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Cytotoxic Activities of *Cyamopsis tetragonoloba* L. Seed Extracts Against huh-7 Human Liver Cancer Cells

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Abstract

Finding of new effective anti-cancer agents has become a top goal around the world. Thus, the objective of this study was to investigate the cytotoxic activities of guar seeds methanolic extract (GSME), guar seeds oil (GSO), guar gum (GG) and guar saponin (GSP) from seeds against huh-7 cancer cell line. For 72 hours, cells were exposed to 0.01, 0.1, 1.0, 10, 100 µg/ml of various guar seeds extracts (GSME, GSO, GG, and GSP), then the SRB assay was performed three times in triplicate to determine cell viability %, cell growth inhibition % and half-maximal inhibitory concentration (IC_{50}). In addition, phase contrast inverted microscopy was used to examine the morphological changes in the cells. The huh-7 cells were shown to be cytotoxic to all guar seed extracts. Among the various examined preparations, guar seed saponin (GSP) was shown to be the most cytotoxic substance at a concentration of 100 µg/ml towards huh-7 cells. As a result, the guar seed tested extracts could potentially be used in the creation of anticancer drugs.

Keywords: *Cytotoxicity, liver cancer cells, guar seeds.*

Introduction

Legume seeds have gained popularity as functional foods due to their high levels of amino acids, fiber, minerals, vitamins, flavonoids, and phenolic acids (Prabaharan, 2011; Bouchenak and Lamri-Senhadji, 2013). The guar bean, often known as the cluster bean (*Cyamopsis tetragonoloba* L), is a legume and a tropical African and Asian plant that belongs to the *Fabaceae*, family. It is a well-known traditional plant that has been utilized in folk medicine for centuries and can be used as an appetizer, cooling agent, digestive aid, laxative, anti-ulcer, cytoprotective and others (Mukhtar *et al*., 2006). Furthermore, guar beans could be a rich source of extra phytochemicals (Wang and Morris, 2007). Different concentrations of guar seeds methanol extracts showed antibacterial efficacy against various bacterial strains (Hassan *et al.,* 2010). Phytochemicals such as quinone, steroids, flavonoids, cardiac glycoside, and terpenoids have been identified in the hexane extract of guar seeds (Ganatra *et al*., 2013). One of the major phytochemicals produced by *Cyamopsis tetragonoloba* seeds is guar gum. It's a non-ionic branching polysaccharide with a simple structure (Frias and Sgarbier, 1998). Guar gum has been shown to have the ability to bind harmful chemicals and transport them out of the body, as well as to drastically lower blood sugar, cholesterol, triglycerides, and lipid levels in both

normal and diabetic animals (Bhandari and Prasad, 1991; Frias and Sgarbier, 1998). Guar gum and its derivatives can be used as a food functional supplement to produce possible cancer chemopreventive and anti-inflammatory properties in risk communities (Gamal-Eldeen *et al.,* 2006). Guar also contains saponins which are glycoside substances formed of a fat-soluble nucleus (aglycone) and water-soluble sugars (glycone). The aglycone is either a triterpenoid (C-30) as in guar or alkaloid steroid (C-27) as in other plants. At various carbon positions, one or more side-chains of water-soluble sugars (glycone) are connected to the aglycone nucleus via ester bonds (Hassan *et al*., 2010). Saponins exhibit a variety of biological properties, including antibacterial (Sen *et al.,* 1998) and hemolytic (Khalil and EI-Adawy, 1994) activities. Furthermore, plant saponins were found to have a cytotoxic effect on different of cancer cell lines (Alam *et al.,* 2017). Uncontrolled cell proliferation is one of the pathological features of cancer (Li *et al*., 2017), and inhibiting cell cycle progression is considered a realistic method to eradicating cancer cells (Otto and Sicinski, 2017). Many studies have linked legume dietary to health benefits such as protection against cardiovascular disease, breast cancer, colon cancer, and other cancers (Messina, 1999; Mathers, 2002). The anti-cancer activity of guar seed extract has been revealed in PC-3 and human colorectal carcinomas (HCT116 and CACO-2) (Badr *et al*., 2014). The search for new effective anti-cancer drugs has become a major priority in cancer treatment around the world. The anticancer properties of various guar seed extracts have been studied in limited research. Therefore, the present work aimed to investigate the cytotoxicity of GSME, GSO, GG, and GSP against huh-7 human liver cancer cell line.

Material and Methods

Plant material

The guar seeds (*Cyamopsis tetragonoloba* L.) were collected from the Crops Research Institute, Agriculture Research Center, Giza, Egypt. The seeds are render them dust free, washed under running water, dried, and then crushed using grain mill (Moulinex, France) to a very fine powder at the Department of Agricultural Chemistry, Minia University, El-Minya, Egypt and stored until further use.

Methanolic extract of guar seeds

In a brief, the guar seeds methanol extract was prepared by extracting 20 g of guar seeds powder in 100 ml methanol and shaking it for 24 hours on a rotary shaker. The extract was filtered, then centrifuged for 15 minutes at 5000 g and dried (Badr *et al*., 2014).

Guar seeds oil extraction

Soxhlet apparatus was used to extract the oil content. In a brief, 10 g of guar seed powder was extracted with 200 ml n-hexane at 69^0C for 6 hours in a Soxhlet extractor. The solvent was removed from the obtained oil by rotary evaporation (AOAC, 2000).

Guar gum extraction

The endosperm of guar seeds was carefully separated from the germ and hull, then suspended in absolute ethyl alcohol in a 1:3 (seeds: ethanol) ratio at 70^0C for 15 minutes

to inactivate enzymes and remove low-molecular-weight molecules (Egorov, *et al.,* 2003). The ethanol was removed carefully, and 1:5 (endosperm: water) distilled water was added, and the suspension was allowed to settle for about 24 hours. Then, in a blender, water was added in a 1:10 ratio (suspension: water) and blended for 5 minutes. The mixture was filtered using a nylon net, then centrifuged at 3800 g for 20 minutes at 20^0C. The galactomannan was precipitated by mixing the supernatant with absolute ethyl alcohol in a 1:2 ratio. The ethanol was decanted, and the precipitated galactomannan was air-dried and stored until use (Cerqueira *et al*., 2009).

Saponin isolation

Saponin was extracted for 3 hours by refluxing 25 g of guar seed powder with 250 ml CH_3CH_2OH/H_2O (1:1 v/v). The refluxed extract was cooled and filtered using filter papers with pore sizes of 150 and 125 µm. Ethanol was separated from the filtered extract by evaporating it under reduced pressure in a rotary evaporator until two-thirds of the original volume was gone. Using a separatory funnel, the remaining aqueous extract was fractionated with butanol (1:1 v/v) overnight at room temperature. To improve the yield of saponin extract, higher butanol extract was collected in a glass flask, and lower aqueous extract was collected and separated with butanol two more times. Butanol extracts were consolidated, dried in air, weighed, and kept at room temperature (Hassan *et al.,* 2010).

Cultivation of cells

Nawah Scientific Inc. provided the Huh7 liver cancer cell line (Mokatam, Cairo, Egypt). At 37°C, cells were cultured in DMEM media supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin, and 10% heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere. The vitality of the cells was evaluated before to the starting of the procedures, and they were found to be more than 95% viable.

Cytotoxicity assay

The SRB assay was used to determine cell viability. In 96-well plates, aliquots of 100 µL cell suspension (5 x 10^3 cells) were incubated in full medium for 24 hours. Another aliquot of 100 µL media with GSME, GSO, GG, and GSP dissolved in DMSO at various concentrations (0.01, 0.1, 1, 10, 100 µg/ml) was administered to the cells. After 72 hours of exposure to guar seed extracts, the cells were fixed by replacing the medium with 150 µL of 10% TCA and incubating for 1 hour at 4°C. After removing the TCA solution, the cells were washed 5 times with distilled water. 70 µL SRB solution (0.4 % w/v) was added in aliquots and incubated at room temperature for 10 minutes in the dark. Plates were washed 3 times using 1% acetic acid and left to air-dry for 24 hours. The absorbance was measured at 540 nm using a BMG LABTECH®-FLUO star Omega microplate reader (Ortenberg, Germany) after 150 µL of TRIS (10 mM) was added to dissolve protein-bound SRB stain (Skehan *et al.,* 1990).

% Cell growth = Absorbance sample (treated cells) / Absorbance control (untreated) × 100
% Growth inhibition = 100 - % Cell growth

Furthermore, linear regression analyses were used to calculate the IC_{50} values (the concentration that inhibits cell growth by 50%).

Cellular morphological changes

A contrast inverted microscope at 40X magnification was used to observe morphological changes in huh-7 human liver cancer cells subjected to different doses of guar seed extracts. To detect the morphological alterations, the treated cells were compared to the control cells.

Statistical analysis

The statistical analysis was done using GraphPad Prism® 8 software, that included one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests with a 95 % confidence interval of $P < 0.05$. The comparison between the control and treated cells was considered statistically significant.

Results

Cytotoxic activity

The percentage of huh-7 cell growth was measured after the cells were exposed to various concentrations of GSME, GSO, GG, and GSP at concentrations of 0.01, 0.1, 1, 10, 100 µg/ml for 72 hours, and the results are shown in Table 1. Guar seed extracts prompted a statistically significant ($p < 0.05$) reduction in the percentage of cell growth in a concentration and extract dependent manner. When huh-7 cells were subjected to 0.01 µg/ml and higher doses up to 100 µg/ml of GSME, GSO, GG, and GSP for 72 hours, all of these extracts were found to be cytotoxic for huh-7 cells, as indicated in Table 1. In comparison to the control cells, GSME administration significantly ($P < 0.05$) reduced the percentage of cell growth at concentrations of 10 and 100 µg/ml, which were found to be 96.84 and 94.21%, respectively. At dosage of 100 µg/ml, GSO treatment resulted in a significant ($P < 0.05$) decrease in percent cell growth, which was 93.19%. Furthermore, administration of GG and GSP at concentrations ranging from 0.10 to 100 µg/ml resulted in a significant $P < 0.001$ reduction in the percentage of huh-7 cell proliferation. In addition, the lowest percentage of cell growth was observed (87.87%) at a concentration of 100 µg/ml of GSP, while the highest percentage of cell growth was found (99.03%) at a concentration of 0.01 µg/ml of GSO (Table 1).

Table 1. *In vitro* cytotoxic activity of guar seed extracts against huh-7 human liver cancer cells in the SRB assay

Concentration (µg/ml)	Guar seed extracts			
	GSME	GSO	GG	GSP
	Cell growth %	Cell growth %	Cell growth %	Cell growth %
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.01	98.09 ± 0.51	99.03 ± 0.91	98.36 ± 0.14	96.33 ± 0.25**
0.10	97.44 ± 1.01	99.01 ± 0.31	95.36 ± 0.85**	94.05 ± 0.01****
1.00	97.14 ± 0.28	98.85 ± 0.15	94.06 ± 1.20***	93.71 ± 0.56****
10	96.84 ± 0.62*	95.55 ± 2.56	91.71 ± 0.14****	91.08 ± 1.14****
100	94.21 ± 0.85***	93.19 ± 0.50*	88.07 ± 0.78****	87.87 ± 0.30****

The values are presented as mean ± SEM, and P values are symbolized by stars, *P < 0.05, **P < 0.01,***P < 0.001, ****P < 0.0001 compared to control cells. The viability of the control cells (untreated) was fixed at 100% and in relation to this, the percentage viabilities of the other cells were calculated. GSME = guar seeds methanolic extract, GSO = guar seeds oil, GG = guar gum, GSP = guar saponins

In addition, after 72 hours of incubation with various doses of guar seed extracts, the inhibition percentage of cell growth in huh-7 cells was calculated, and the results are shown in Figure 1. The results showed that GSME at a concentration of 10 and 100 µg/ml can significantly (P < 0.001) decrease cell growth by 3.15 and 5.79 % after 72 hours of incubation, respectively, when compared to untreated cells (Figure 1A). On the other hand, GSO at a dose of 100 µg/ml only, significantly (P < 0.05) reduced cell proliferation by 6.81 % (Figure 1B). GG and GSP at concentrations of 0.1, 1.00, 10, 100 µg/ml significantly (P < 0.0001) inhibited cell proliferation by 4.65, 5.95, 8.29, 11.94, 5.95, 6.29, 8.92, 12.13 %, compared to the control cells (Figure 1 C, D). Additionally, the strongest inhibition percentage of huh-7 cells (12.13 %) was recorded at a concentration of 100 µg/ml of GSP (Figure 1D), all guar seed extracts had IC_{50} values greater than 100 µg/ml (Figure 1 A, B, C, D).

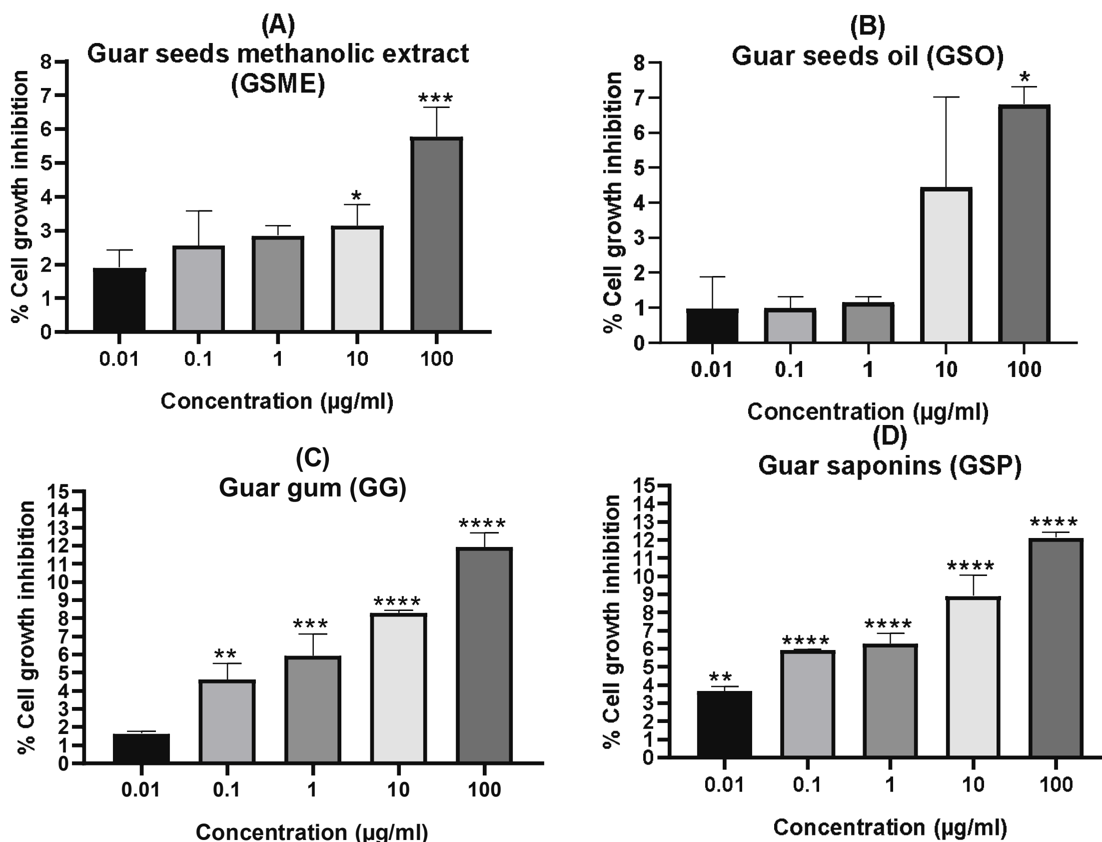

Figure 1. Cell growth inhibition of huh-7 human liver cancer cells induced by *Cyamopsis tetragonoloba* seed extracts in the SRB assay

The values are presented as mean ± SEM, and P values are symbolized by stars, *P < 0.05, **P < 0.01,***P < 0.001, ****P < 0.0001 compared to control cells. The inhibition of the control cells (untreated) was fixed at 0 % and in relation to this, the Cell growth inhibition percentage of the other cells were calculated. Estimated IC_{50} values are greater than 100 µg/ml of all guar seed extracts.

Cellular morphological alterations

The morphological changes in the huh-7 cells after exposure to various *Cyamopsis tetragonoloba* seed extracts with different concentrations were observed using a phase contrast microscope. As shown in Figure 2, the morphological changes in huh-7 cells were found to be concentration and extract dependent. Untreated (control) cells proliferated as colonies with polygonal shape and no voids between them, displaying normal cancer cell morphology. The amount of abnormal and lifeless cells expanded with rising guar seed extracts concentrations. Cells exposed to 0.01 µg/ml and higher doses of GSME for 72 hours showed a reduction in normal shape and cellular volume in compared to control cells. The most significant alterations in cell shape and volume were observed at a concentration of 10 and 100 µg/ml of GSME (Figure 2 B, arrow). Also, GSO administration at a concentration of 100 µg/ml resulted in significant degenerative characteristics such as cell shrinkage, cellular volume reduction (Figure 2 C, arrow), and an

increase in the number of floating or lifeless cells (Figure 2 C, dashed arrow). GG-exposed cells at concentrations of 0.1, 1.00, 10, and 100 µg/ml lost their polygonal form (Figure 2 D, dashed arrow), had a poorly defined nucleus (Figure 2 D, arrow), cytoplasmic vesicles (Figure 2 D, bold arrow) and a damaged cell membrane with leaky cell contents (Figure 2 D, zigzag arrow) suggesting necrotic cell death. Additionally, GSP treatment of huh-7 cells at various doses (0.01, 0.1, 1.00, 10, and 100 µg/ml) resulted in cytoplasm and chromatin condensation (Figure 2 E, arrow), cell shrinkage with a damaged membrane, fragmentation (Figure 2 E, zigzag arrow), and the appearance of apoptotic bodies (Figure 2 E, bold arrow). The most severe changes in cellular structures were caused by 100 µg/ml GSP administration, including cell shrinkage and a ruptured membrane, indicating apoptotic cell death.

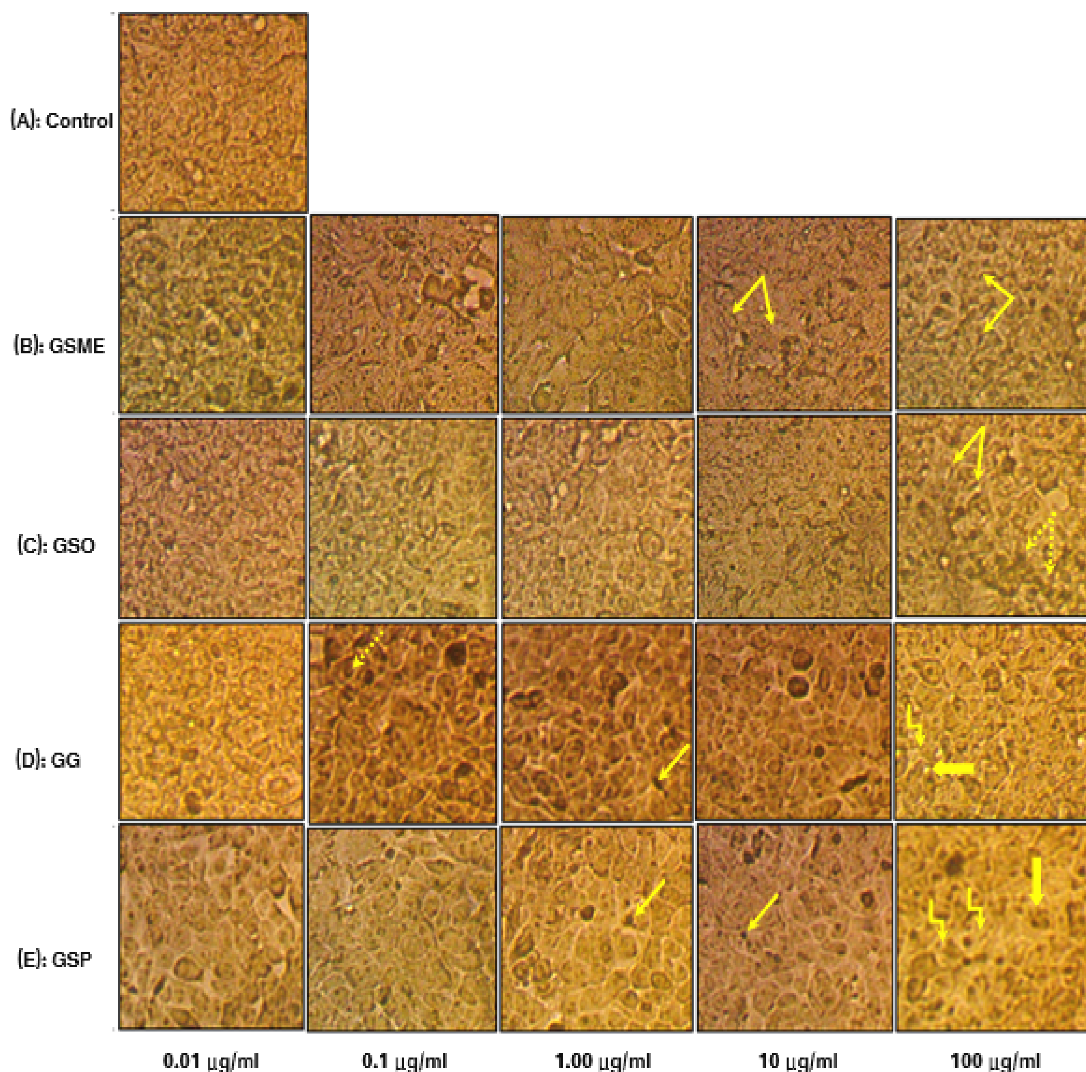

Figure 2. Cellular morphological changes in huh-7 human liver cancer cells induced by various *Cyamopsis tetragonoloba* seed extracts at different concentrations.

(A): Control, (B): GSME = guar seeds methanolic extract, (C): GSO = guar seeds oil, (D): GG = guar gum, (E): GSP = guar saponins. A contrast inverted microscope at a magnification of 40 X was used to capture the images.

Discussion

Modern anti-cancer drug development has become an important factor of cancer therapy projects around the world. Plants have been shown to have anticancer properties that are linked to a range of phytochemicals (Uddin *et al*., 2009). The cytotoxic activity of plant source extracts against human cancer cells has been investigated in many researches (Hamedeyazdan *et al.,* 2012; Zhao *et al.,* 2014). *Cyamopsis tetragonoloba*, also called as Guar, is one such plant in this respect. Little phytochemical biological activity research has been done on this plant. Guar seeds contain a diverse range of natural compounds (Badr *et al*., 2014). Thus, the goal of this study was to examine the cytotoxic properties of several guar seed extracts (GSME, GSO, GG, and GSP) against the huh-7 cancer cell line. The percent of cell growth and the percent of cell growth inhibition in the SRB assay were used to measure the cytotoxic responses of different guar seed extracts. In addition, the morphological alterations in huh-7 cancer cells induced by several guar seed extracts were examined.

Guar bean contains a lot of protein as well as essential amino acids such isoleucine, leucine, phenylalanine, and tyrosine (Saeed *et al.,* 2017). Guar legume seeds are high in flavonoids, phenolic acid, and vitamins, including polyphenols such as gallic acid, gallotannins, myricetin-7-glucoside-3-glycoside, chlorogenic acid, stigmasterol, and other compounds (Tripathi and Pandey, 2016). Different phenolic compounds and fatty acids were identified in the methanolic extract of guar seeds using GC-MS (Badr *et al.,* 2014). Guar seeds methanolic extract (GSME) was found to have cytotoxic action at concentrations of 10 and 100 µg/ml. against huh-7 cancer cells in the current investigation, reducing the percentage of cell proliferation (Table 1) and causing various morphological changes in cellular form and volume (Figure 2 B). In a comparable investigation, the methanolic extract of guar seeds was found to have anticancer properties against CACO-2, HCT116, and PC3 cancer cell lines, with different IC_{50} values (Badr *et al.,* 2014).

Many physiological activities produce metabolites that form reactive oxygen species (ROS), which are extremely unstable due to their unpaired electrons (Ames *et al.,* 1993). ROS can induce a range of underlying disorders in the body, including cardiovascular and cerebrovascular diseases, arthritis, diabetes, skin diseases, ageing processes, and even tumors (Tanzadehpanah *et al.,* 2012). Cancer cells usually create significant amounts of reactive oxygen species (ROS), due to their extremely proliferating nature. As a result, increasing ROS generation needs an increase in antioxidant production (Lieu *et al*., 2020). According to previous studies, excessive levels of essential amino acids and polyphenols in guar seeds may be associated to anticancer properties (Tripathi and Pandey, 2016, Saeed *et al.,* 2017), due to their antioxidant activity (Joondan *et al.,* 2019).

The fatty acid content of guar seeds was determined by Badr *et al.,* 2014 and discovered that cis-linolenic acid is the most prevalent fatty acid in

guar seeds, followed by cis-oleic acid and palmitic acid. At 5.1 %, both linolenic and stearic acids had a similar ratio. Because linolenic acid's metabolic conversion efficiency to n-3 polyunsaturated fatty acids (PUFA) is low in humans, n-3 PUFA are currently considered dietary requirements (Hassan *et al*., 2010). This n-3 PUFA serves a variety of functions in human health that are unrelated to its conversion to n-3 PUFA (Artemis and Simopoulos, 2004).

Certainly, n-3 PUFAs have anti-cancer properties and could be used to prevent or treat cancer (Laviano *et al*., 2013, Bhagat and Das, 2015, Murray *et al*., 2015). The majority of investigations, whether *in vitro* or *in vivo*, have shown that n-3 PUFAs protect against cancer incidence (Chapkin *et al*., 2014, Gu et al., 2015). However, some studies challenge the efficacy of these substances in preventing cancer, while others claim that consuming more n-3 PUFAs can cause some types of cancer (Serini *et al.,* 2011, Brasky *et al.,* 2013, Kiyabu *et al*., 2015). As a consequence, the putative preventative role of n-3 PUFAs has sparked much attention and controversy. GSO was shown to significantly reduce huh-7 cell proliferation at a concentration of 100 µg/ml only by 6.81 % in the current study (Figure 1C). According to the previous study, GSO anti-cancer potential could be due to its high level of polyunsaturated fatty acids (PUFA) (Badr *et al.,* 2014).

Polysaccharides have been shown to have anti-cancer properties and suppress tumor growth in several investigations (Rapa *et al*., 2019, Thakur *et al.,* 2020). Guar gum is a branching polysaccharide derived from *Cyamopsis tetragonoloba* seeds (Frias and Sgarbier, 1998). Gamal-Eldeen *et al.* (2006), investigated the cytotoxic effect of guar gum and guar gum sulfation in human cancer cell lines, and found that they had considerable antiproliferative activity against the HepG2 cells, which they attributed to antioxidant and anti-inflammatory activities. Accordingly, in the current study, guar gum has anti-cancer action against huh-7 cells at varied doses (0.1-100 µg/ml) by lowering the percentage of cell proliferation (Table 1) and producing various morphological changes (Figure 1D), as a result of its antioxidant and anti-inflammatory properties.

Guar is a well-known traditional plant used in folkloric medicine, containing several vital minerals and phytochemicals such as saponin and flavonoids (Mukhtar *et al.,* 2006). Saponins are secondary metabolites that are predominantly present in plants (Francis *et al*., 2002). Their biological function is yet unknown, however it is thought to be related to the plant organisms defense against predators (Szakiel *et al*., 2011). Plant saponins in several studies were shown to have the ability to destroy cancer cells via an apoptotic mechanism (Liua *et al*., 2000, Yu *et al*., 2015, Alam *et al*., 2017). GSP also showed anti-cancer action against huh-7 cells in the current investigation at all concentrations (0.01-100 µg/ml), with percent cell growth suppression ranging from 3.67 to 12.13% (Figure 1D). The growth-inhibiting effects of GSP were accompanied with morphological changes in the cells, such as the appearance of apop-

totic bodies, cell shrinkage, and a ruptured membrane, all of which indicated apoptotic cell death (Figure 2 E).

In conclusion, the current study examined the cytotoxic activities of various guar seed extracts against huh-7 cancer cells for the first time. Various guar seed extracts were found to be cytotoxic towards huh-7 cells. Among the various tested extracts, guar seed saponin (GSP) revealed to be the most cytotoxic to huh-7 cells at a concentration of 100 µg/ml. More research is needed to investigate into the cytotoxic effects of guar seed extracts at various concentrations and to learn more about the mechanism of action of these preparations against huh-7 cancer cells.

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الأنشطة الخلوية السامة لمستخلصات بذور الجوار ضد خلايا سرطان الكبد huh-7

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الملخص

أصبح العثور على عوامل جديدة فعالة مضادة للسرطان هدفًا رئيسيًا في جميع أنحاء العالم. لذا كان الهدف من هذه الدراسة هو فحص الأنشطة الخلوية السامة للمستخلص الإيثانولي لبذور الجوار، زيت بذور الجوار، صمغ الجوار بالإضافة الي الصابونين المستخلص من بذور الجوار ضد خلايا سرطان الكبد huh-7. تم تعريض الخلايا لمستخلصات بذور الجوار المختلفة بتركيزات ٠,٠١، ٠,١، ١، ١٠، ١٠٠ ميكروجرام/ مل لمدة ٧٢ ساعة ثم بعد ذلك تم اجراء اختبار الـ SRB ثلاث مرات بثلاث مكررات لقياس النسبة المئوية لحيوية الخلايا، والنسبة المئوية لتثبيط نمو الخلايا، والتركيز المثبط لنمو ٥٠% من الخلايا. بالإضافة الي ذلك تم استخدام الفحص الميكروسكوبي لفحص التغيرات الخلوية المورفولوجية. وقد تبين من هذه الدراسة أن جميع مستخلصات بذور الجوار ذو سمية خلوية لخلايا huh-7. كما أظهر الصابونين المستخلص من بذور الجوار أعلى سمية خلوية ضد خلايا huh-7 بتركيز ١٠٠ ميكروجرام/ مل. ونتيجة لذلك، يمكن استخدام مستخلصات بذور الجوار المختبرة في صناعة الأدوية المضادة للسرطان.