Available online on 15.10.2023 at http://ajprd.com

Asian Journal of Pharmaceutical Research and Development

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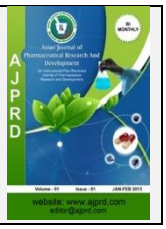

Open Access **Research Article**

The Cytotoxic Activities of n-hexane Extract of Pumpkin Seed (*Cucurbita moschata*) on WiDr Colon Cancer Cell and HeLa Cervical Cancer Cell

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A B S T R A C T

Colon cancer and cervical cancer are types of cancer with a high prevalence, contributing 10% and 3.1% respectively of the total new cancer cases. Nutrition and dietary factors play an important role in human carcinogenesis. Natural plants contain substances that can be beneficial to human health. One such plant is the pumpkin (*Cucurbita moschata*). Pumpkin seeds are thought to have many biological activities for health, such as antioxidant, anticancer, and anti-inflammatory effects. This study aimed to determine the cytotoxic activity of n-hexane extract of pumpkin seed against colon (WiDr) and cervical (HeLa) cancer cells using the MTT Assay method. The method used in this study consisted of the preparation of plant extracts, phytochemical screening, total content of metabolites. The results proved that the n-hexane extract of pumpkin seed was observed containing flavonoids, alkaloids, and steroids. The content of highest total content, namely flavonoid compounds as much as 616.46 mg/L. The n-hexane extract of pumpkin seed showed cytotoxic activity for WiDr cells and HeLa cells with an IC_{50} value of 193.44 µg/mL and 237.702 µg/mL, respectively. The results of this study can be concluded that n-hexane extract of pumpkin seed can be used as a chemopreventive agent, due to the moderate cytotoxic test results.

Keywords: pumpkin seeds (*Cucurbita moschata*), n-hexane, cytotoxic.

A R T I C L E I N F O: Received 10 July 2023; Review Complete 4 Sept 2023; Accepted 25 Sept 2023; Available online 15 August 2023

Cite this article as:

Krisridwany A, Ningrum DWC, Susilowati F, Octavia R, Winanta A, The Cytotoxic Activities of n-hexane Extract of Pumpkin Seed (*Cucurbita moschata*) on WiDr Colon Cancer Cell and HeLa Cervical Cancer Cell, Asian Journal of Pharmaceutical Research and Development. 2023; 11(5):01-06. DOI: http://dx.doi.org/10.22270/ajprd.v11i5.1284

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INTRODUCTION

Cancer is a health problem and a non-communicable disease that still has a reasonably high prevalence in Indonesia and worldwide. According to data from GLOBOCAN, there were 19.3 million new cancer cases worldwide in 2020, resulting in 10 million cancer-related deaths. Colon cancer and cervical cancer are among the types of cancer with a high prevalence, accounting for 10% and 3.1% of total new cancer cases, respectively[1]. Oxidative stress is one of the leading causes of many diseases, including cancer. High levels of oxidative stress can reduce the body's antioxidant defense against angiogenesis and metastasis in cancer cells, which are the primary factors in cancer development[2]. Nutrition and dietary factors play a significant role in human carcinogenesis. A diet rich in plant-based foods, whole grains, and antioxidants, and low in saturated fat, such as the traditional mediterranean diet, is associated with a lower risk of cancer [3].

Natural plants contain substances that can benefit human health. One of the examples is the pumpkin (*Cucurbita moschata*), which has been found to have anticancer activity in previous studies. Pumpkin seeds are thought to have many biological activities for health, such as antioxidant

activity, anticancer, and anti-inflammatory effects[4–6]. Pumpkin seeds contain various compounds, including alkaloids, steroids, flavonoids, triterpenoids, saponins,resins, phenolics, lecithin, stearin, cucurbitacin, phytosterols, squalene, tyrosol, luteolin, tocopherol, fatty acids, vanillin, and sinapic acid [7]. Pumpkin seed oils from Egypt and Europe have shown anticancer activities against Hep-G2 (liver cancer cells), MCF-7 (breast cancer cells), and Caco-2 (colon cancer cells) [8].Previous research suggests that pumpkin has the potential to be developed as a companion supplement for cancer patients. In Indonesia, pumpkin seeds are often discarded and are still underutilized in research. This study aims to optimize the potential of Indonesian pumpkin seeds by extracting the seeds with n-hexane and testing their anticancer activity against WiDr colon cancer cells and HeLa cervical cancer cells by in vitro method.

MATERIALS AND METHODS

Plant and Chemicals materials

Pumpkin seed (*Cucurbita moschata*) from Indonesia, WiDr (colon cancer cells) and HeLa (cervical cancer cells), n-hexane, 20% NaOH, metallic Mg (Magnesium), HCl (Chloric Acid), Lieberman reagent, Dragendroff reagent, Mayer's reagent, H_2SO_4 (Sulfuric Acid),1% $FeCl_3$ (Iron(III) Chloride), Diethyl Ether, Petroleum Ether, N_2, Sodium Methanoate, Boron Trifluoride Methan oate, Heptane, Saturated NaCl, Roswell Park Memorial Institute (RPMI), Phosphate Buffer Saline (PBS), Trypsin EDTA, Dimethyl Sulfoxide (DMSO), MTT, SDS 10%, Foetal Bovine Serum (FBS) 15 % v/v, penisilin-streptomisin 1.5 % v/v, and Doxorubin.

Preparation plant extracts

Pumpkin seeds that have been separated from the fruit are cleaned and dried in the oven at 60°C for 48 hours. Then, they are ground into a fine powder. The extraction process was carried out using the soxhletation method. 100 grams of pumpkin seed powder and 450 mL of n-hexane solvent were soxhleted at 70°C for 6 hours. The mixture was then heated in a water bath at 65°C to obtain the extract.

Phytochemical screening

Several reaction tubes were prepared for phytochemical screening tests of flavonoid, alkaloid, tannin, steroid, and triterpenoid compounds. Two ml of N-hexane extract of pumpkin seed was added to each tube, followed by the addition of a few drops of 20% NaOH for flavonoid testing. The formation of a yellow solution indicates the presence of flavonoids in the extract[9]. 2-3 mL of the extract is added with a few drops of Dragendorff reagent. The results showed a positive presence of alkaloids, marked by the formation of an orange-brown precipitate [10]. Steroid and triterpenoid testing were done by adding chloroform + acetic anhydride + concentrated sulfuric acid through the wall of the tube. The formation of yellow with green fluorescence indicates the presence of steroids, while the formation of a reddish violet colour indicates the presence of triterpenoids in the extract [11]. Tannin phytochemical screening was carried out by adding 2-3 drops of 1% $FeCl_3$. The formation of a bluish-black or greenish-black precipitate indicates the presence of tannins in the extract [12].

Determination of total flavonoid content

Standard curves were made using quercetin. For the total flavonoid test, 2 mL of HCl was added to 0.10 mL of the n-hexane extract of pumpkin seeds. The polar phase was collected, 1 mL of ether was added, followed by extraction and collection of the organic phase. Then, 0.3 mL of 5% sodium nitrite was added, followed by 10% $AlCl_3$ and 2 mL of 1M NaOH. The solution was made up to 10 mL with distilled water using a measuring flask, and the absorbance was measured using UV-Vis spectrophotometry at a wavelength of 510 nm [13].

Determination of total alkaloid content

Standard curves are made using Quinine. As much 0.250 mL volume of n-hexane extract from pumpkin seeds was taken, and then 5 mL of 2N HCl was added to it. The solution was washed with 10 mL of chloroform three times in a separatory funnel, and the chloroform phase was discarded. Then, 0.1 N NaOH was added, followed by 5 mL of phosphate buffer. The solution was then extracted with 5 mL of chloroform and stirred with a magnetic stirrer at 500 rpm for 15 minutes. The chloroform phase was collected and evaporated with nitrogen gas. After that, chloroform was added to the solution to make a volume of 10 mL, which was then diluted ten times and analyzed by UV-Vis spectrophotometry at a wavelength of 470 nm.

Determination of total phenolic content

Standard curves were prepared using gallic acid. The n-hexane extract of pumpkin seed was mixed with 0.5 mL of folin-ciocalteu reagent and 7.5 mL of water distilled, then left for 10 minutes at room temperature. After that, 1.5 mL of 20% sodium carbonate was added, followed by water distilled to make a volume of 10 mL. The solution was then diluted as needed and analyzed by UV-Vis spectrophotometry at a wavelength of 760 nm [14].

In Vitro Cytotoxicity

The tools were sterilized in an autoclave at 121˚C and 15 lb of pressure. A complete medium was prepared to contain RPMI, 15% FBS, 0.25% Fungizone (Amphotericin B), and 1.5% penicillin-streptomycin. HeLa and WiDr cells in ampoules were taken from a liquid nitrogen tank, melted at 37°C, and sprayed with 70% alcohol. Cell preparation was carried out in a tissue culture flask until 80% confluence was achieved, and then the cells were harvested and counted using a hemocytometer. Pumpkin seed n-hexane extract was dissolved in DMSO at a less than 0.2% concentration. Then a concentration series of 500, 250, 125, and 62.5 µg/mL was made in the culture medium. Similarly, a doxorubicin concentration series of 20, 10, 5, and 2.5 µg/mL was made. Cells were distributed into 96-well plates, incubated for 24 hours, washed, and given the MTT reagent. The cells were then incubated for 3 hours. Live cells reacted with MTT to form purple formazan crystals, dissolved in SDS, and left to stand for 24 hours. The solution was then read using an ELISA reader at a wavelength of 595 nm. Data analysis was performed based on the IC_{50} value.

RESULT AND DISCUSSION

Plant extracts

Extraction is the initial method of separating an active substance from a mixture using a solvent. In this study, extraction was carried out using the soxhletation method. Pumpkin seed powder extracted using the soxhletation method with n-hexane solvent obtained results with an extract weight of 36 grams with a yield of 36%.

Phytochemical screening

Phytochemical analysis was conducted to determine the presence of secondary metabolites in the extract [15]. Results of the analysis showed that the N-hexane extract of pumpkin seeds contained compounds belonging to the flavonoid, alkaloid, and steroid groups (Table 1). Tannin compounds, however, were not detected in the extract due to various factors that may have affected the phytochemical screening process, including pre-extraction factors such as plant parts used, Simplicia particle size, moisture content, and drying method, as well as extraction method, type of solvent used, pH, solvent temperature, and long extraction time[16,17]. Flavonoids play a crucial role in cancer and have the potential as cytotoxic agents[18]. Alkaloids, on the other hand, have a high potential as anticancer agents and are known to be the basis of drug development for various diseases, such as antitumor, anti-inflammatory, and antibacterial[19,20]. Steroid compounds have also been found to possess pharmacological activities, such as antioxidant, neuroprotective, and antihypercholesterolemic activities [21].

Table 1: Results of the Phytochemical Screening N-hexane Extract of Pumpkin Seed

Compounds	*Reagent*	*Result*	*Information*
Flavonoids	NaOH 20%	yellow solution	+
Alkaloids	Dragendorff	orange-brown precipitate	+
Tannins	$FeCl_3$ 10%	Fawn	-
Triterpenoids and Steroids	Chloroform + acetic anhydride + concentrated sulfate acid through the tube wall	yellow with green fluorescence	+ (steroids)

*Note: (+): presence, (-): absence of phytochemicals.

Total flavonoid content

The determination of flavonoid levels in this study was carried out using the colorimetric method with aluminum chloride. The principle of this method is that the N-hexane extract of pumpkin seeds reacts with aluminum chloride to form a stable complex with the carbonyl group at C4 and the hydroxyl groups at C3 and C5 of flavone and flavonoid compounds, as well as with the ortho hydroxyl groups on the A or B ring of flavonoids [22,23]. The standard used to determine flavonoid levels were quercetin, which has a wavelength of 510 nm. Quercetin was chosen as a standard because it is a flavonoid compound that can react with aluminum chloride to form a complex [24]. According to Table 2, flavonoid compounds had the highest total content compared to other secondary metabolites, with a total amount of 616.46 mg/L.

Total alkaloid content

The determination of the total alkaloid content of N-hexane Extract of Pumpkin Seed was carried out using the UV-Vis spectrophotometry method. Firstly, the N-hexane extract from pumpkin seeds was obtained using a chloroform solvent, which resulted in two phases. The lower phase contained the chloroform component, while the upper phase consisted of hydrochloric acid. The alkaloids present in the N-hexane Extract of Pumpkin Seed were made to react with NH3 and attract H+ to form free alkaloids in chloroform. The ammonia was separated in another phase and then evaporated to produce a residue, which is the total alkaloid. The total alkaloid content of N-hexane Extract of Pumpkin Seed was found to be 1.77%. However, the total alkaloid levels in this study were not as high as the total levels of flavonoids. Quinine, a natural alkaloid compound derived from the Cinchona plant, was used as the standard compound in this study[25].

Total phenolic content

The standard solution used is gallic acid, which is a phenolic compound derived from hydroxybenzoic acid. Gallic acid is a stable phenolic compound with high solubility and relatively inexpensive compared to other compounds [26]. The N-hexane extract of pumpkin seeds and gallic acid were reacted using the Folin-Ciocalteu reagent to produce a yellow color indicating the presence of phenolic group compounds. Na_2CO_3 was then added to the mixture to create an alkaline atmosphere, which facilitated the formation of oxotungstate and oxomolybdate. Proton dissociation occurred in the phenolic compounds, resulting in phenolic ions, as indicated by the production of a blue color in the sample. The more intense the blue color, the higher the total phenol content in the extract [27]. The level of total phenolic compounds in this study was 322.99 mg/L.

Table 2: Results of the Total Flavonoid, Alkaloid and Phenolic Content

No	*Parameters*	*Result*
1.	Total Flavonoid	616.46 mg/L
2.	Alkaloid total equivalent to Quinine	1.77 %b/v
3.	Phenolic total equivalent to Gallic Acid	322.99 mg/L

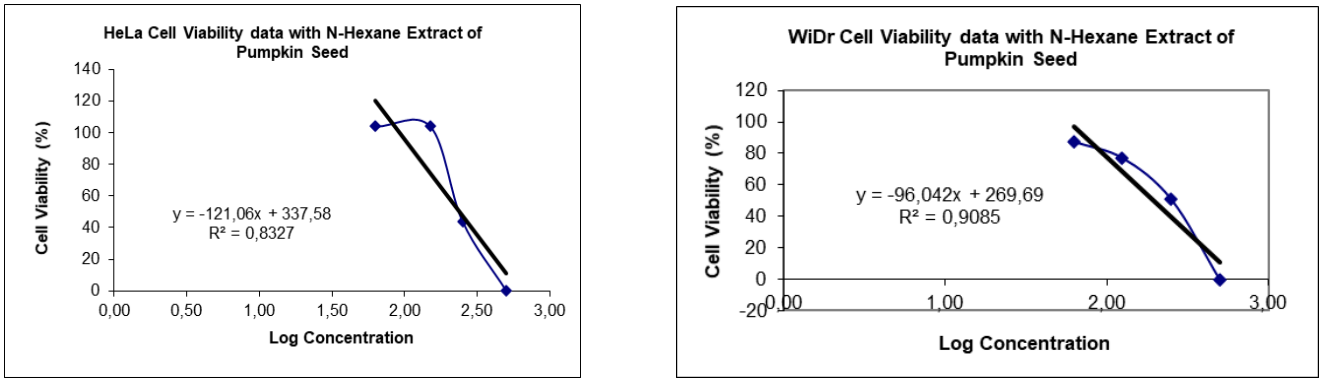
In Vitro Cytotoxicity

The in vitro cytotoxic test was carried out on HeLa cervical cancer cells and WiDr colon cancer cells to determine the ability n-hexane extract of pumpkin seed to inhibit cancer cells using the MTT method ((3-(4,5-dimethylthyazol-2-yl))-2,5-diphenyl tetrazolium bromide) assay. In this study, doxorubicin was used as the positive contro. The use of doxorubicin as a positive control is due to its broad-spectrum chemotherapeutic properties, which make it widely used in the treatment of various types of cancer, including cervical cancer and colon cancer[28]. The parameters used were based on the IC_{50} value, which indicates the concentration of a sample required to produce cell proliferation barriers of50%. The MTT test results in (Table 3) show that the IC_{50} value of the N-hexane Extract of Pumpkin Seeds against WiDr cells is 193.44 µg/mL, with a linear result of y = -96.04x + 269.6, R2 = 0.908. Meanwhile, the results of the MTT test on N-hexane Extract of Pumpkin Seed against HeLa cells showed linear results, with an IC_{50} of 237.7 µg/mL, y = -121.0x + 337.5, R2 = 0.832. The results of this study indicate that the cytotoxic activity of N-hexane Extract of Pumpkin Seed on HeLa and WiDr cells was moderate or moderately toxic, with IC_{50} values ranging from 100 to 500 µg/mL.

The drug doxorubicin in this study has a stronger cytotoxic potential than the N-hexane Extract of Pumpkin Seed, with a large IC_{50} value in WiDr cells of 0.0314 µg/mL and in HeLa cells of 0.0002 µg/mL. Nevertheless, the results of this study suggest the cytotoxic potential of N-hexane Extract of Pumpkin Seed, which can be used as a chemopreventive agent because its IC_{50} value falls within the moderate category. The cytotoxic potential of pumpkin seeds has been proven in a previous study conducted by Al-Okbi, et al (2016) showing that there is potential for cytotoxic activity in Egyptian and European pumpkin seed oil against some cancer cells with the result of the study being an IC_{50} value of 0.517 µg/ mL and 0.483 µg/mL against HepG-2 cells; IC_{50} value of 0.483 µg/mL and 0.517 µg/mL for MCF-7 cells; and IC_{50} values of 0.483 µg/mL and 0.483 µg/mL for Caco-2 cells [8]. The cytotoxic potential of the N-hexane Extract of Pumpkin Seed is due to the content of secondary metabolites. The potential for cytotoxic activity by flavonoids is due to their ability to inhibit the activity of DNA topoisomerase I/II[29]. The potential for cytotoxic activity by alkaloids is due to the ability to inhibit the topoisomerase II. The potential for cytotoxic activity by steroids is thought to be due to the presence of inhibitory enzymes, namely aromatase and sulfatase inhibitors [30].

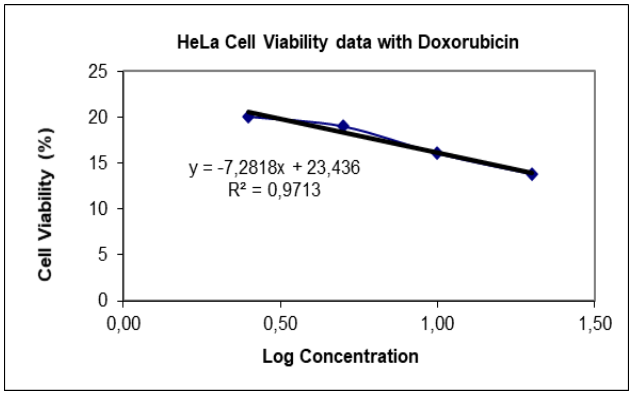
Table 3: Cytotoxicity Activity of N-hexane Extract of Pumpkin Seed and Doxorubicin

Cell Line	*IC_{50} Value (µg/mL)*		*Linear Equation*	
	N-hexane Extract of Pumpkin Seed	*Doxorubicin*	*N-hexane Extract of Pumpkin Seed*	*Doxorubicin*
WiDr	193.44	0.0314	y = -96.04x + 269.6 R2 = 0.908	y= -10,75x + 33,85 R2 = 0,935
HeLa	237.7	0.0002	y = -121.0x + 337.5 R2 = 0.832	y= -7,281x + 23,43 R2 = 0,971

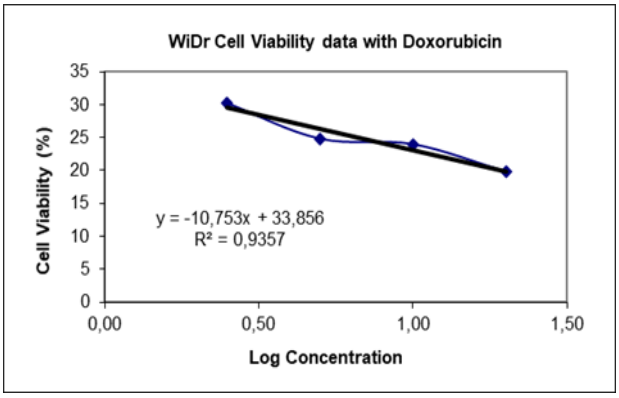


(a) (b)

Figure 1: Graphic of HeLa Cell Viability (a) and WiDr Cell Viability (b) on N-hexane Extract of Pumpkin Seed



(a)



(b)

Figure 2: Graphic of HeLa Cell Viability (a) and WiDr Cell Viability (b) on Doxorubicin

CONCLUSION

In the present study the n-hexane extract of pumpkin seed contains flavonoids, alkaloids, and steroids. The compound that observed in the n-hexane extract of pumpkin seed belongs to the flavonoid group. The cytotoxic activity of the n-hexane extract of pumpkin seed against HeLa and WiDr cells was found to be moderate cytotoxicity that provided potency as a chemopreventive agent. Further research is necessary to isolate, identify, and determine the specific compounds from pumpkin seeds extract that provide more potent cytotoxic activities.

ACKNOWLEDGEMENTS

This work supported by Lembaga Riset dan Inovasi (LRI) Universitas Muhammadiyah Yogyakarta and Department of Pharmacy, Faculty of Medicine and Health Science, Universitas Muhammadiyah Yogyakarta, Indonesia

CONFLIC OF INTEREST

The authors declare no conflict of interest.

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