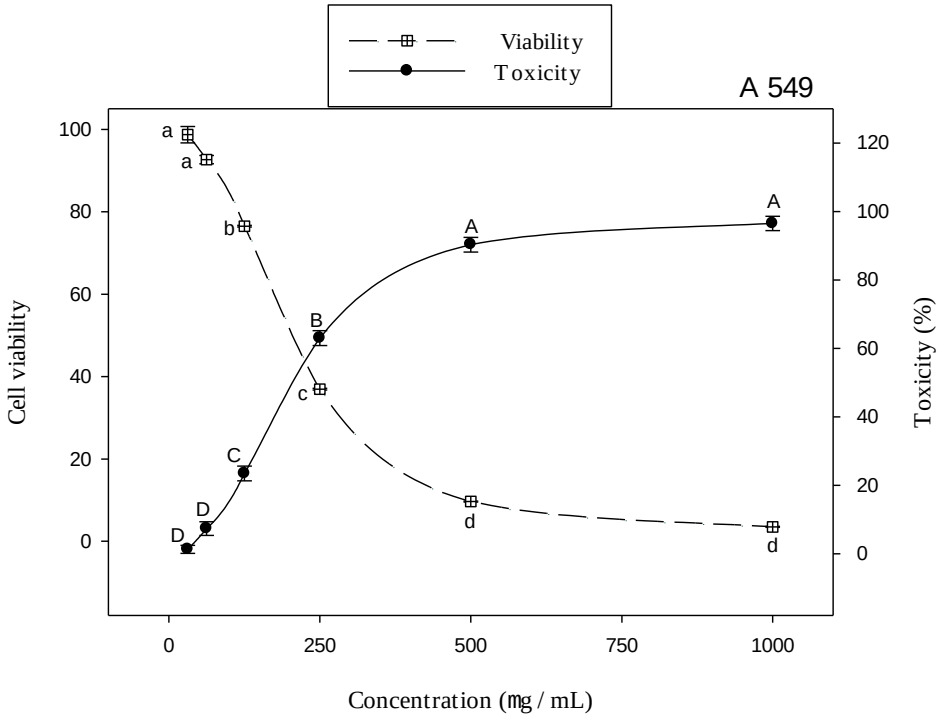

Fig. 3. Toxicity (%) and cell viability (%) of A549 cell line treated with pumpkin seed aqueous extract (PAE) at different concentration

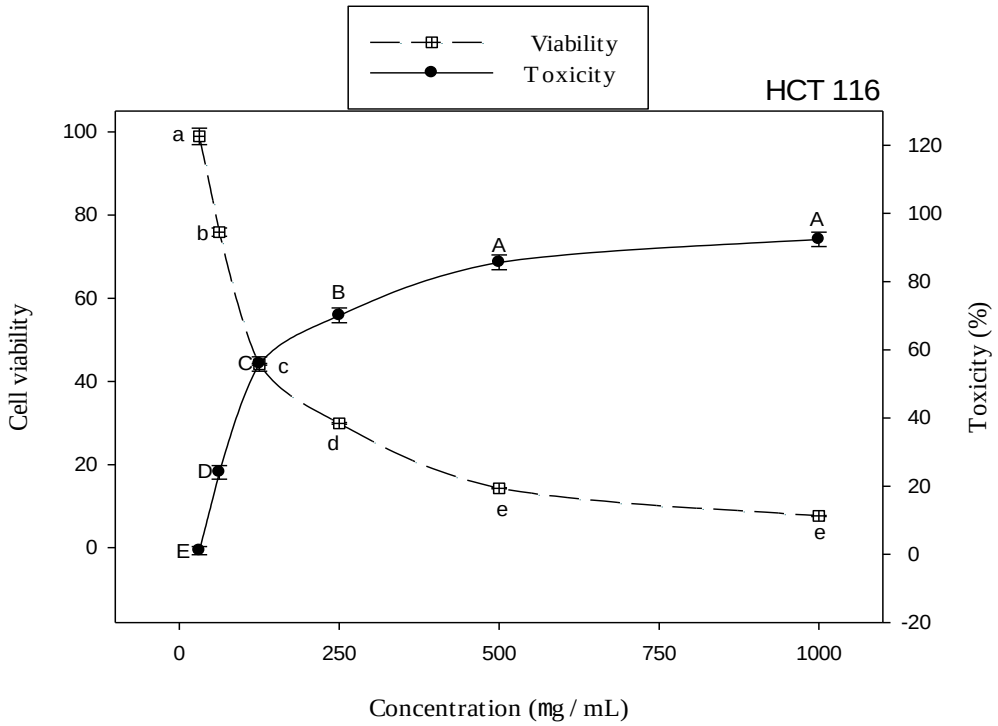

Fig. 4. Toxicity (%) and cell viability (%) of HCT 116 cell line treated with pumpkin seed aqueous extract (PAE) at different concentration

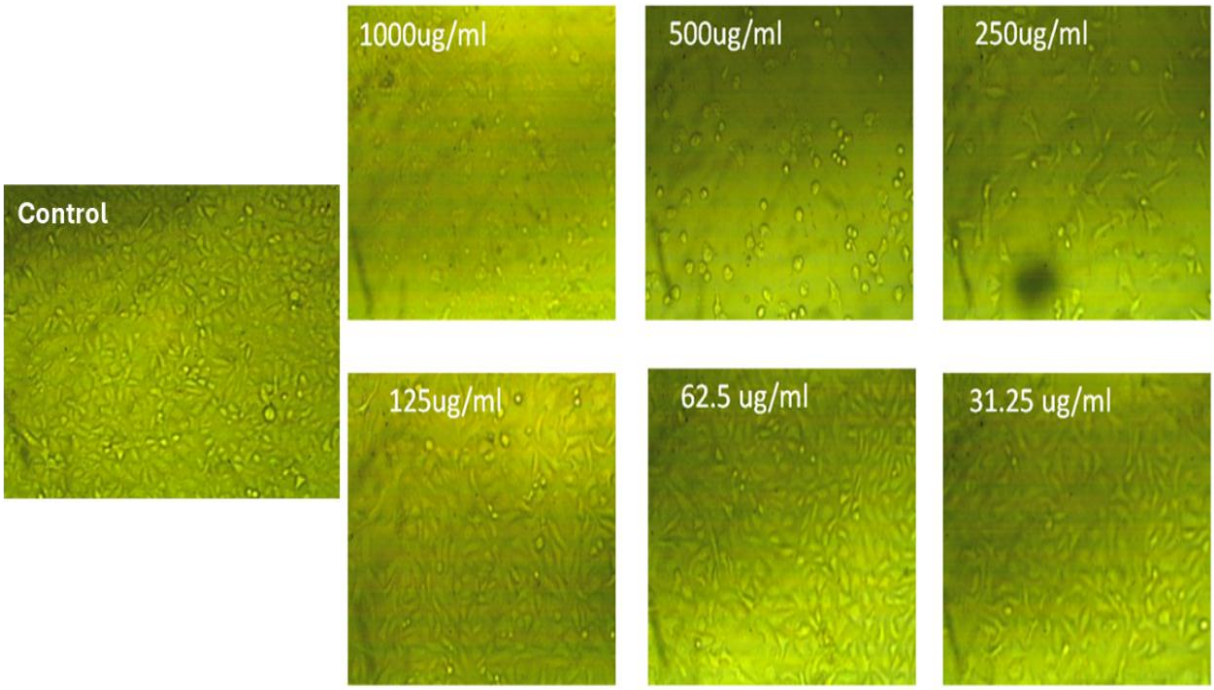

Fig. 5. Morphological changes in HCT 116 cell lines treated with different concentrations from pumpkin seed aqueous extract (200X) compared to control

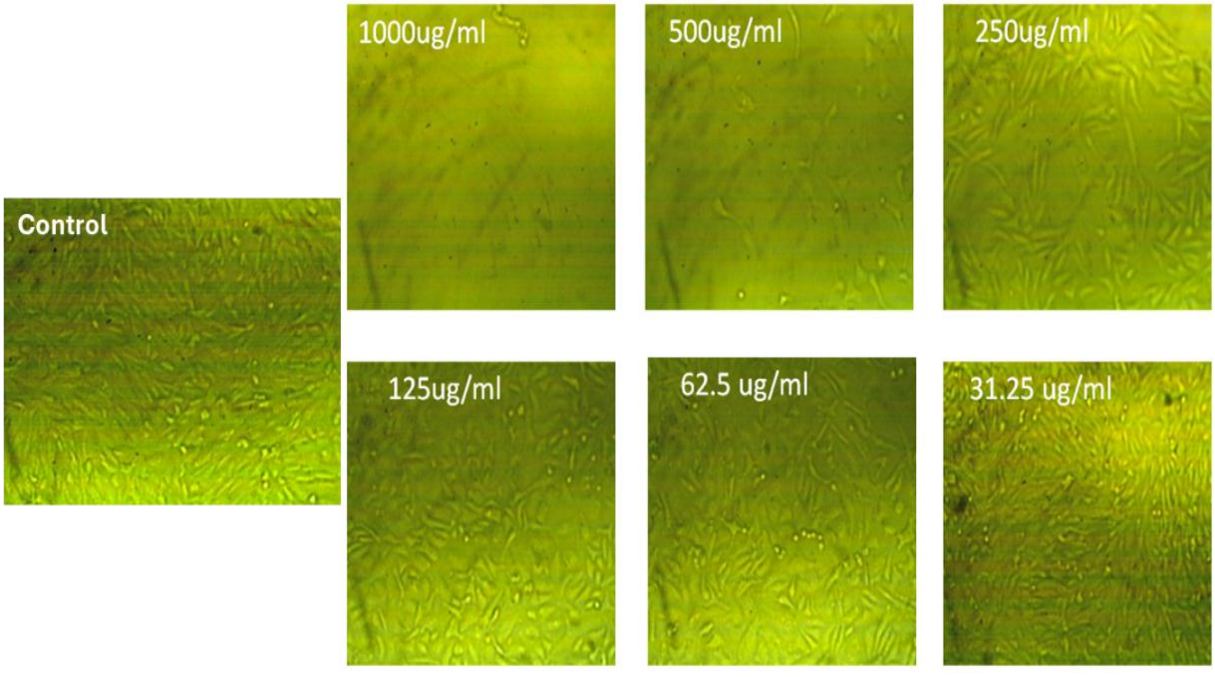

Fig. 6. Morphological changes in A549 cell lines treated with different concentrations from pumpkin seed aqueous extract (200X) compared to control

Conclusions

Pumpkin seeds are a valuable source of medicinal compounds that have been traditionally used for numerous applications. Based on the results obtained in this study, it can be concluded that PAE showed highly effective as an antioxidant and anticancer. Therefore, PAE can be used as a source to produce pharmaceutical and medicinal products.

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فعاليـــة المستخلـص المـائـي لبـــذور اليقطيـن كعـامـــل مضـــاد للسـرطـــان

ألاء إبراهيم محمد عبدالحق الشرقاوى – على عثمان – عبدالعليم اسماعيل السيد – محمود ذكي سطوحى

قسم الكيمياء الحيوية - كلية الزراعة - جامعة الزقازيق - مصر

فـى هـذه الدراسـة تـم عمـل مستخلص مـائي مـن بـذور نبـات اليقطين وتقـدير محتـواة مـن المركبـات الفينوليـة ومركبـات الفلافونويـد الكليـة وتعريـف المركبـات الفعالـة بواسـطه جهـاز التحليـل الكروماتوجراى السـائل عـالى الأداءHPLC. علـى الجانب الأخر تم تقدير النشاط المضاد للأكسدة والمضاد للسرطان.أوضحت النتائج أن كل 100 جرام من البذور يعطـى 13 جرام مستخلص وأن كمية المركبات الفينولية الكلية 144.49 ملليجرام مكافئ من حامض الجاليك لكل جرام مستخلص بينما كميـة مركبـات الفلافونويـدات الكليـة 11.84 ملليجـرام مكـافئ من الكيريستين لكـل جـرام مستخلص. سـجلت نتـائج التحليـل الكروماتوجرافى السـائل أعلـى تركيـز فـى حالـه حمـض الايلاجيـك (37 ميكروجـرام لكـل جـرام مسـتخلص) يليـه حمـض السينامـيك (36.58 ملليجـرام لكل جرام مستخلص). تم تقييم النشاط المضاد للسرطان معمليا ضد الخلايا السرطانية البشرية (HCT116 و A-549) باستخدام اختبار .MTT أظهرت نتائج اختبار MTT أن مستخلص بذور الحنظل يثبط تكاثر خلايا HCT116 و A-549 بطريقة تعتمد على التركيز. حيث سجلت قيم IC50 (213.59 و 208.72 ميكروجرام لكل ملل مستخلص ضد HCT116 و A549 على التوالى. وبالتالى يوصى باستخدام بذور نبات اليقطين لانتاج المنتجات الصيدلانية واستخدامها طبيا.

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