

IN VITRO CYTOTOXIC ACTIVITY OF SECOISOLARICIRE SINOL DIGLUCOSIDE ON HT-29, PA-1 CELL LINES, AND α -AMYLASE INHIBITORY ACTIVITY

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ABSTRACT

Objective: The present study was conducted to evaluate the *in vitro* cytotoxic activity and α -amylase inhibitory activity of secoisolariciresinol diglucoside (SDG).

Methods: The cytotoxic activity was conducted on HT-29 (human colon cancer cell line) and PA-1 (human ovarian cancer cell line) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and the α -amylase inhibitory activity using acarbose as a standard. Both the tests were evaluated at different concentrations, 3.125–100 μ g/ml and 50–2000 μ g correspondingly and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The effect of the samples on the proliferation of HT-29 and PA-1 was expressed as the percentage cell viability.

Results: SDG exhibited a considerable dose- and time-dependent inhibition on both HT-29 and PA-1 and also observed a concentration-dependent α -amylase inhibitory activity that leads in reduction of starch hydrolysis and hence eventually to lowered glucose levels.

Conclusion: The present *in vitro* study concluded that SDG can be a potent anticancer and moderate hyperglycemic component.

Keywords: Secoisolariciresinol diglucoside, HT-29 cell lines, PA-1 cell lines, α -amylase inhibition.

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INTRODUCTION

Cancer is a communal health problem all over the world which may affect many different parts of the body. If the process is not controlled, it could progress until it causes the death of the organism [1]. Plants are the source of medicine from thousands of years and phytochemicals are continued to play an essential role in medicine. The use of medicinal plant extracts for the treatment of human diseases is an ancient practice; this has greatly increased in recent years. For a long time, plants are being used in the treatment of cancer [2]. Usage of medicinal plants for infection as curative by man is an old practice. In the past time, man searches for drugs from different sources such as preserved monuments, writer documents, and from plants [3]. Since early times, plants were playing an important and key role in food and drug administration [4]. Medicinal plants are considered as a healthy source of human life due to their therapeutical capacities in the treatment of different diseases [5].

Natural products and its related drugs are used to treat about 87% of all categorized human diseases including bacterial infections, cancer, and immunological disorders [6]. About 25% of prescribed drugs in the world originate from plants [7] and over 3000 species of plants have been reported to have anticancer properties [8]. About 80% of the populations in developing countries rely on traditional plant-based medicines for their primary health-care needs [9]. Natural products possess immense pharmacological significance in the development of drugs and were discovered through plant bioprospecting. Majority of the drugs, such as paclitaxel, etoposide, camptothecin, vinca alkaloids, indole alkaloids, podophyllotoxin derivatives, etoposide, and teniposide, at present used in clinical cancer chemotherapy, were originally derived from plants [10].

Phytochemicals with various bioactivities including antioxidant, anti-inflammatory, and anticancer properties are extensively extracted

from many plant species. To date, the potential of using novel bioactive plant extracts for cancer prevention is still dominating over synthetic chemicals present in drug production [11-13]. Numerous studies indicated that in many types of cancer cells, dietary agents would induce apoptosis and cell cycle arrest without affecting the normal cells [14,15]. Pharmacological research on the medicinal properties of phytochemicals has become mandatory, to establish the claimed medicinal properties of herbs [16]. The World Health Organization estimated that about 80% of the populations in developing countries rely on traditional medicines, mostly plant drugs, for their primary health-care needs [17,18].

Secoisolariciresinol diglucoside (SDG) is the major lignin found in *Linum usitatissimum* [19], a range of studies on SDG supported its antioxidant [20,21] and anticancer [22] properties. Mammalian lignans show structural similarities to estradiol [23]. Experimental studies have been shown that flaxseed and SDG are shown to be protective against azoxymethane-induced colon carcinogenesis in rats [24,25]. Aberrant crypt foci (ACF) of the colon are possible precursors of adenoma and cancer and were described as lesions consisting of large, thick crypts in methylene blue-stained specimens of colon from mice treated with a carcinogen [26].

In animals, α -amylase inhibitors drop the high glucose levels that can occur post-meal by decelerating the speed with which α -amylase can transform starch to simple sugars [27]. α -Amylase inhibitors offer an effective strategy to lower the levels of slowing the speed with which α -amylase can convert starch to simple sugars until the body can deal with it. This is of particular importance in those with diabetes, where low insulin levels prevent extracellular glucose from being cleared quickly from the blood. The finest approach for controlling postprandial hyperglycemia is to reduce the carbohydrate hydrolyzing enzymes such as α -amylase and α -glucosidase in the digestive system [28].

However, this *in vitro* study outlines the cytotoxic activity of the SDG against HT-29 and PA-1 cell lines since both cell lines were not studied much and also not much data have been available. The 50% inhibitory concentration (IC₅₀) was calculated and represented, also evaluated the α-amylase inhibitory activity of SDG.

METHODS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent, dimethyl sulfoxide (DMSO), carbon dioxide (CO₂) incubator, and Spectrostar Nano enzyme-linked immunosorbent assay (ELISA) microplate reader were used. Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS), Pen strip, Trypsin procured from Invitrogen, α-amylase purchased from Loba, starch, dinitrosalicylic acid, Na₂SO₃, and NaOH used were obtained from Sisco Research Laboratories, SDG isolated from flaxseed [29].

Cell lines and culture medium

HT-29 (human colon cancer cell line) was procured from ATCC, human ovarian teratocarcinoma cell lines (PA-1) were procured from NCCS, stock cells were cultured in medium supplemented with 10% inactivated FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with Trypsin Phosphate Versene Glucose solution (0.2% trypsin, 0.02% ethylenediaminetetraacetic acid, and 0.05% glucose in phosphate buffer saline [PBS]). The cell viability was checked and centrifuged. Further, 50,000 cells/well of Jurkat were seeded in a 96-well microtiter plate and incubated for 24 h, 48 h, and 72 h at 37°C, 5% CO₂ incubator.

Preparation of test solutions for cytotoxic study

A 3–100 µg/ml of serial dilutions of SDG were prepared to carry out cytotoxic studies.

Cytotoxic study by MTT assay

Cytotoxicity of the SDG on HT-29 cells and PA-1 cells was evaluated by studying the cell viability by MTT assay [30,31]. The monolayer

cell culture was trypsinized and the cell count was adjusted to 1.0×10⁵ cells/ml using the media containing 10% FBS. Added 100 µL of the diluted cell suspension (50,000 cells/well) to each well of the 96-well microtiter plates. After 24 h of incubation, when a partial monolayer was formed, the supernatant was flipped off. The monolayer was washed with medium once and 100 µL of different test concentrations of 3.125 µg/ml, 6.25 µg/ml, 9.5 µg/ml, 25 µg/ml, 50 µg/ml, and

Table 1: *In vitro* cytotoxicity of secoisolariciresinol diglucoside on HT-29 cell line

Concentration (µg/ml)	% cell viability (mean)			IC ₅₀		
	24 h	48 h	72 h	24 h	48 h	72 h
3.125	95.55	97.43	96.51			
6.25	93.55	94.60	94.49			
12.5	90.45	88.52	84.67	>100	>100	75.58
25	88.47	82.10	71.79			
50	82.83	71.20	53.07			
100	70.62	50.40	43.70			

Cell viability percentage is presented as mean of triplicate

Table 2: *In vitro* cytotoxicity of secoisolariciresinol diglucoside on PA-1 cell line

Concentration (µg/ml)	% cell viability (mean)			IC ₅₀		
	24 h	48 h	72 h	24 h	48 h	72 h
3.125	97.52	92.08	90.34			
6.25	86.56	83.86	79.94			
12.5	79.33	75.73	70.58	112.70	72.69	57.49
25	73.12	69.59	65.78			
50	58.54	52.71	49.97			
100	51.59	44.38	41.77			

Cell viability percentage is presented as mean of triplicate

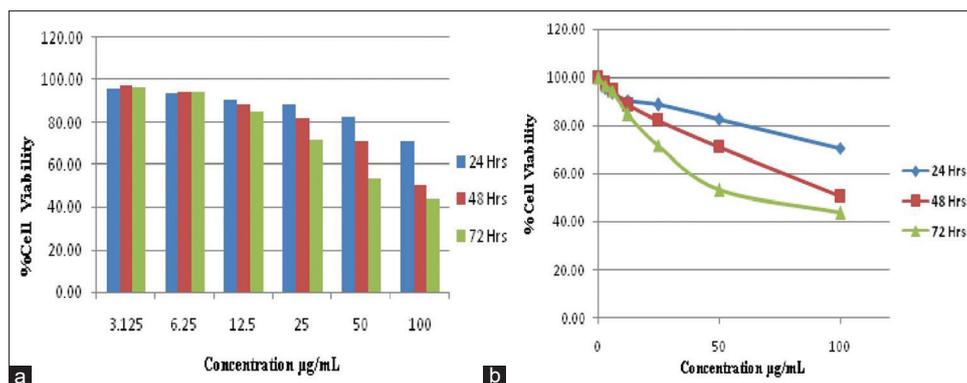


Fig. 1: (a and b) Cytotoxicity of secoisolariciresinol diglucoside on HT-29 cell line

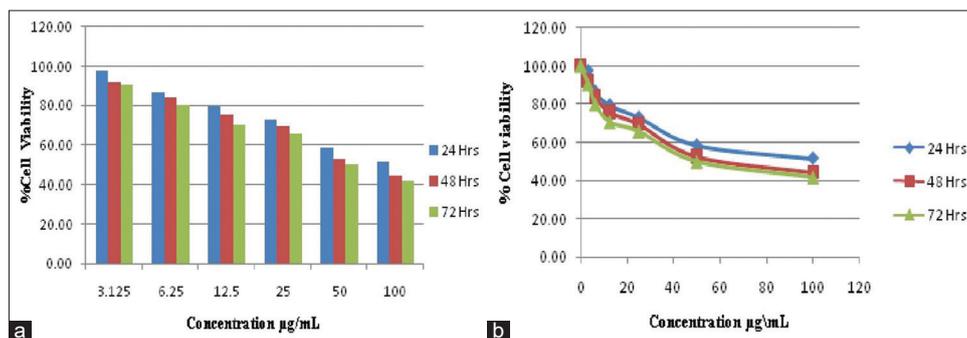


Fig. 2: (a and b) Cytotoxicity of secoisolariciresinol diglucoside on PA-1 cell line

100 µg/ml of SDG were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for another 3 days in 5% CO₂ atmosphere. Then, 100 µL of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated further for 4 h at 37°C in 5% CO₂ atmosphere [32]. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to dissolve the formed formazan. The absorbance was measured at different interval time points of 24 h, 48 h, and 72 h of incubation periods for both the cell lines. The absorbance was measured using Spectrostar Nano ELISA microplate reader at a wavelength of 570 nm for viable cells. The percentage growth inhibition was calculated and the concentration of SDG needed to inhibit cell growth by 50% (IC₅₀) determined graphically. The absorbance was measured using wells without sample containing cells as blanks.

Table 3: α-amylase inhibition activity of SDG

Concentration	Control Abs	SDG Abs	Acarbose Abs	% inhibition	
				SDG	Acarbose
50 µg	0.184	0.192	0.263	4	30
100 µg		0.198	0.289	7	36
250 µg		0.234	0.328	21	44
500 µg		0.247	0.396	26	54
1000 µg		0.252	0.574	27	68
2000 µg		0.279	0.862	34	79

SDG: Secoisolariciresinol diglucoside

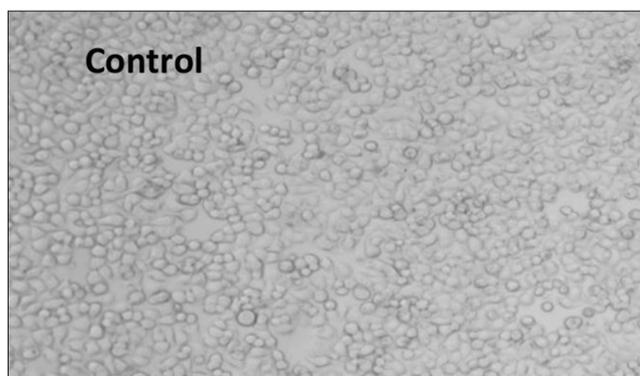


Fig. 3: Morphology of secoisolariciresinol diglucoside-treated HT-29 cells (control)

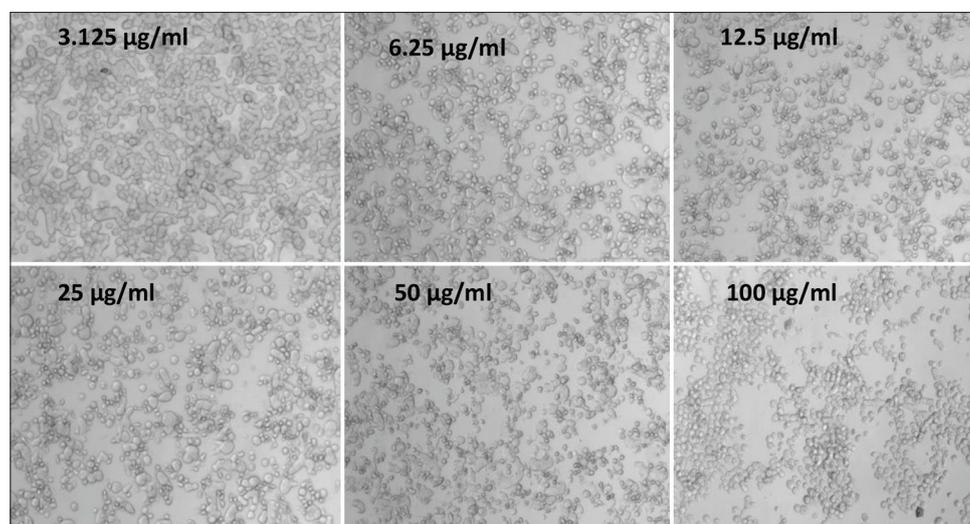


Fig. 4: Morphology of secoisolariciresinol diglucoside-treated HT-29 cells (24 h)

Preparation of test solutions for cytotoxic study

For α-amylase inhibition assay, various concentrations of sample solutions 50 µg, 100 µg, 250 µg, 500 µg, 1000 µg, and 2000 µg were prepared.

α-amylase inhibition assay

The assay was carried out using transformed method of Sudha *et al.* International Society for Complementary Medicine Research [33]. A 0.1 mg/ml concentration of α-amylase was dissolved in (PBS, 0.02 mol/L, pH 6.8). A 0.25 ml of α-amylase solution was added to 0.25 ml of each sample concentration and incubated at 37°C for 5 min. The reaction was originated by adding 0.5 ml of 1.0% (w/v) starch substrate solution and incubated for 3 min at 37°C. The reaction was halted by adding 0.5 ml 3,5-dinitrosalicylic acid (which was prepared by mixing 1% dinitrosalicylic acid, 0.05% Na₂SO₃, and 1% NaOH solution) reagent to the reaction mixture and heated at 100°C for 5 min.

RESULTS AND DISCUSSION

Cytotoxic activity

The effect of the samples on the proliferation of HT-29 and PA-1 was expressed as the percentage cell viability, using the following formula:

$$\% \text{ Cell viability} = (\text{OD of the sample} / \text{OD of the Control}) \times 100$$

The cytotoxic potential of SDG was examined by MTS assay. The numbers of viable HT-29 and PA-1 cells were decreased after treatment with SDG (Figs. 3-10). Former studies proposed that EL and ED significantly reduced the proliferation of human colon tumor cell lines LS174T, Caco-2, HCT-15, and T-84 after incubation with various levels of EL and ED for 8–10 days [34]. A study indicated flaxseed decreases the risk for colon carcinogenesis, the total number of ACs and foci was significantly reduced by 41–53% and 48–57%, respectively [24]. Another study proven that flaxseed and defatted flaxseed have a colon cancer-protective effect and that this effect is due, in part, to standard deviation (SD) and not the oil at 2.5–5% levels of intake. The colon cancer-protective effects of flaxseed, defatted flaxseed, and SD are associated with a dose-dependent increase in cecal activity of P-glucuronidase and urinary lignan excretion, however, not to short-chain fatty acids production or pH [35]. In a 28-day feeding study on carcinogen-treated rats, flaxseed and defatted flaxseed have been shown to significantly reduce epithelial cell proliferation and the number of ACs and ACF which are thought to be early markers of colon cancer risk. The effect of flaxseed at 5 and 10% levels was not dose related. The study on

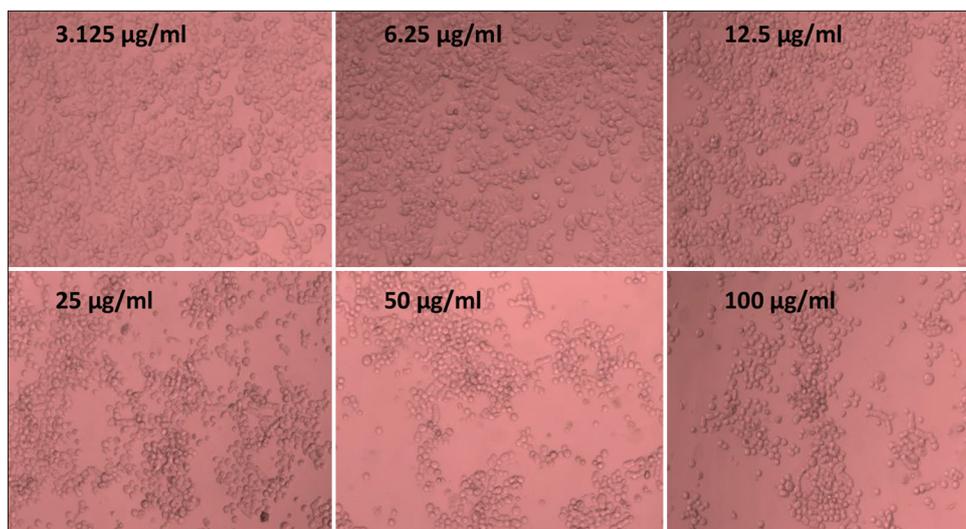


Fig. 5: Morphology of secoisolariciresinol diglucoside-treated HT-29 cells (48 h)

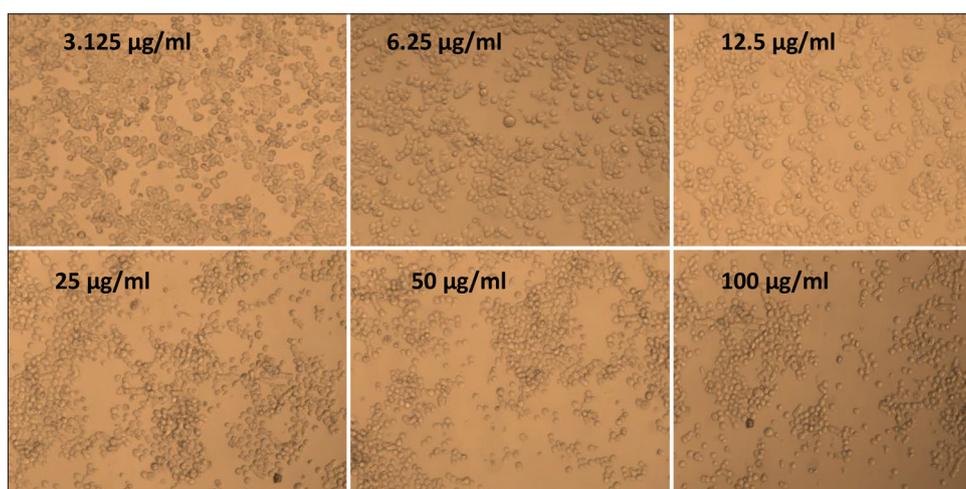


Fig. 6: Morphology of secoisolariciresinol diglucoside-treated HT-29 cells (72 h)

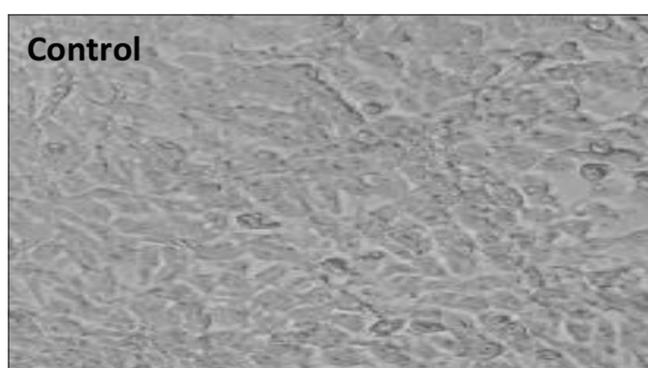


Fig. 7: Morphology of secoisolariciresinol diglucoside-treated PA-1 cells (control)

rye bran, flaxseed, and SDG has been shown to be protective against azoxymethane-induced colon carcinogenesis in rats [24,25].

Unlike the previous studies, the current *in vitro* study has been carried out only up to 72 h, at lower concentrations of SDG which was isolated

from the defatted flaxseed indicated a dose- and time-dependent cytotoxic activity.

IC_{50} – A concentration of an inhibitor where the response (or binding) is reduced by half of its maximum. IC_{50} values for the present cytotoxicity studies were derived from a non-linear regression analysis (curve fit) based on sigmoid dose-response curve. The IC_{50} values of HT-29 cell lines were >100 µg/ml for 24 h, 192.91 µg/ml for 48 h, and for 75.58 µg/ml 72 h. Whereas for PA-1, the IC_{50} values were 112.70 µg/ml, 72.69 µg/ml, and 57.49 µg/ml for 24 h, 48 h, and 72 h, respectively.

As shown in Figs. 1 and 2, SDG shows significant concentration-dependent inhibition on propagation and viability of the colon cancer cell line (HT-29) and ovarian cell lines (PA-1). IC_{50} values are summarized in Tables 1 and 2 which are clearly indicating that SDG is proficient to inhibited the growth of two human cancer cell lines, namely, HT-29 and PA-1. Morphology of the SDG-treated HT-29 cells is presented in the consequent, Figs. 3-10.

α -amylase inhibition activity

After cooling the reaction mixture to room temperature, the absorbance was recorded by ELICO, SL-210 spectrophotometer. The absorbance (Abs) recorded at 540 nm by keeping diluents (PBS) as a control.

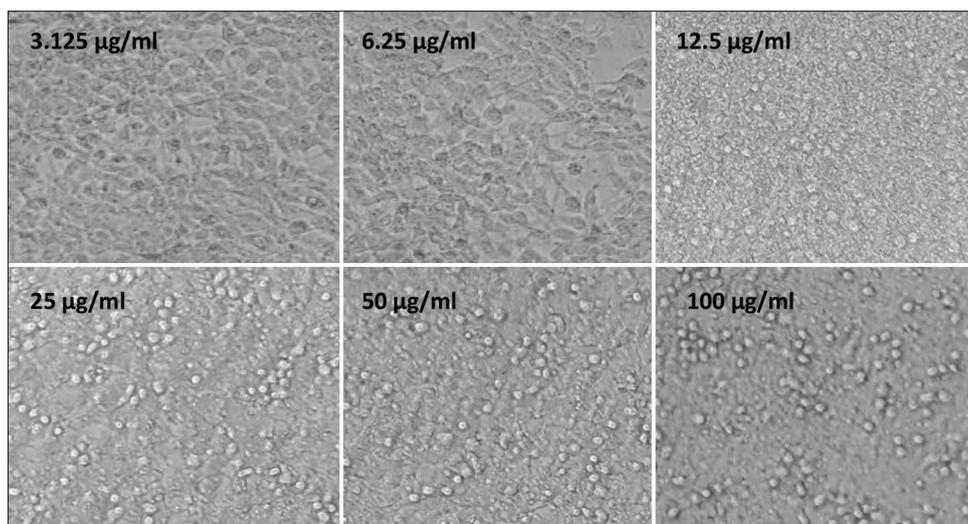


Fig. 8: Morphology of secoisolariciresinol diglucoside-treated PA-1 cells (24 h)

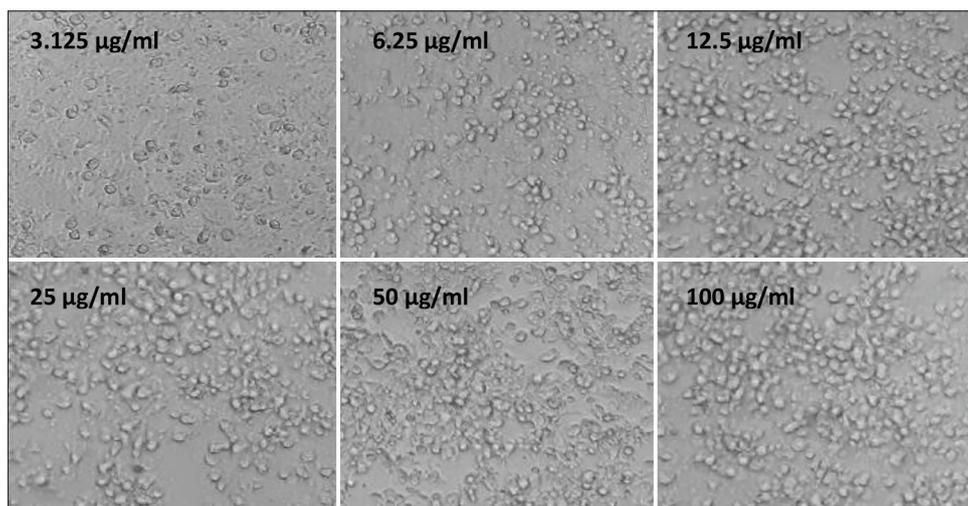


Fig. 9: Morphology of secoisolariciresinol diglucoside-treated PA-1 cells (48 h)

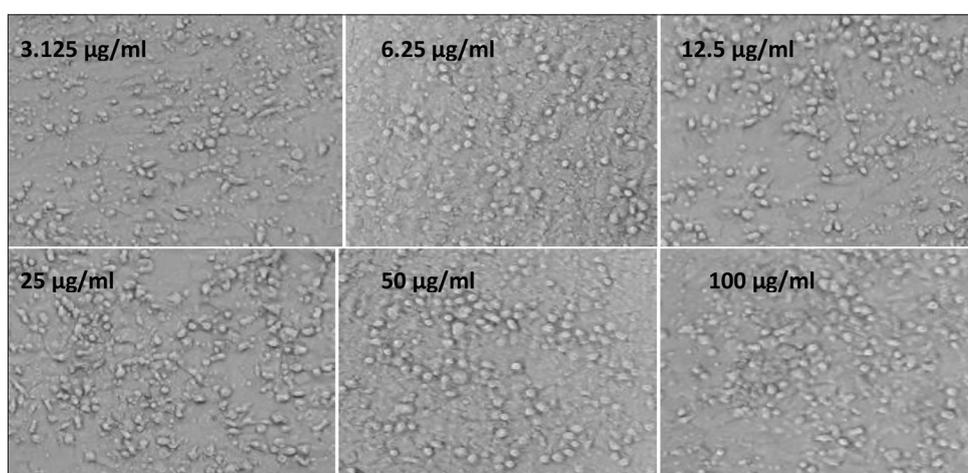


Fig. 10: Morphology of secoisolariciresinol diglucoside-treated PA-1 cells (72 h)

The inhibition percentage was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{[\text{Abs1} - \text{Abs2}]/\text{Abs1} \times 100}{\text{where, Abs1=sample and Abs2=control.}}$$

The previous studies shown α -amylase inhibitory potential of flaxseed extracts of isopropanol, acetone, and methyl t-butyl ether [30]. However, the current study demonstrated the α -amylase inhibitory of pure SDG which is exhibiting relatively similar inhibition to that of acetone

fraction ($\approx 30\%$) Table 3. SDG has shown to be efficient in preventing or delaying the progress of diabetes mellitus in animal models, which was considered to be attributable to its strong antioxidant activity [36,37]. SDG was also shown to suppress the expression of the phosphoenolpyruvate carboxykinase gene that codes for the rate-limiting enzyme responsible for gluconeogenesis in the liver [38]; these studies have been proved that SDG prevented the development of diabetes.

CONCLUSION

Based on the above results, it is concluded that SDG isolated from flaxseed exhibited cytotoxic activity against colon and ovarian cancer cells and moderate hyperglycemic activity by inhibiting α -amylase. It is showed a considerable dose-dependent inhibition. α -amylase inhibitors from plant sources possess natural therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch and delaying carbohydrate absorption in the small intestine and may have potential for use in the treatment of diabetes mellitus and obesity. However, clinical studies need the confirmation of SDG as safe and effective with supporting evidence from human and animal studies for future research.

AUTHORS' CONTRIBUTIONS

The author declares that this work was done by the author named in this article.

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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