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Anti-proliferative Effect of Chickpea Extract on Hela, MCF-7, Saos and Fibroblast Cell Lines

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Authors' contributions

This work was carried out in collaboration between all authors. Author MME designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LB, ZA and FE managed the analyses of the study. Author AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: The present study aimed to investigate the proliferation inhibitory effects of seed protein extract from chickpeas (*Cicer arietinum* L.*)* on cancer cell lines.
Materials and Methods: The chickpea water, soluble proteins from ammonium sulphate precipitates, was called R1 and R5. The anti-proliferative properties of all fractions were evaluated by MTT assay. Morphological analysis using fluorescence microscopy was performed to show the ability of the extract to induce cell apoptosis.
Results: MTT assay revealed that the protein extract fractions of R3 and R4 had strong anti-proliferative properties against Hela (cervical cancer cells), MCF-7 (breast cancer cell) and Saos

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(Sarcoma osteogenic) at all levels of concentration (0, 25, 62.5, 125, 250, 500 µg/ml) and they were tested at different times (24, 48, and 72 h). In addition, the data further revealed that the chickpea proteins showed the greatest activity against Hela and had the lowest effect on fibroblast. **Conclusion:** The results also demonstrated that chickpea proteins can be a source of bio- active components and can also be regarded as an alternative source of a new anticancer drug.

Keywords: Anti-proliferative; cell line; chickpea; plant extract protein; apoptosis.

1. INTRODUCTION

Naturally, plants contain some products with biological activities [1-3]. These components are found in many plant family such as: Asteraceae, Rubiaceae, Violaceae, Fabaceae or Leguminosae [4]. Legume families include plants such as: faba, peas, beans, lentils, and other pod beans. Each contains high amounts of protein and peptides that can play an important role in the world's nutrition [5]. In addition to their dietary features, these plants have components such as antimicrobial proteins and peptides (AMPs) that can act as a natural defense against invading pathogens such as: viruses, bacteria and fungi [6]. It the same line, it has been found that the consumption of beans can decrease the prevalence of cancers [7,8]. Recently, there has been an increased tendency to explore natural proteins and peptides, which have cytotoxic and anticancer activities [9-11]. For instance, plant cyclopeptid RA-V, which was isolated from *Rubia yunnanensis*, inhibited the growth of human breast cancer cells and murine breast cancer [12]. Also, the protein-extract from *Bidens alba* (Asteraceae) was applied against human colorectal carcinoma SW480 cells [13]. The results showed DNA damages and apoptosis-related cellular morphologies and intracellular reactive oxygen species increased. *Phaseolus* seeds are rich in bioactive components which exhibit metabolic roles in humans and animals. Its biological activities are the antioxidant activity, the reduction of cholesterol, the reduction of low-density lipoproteins, and the antimutagenic and anti-proliferative properties [14]. The herbal plant *Kedrostis foetidissima* extract also proved to have an anti-proliferative effect on the breast cancer cell lines, MCF-7 and YMB-1 cells [15].

So far, the antitumor mechanisms of some plant proteins and peptides have been studied [16-18]. These mechanisms comprise of cell apoptosis, disrupting signal transduction, inhibiting angiogenesis, changing cell cycle, and microtubule polymerization and depolymerization [19]. It is believed that there is an urgent need for further comprehensive investigations to identify more plant proteins and peptides with antitumor activities, which may be used as anticancer agents in clinical treatment. Thus, this study strove to investigate the anti-proliferative potentials of chickpea (*Cicer arietinum L*.) extracts on three cell lines, Hela (cervical cancer cells), MCF-7 (breast cancer cell), and Saos (Sarcoma osteogenic).

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Chickpea (*Cicer arietinum L*.) seeds were purchased from a local market in Babol, a city in north Iran and were verified by Iranian research institute of plant protection, Tehran, Iran. All reagents were purchased from Merck in Germany; MTT 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide), DMSO, from Sigma– Aldrich, UK. All other chemicals used in the experiments were of analytical grade.

2.2 Partial Isolation of Chickpea Protein Extract

Chickpeas owe a lot to the extremely hard nature of their seeds. They were soaked in an extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, pH 7) at 4℃ a day. They were grained, homogenized, and then filtrated using gauze. The water-soluble protein-extract was centrifuged at 3,000 xg at 4℃ for 10 min. The supernatant was precipitated by adding 60% relative saturation of ammonium sulfate, and the precipitated proteins were gathered through centrifugation, at 10,000 xg for 30 min at 4℃. The pellet was also suspended in distilled water.

2.3 The Bradford Protein Assay

The Bradford protein assay was used to determine the total protein concentration of a sample. The acidic solution of Coomassie Brilliant Blue G-250 absorbs at 465 nm. it shifted to 595 nm [20] when proteins bounded with Coomassie. Bovine Serum Albumin (BSA) was

prepared in a different concentration (0.1- 1 mg/ml) in order to draw the standard curve. The protein concentration in each sample was determined by the standard curve. The Bradford assay was repeated multiple times, and each time it was repeated three times. Although the result is only for one test with three replicates.

2.4 MTT Assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) colorimetric assay was used to determine cell proliferation. Hela, MCF-7, Saos and fibroblast cells were seeded in a 96-wells flat bottom microplate (6×10^3 cells/ well). After 24 h, the cultured media was replaced with fresh media as well as different concentrations (0, 25, 62.5, 125, 250 and 500 µg/ml) of fraction R3 or R4, which were incubated for 24, 48, 72 h at 37℃. After incubation, 50 µl of MTT solution (5 mg/ml) in PBS was added to each well. After 4 h of incubation at 37℃, the Formazan precipitate was dissolved, and the absorbance was measured at 570 nm.

The following equation was used to calculate the cell proliferation inhibition ratio [21]. The proliferation inhibition ratio is calculated by (%) = $\frac{(A-B)}{A}\times 100$, in which A is the control absorbance (without treatment) and B is the samples absorbance with fraction R3 in 570 nm.

2.5 Statistical Analysis

The experiment was carried out three times. IBM SPSS Statistics 19 was used for the statistical analyses. The results were presented as mean ± standard deviation (SD). Data were compared usingthe post hoctest (Tukey's method) for one way analysis of variance (ANOVA) test and paired sample T -Test. All the statistical differences were considered significant at $p < 0.05$.

3. RESULTS

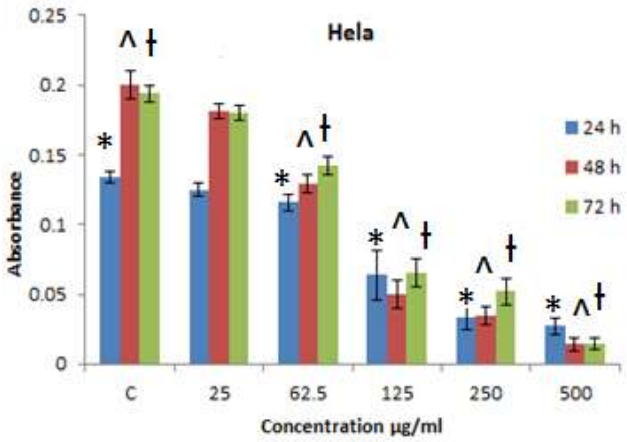
The chickpea proteins were precipitated by ammonium sulfate in 5 steps (5%, 10%, 20%, 40% and 60%), which were called R1 to R5. Bradford assay was used to measure the total protein concentration of each step (Table 1).

The anti-proliferative effect of the crude extract and the fractions from ammonium sulfate precipitation on Hela, MCF-7, Saos and fibroblast revealed that the proliferation was reduced. SinceR3 were found to be more effective, they were used in subsequent experiments (Fig. 1). (The result related to R4 fractions were shown in the supplement).

The statistical analyses proved that the treatment of Hela cells with R3 led to a statistically significant difference compared to the respective controls for all concentrations (P= 0.000), except

Table 1. The total protein concentration of each step was measured via Bradford assay

Chickpea protein	Crude extract	R1	R2	R3	R4	R5
Concentration (mg/ml)	3.34	3.77	6.21	28.22	15.53	3.14



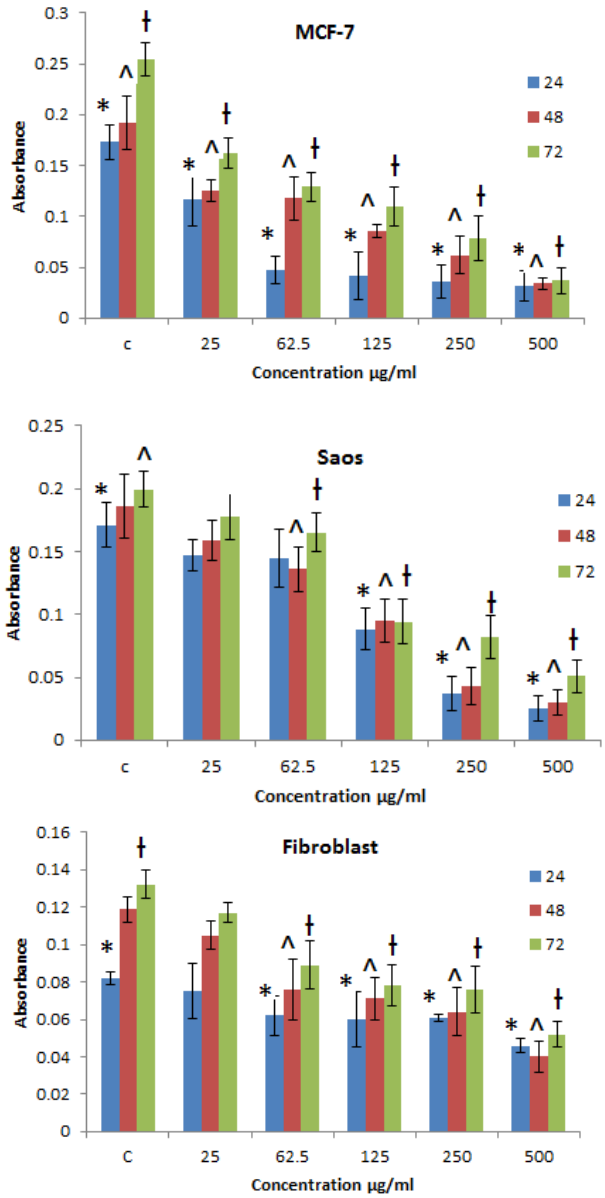


Fig. 1. The anti-proliferative effect of the fractions R3 fraction, from ammonium sulfate precipitation on Hela, MCF-7, Saos and fibroblast revealed that the proliferation was reduced. Means and standard errors of the mean obtained from three independent experiments are expressed. Statistically, analysis were significant inrespect to the control group (*P* <.05, the post hoc test for one- way analysis of variance (ANOVA) test)*, 24h, ^, 48 h and †, for 72 h incubation

for 25 µg/ml after 24, 48, and 72 h (P= 1.000, 1.000 and 0.148, respectively). Anti-proliferative effect decreased with increasing concentration, and it depends on concentration. The results also showed that the difference between concentrations 125 and 500 µg/ml was statistically significant (P= 0.045), but the difference between 125 and 250 (P= 0.171) after 24 h and also25 µg/ml and 62.5 µg/ml (P= 1.000), 125 and 250 (P= 0.990) for 48 h, and 62.5 compared to 125 µg/ml (P= 0.118), 125 µg/ml compared to 250 and 500 (P= 1.000, 0.546, respectively), 250 and 500 (P= 0.975) for 72 h was not statistically significant. It is also worth mentioning that the anti-proliferation activity in each concentration at different times (24, 48 and 72 h) can be found in Table 2.

Table 2. The statistical analyses (P Value) of R3 on Hela using the post hoctest for one way analysis of variance (ANOVA) and paired sample T –Test. The significant was at the 0.05 probability level

Concentration / Time	24 h				
	25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)
	P value				
48 h	0.000	0.000	0.940	0.210	0.000
72 h	0.015	0.000	0.919	0.686	0.000
	48 h				
72 h	0.949	0.003	1.000	1.000	0.992

The statistical analyses also revealed that the anti-proliferation effect of R3 on MCF-7 was significant compared tothe respective controls (P= 0.000), and no significant difference was found for 25 µg/ml compared to 62.5, 125 and 250 µg/ml or 250 and 500 µg/ml of concentration (P= 1.000) after 24 h. Also, the statistical analyses was not significant for 25 and 62.5, 125 µg/ml (P= 1.000, 0.997, respectively) or 62.5 compared to 125, 250 µg/ml or 125 µg/ml compared to 250 and 500 µg/ml (P= 0.950, 1.000, 0.116, independently) or 250, 500 µg/ml (P= 0.939) after 48 h. In addition, for 72 h incubation, the results illustrated that there was no significant difference between 62.5 compared to 25, 125 and 250 µg/ml (P= 0.710, 0.996, 0.085, in the order given). Furthermore, there was no statistical difference between 125,250 µg/ml (P= 0.739) and 250, 500 µg/ml (P= 0.301). It should be mentioned that in each concentration at different times (24, 48 and 72 h), the anti -proliferation activity did not depend on time (Table 3).

The treatment of Saos with R3 indicate that the difference for some concentrations is statistically significant compared to the respective controls (P=0.000), (Fig. 1). Also, for the concentration of 62.5 µg/ ml, the growth of the cells was a little more than 25 µg/ml after 24 h but it gradually decreased within 48 h of incubation and was ultimately equal to that of other concentrations. The results also showed that the difference between concentrations 62.5 µg/ml compared to 125, 250, and 500 µg/ml (P= 0.437, 0.839 and 1.000, respectively) or 250, 500 µg/ml (P= 1.000) was not significant after 24 h. Furthermore, the statistical analysis between 62.5 µg/ml and 125, 250 or 500 µg/ml (P= 0.437, 0.839 and 1.000, respectively), as well as 125 matched to 250 and 500 µg/ml (P= 0.979, 1.000), 250 and 500 µg/ml (P= 1.000) was not significant after 48 h. In addition, statistical comparison between62.5 µg/ml and 125, 250 µg/ml (P= 0.633, 0.247 respectively), and 125 matched to 250 and 500 µg/ml (P= 0.942, 1.000), 250 and 500 µg/ml (P= 0.999) was not significant after 72 h. Table 4 shows that in each concentration at different times (24, 48 and 72 h), the anti -proliferation activity did not depend on time.

The statistical analyses demonstrate that the anti-proliferation effect of R3 on fibroblast was significant compared to the respective controls except 25 µg/ml (P= 0.965). Significant difference was not found with other concentrations such as 25 and 62.5 µg/ml (P=0.069) or 62.5 compared with 125, 250 µg/ml (P= 0.378, 0.767) or 125 and 250, 500 µg/ml (P=1.000, 0.994) or 250 and 500 µg/ml (P=1.000) after 24 h. Statistical analysis after 48 h incubation showed that there was no significant difference between 62.5 µg/ml compared to25 (P=0.140), 125 µg/ml (P=0.994), 250 and 500 µg/ml (P=1.000), 250 and 500 µg/ml (P=0.378). Also, there was no significant difference between 62.5 µg/ml with other concentrations (P>0.05). It should be stated that in each concentration at different times (24, 48 and 72 h), the anti -proliferation activity did not depend on time (Table 5).

Inhibitory proliferative percent was shown in Fig. 2. The lowest and the highest inhibitory of R3 were detected to be on Hela and on fibroblast after 48 h (Fig. 2).

4. DISCUSSION

Some cancer patients are resistant to normal chemotherapy, and there is no effective chemotherapy to treat these patients. Thus, it is interesting and necessary to find an alternative compound from natural sources, especially from plants, which can be employed in the treatment of cancer.

Table 3. The statistical analysis and P value of R3 on MCF-7. The post hoc test for one way analysis of variance (ANOVA) and paired sample T –Test was carried out. Significant was at 0.05 probability level

Concentration / Time	24 h				
	25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)
	P value				
48 h	1.000	0.089	0.245	0.918	1.000
72 h	0.1888	0.000	0.004	0.266	1.000
	48 h				
72 h	0.525	0.710	0.950	0.999	1.000

Table 4. The statistical analyses (P value) of R3 on Saos using the post hoc test forone way analysis of variance (ANOVA) and paired sample T –Test. Significant was at the 0.05 probability level

Concentration / Time	24 h				
	25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)
	P value				
48 h	1.000	0.801	1.000	1.000	1.000
72 h	0.987	0.002	0.773	0.996	1.000
	48 h				
72 h	1.000	0.283	0.979	1.000	1.000

Table 5. The statistical analyses, P value, of R3 on Saos using the post hoc forone way analysis of variance (ANOVA) and paired sample T –Test. Significant at the 0.05 probability level

Concentration / Time	24 h				
	25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)
	P value				
48 h	1.000	0.801	1.000	1.000	1.000
72 h	0.987	0.002	0.773	0.996	1.000
	48 h				
72 h	1.000	0.283	0.979	1.000	1.000

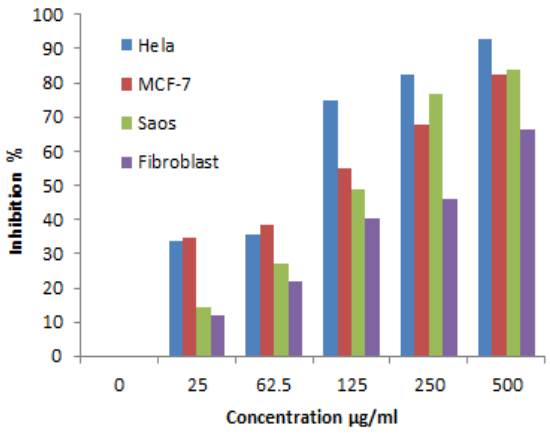


Fig. 2. Inhibitory proliferative percent effect of R3 fraction, from ammonium sulfate precipitation on Hela, MCF-7, Saos and fibroblast after 48 h

In the present study, the chickpea protein extracts were investigated for their possible anti-proliferative potentials on three cancer cell lines: Hela, MCF-7 and Saos and also non- cancer cell line, fibroblast. The study also used two fractions of chickpea seed aqueous extracts (R3 & R4) for anti-proliferation effect with different concentrations 25, 62.5, 125, 250, 500 µg/ml with different incubation times 24, 48 & 72 h. The results showed that chickpea seed aqueous extracts had anti-proliferation effect on Hela, MCF-7, Saos and also fibroblast. The highest observed effect was for Hela and the lowest effect was for fibroblast.

There are many reports shedding light on the fact that protein extracts from plants can display anticancer effects [12,13,22,23]. Chickpeas belong to a large group of plants known as legumes. Legume seeds contain high

amounts of protein and peptides, and some are known for their antitumor activities. It has also been reported that chickpea seed extracts (aqueous and organic extracts, up to 400 μg/ml) can be applied on the cell lines Caco-2 (epithelial intestinal) and J 774 (macrophages). It was also found that the cell growth-promoting and the cell growth-inhibiting effects, and a fraction soluble in ethanol and acetone entirely inhibited the growth of Caco-2 cells. The ethanol and acetone soluble fraction contain not only phospholipids and neutral lipids but also relatively apolar minor or hydrophobic components like isoflavones which may be important as cancer-preventive agents. Also, it contains protease inhibitors, saponins, phytosterols, lectins, and phytates, which have been shown to have anti-cancer effect. In addition other chickpea fraction, the pH 4.3-soluble one, enhanced proliferation of confluent Caco-2 cells were cultured for 6 days. Even if these mechanisms are still not clear. This fraction contains albumins, lectins and protease inhibitors, non-phenolic water-soluble phytochemicals, in addition to phytic acids and saponins. It expected that these agents are cancer-preventive, but in this case they do not have any inhibitory effect on the growth of Caco-2 cells at low density [24]. The findings from previous studies also showed that protease isolated from chickpeas inhibited the growth of MDA-MB-231 breast cancer, PC-3, and LNCaP prostate cancer cells at the concentrations of 25-400 µg/ml in a concentration similar to our studyusing the MTT assay [25]. In contrast to our study, cell viability with chickpea protein extract occurred in a dose-dependent manner with the entire cell lines tested. In support of our findings, chickpea protein extract contained components like lectins in addition to the protease inhibitor. It was shown that the hydrophilic extracts of chickpea seed (Acetone/Water, 50/50, v/v) at the concentrations of 0 - 5 mg/ml had dose-dependent inhibitory effect on MCF-7 [26]. Lectins (plant protein) are believed to have an anticancer effect on human cell line. They can promote cytotoxicity, apoptosis, and inhibition proliferation [27,28].

It seems that the anticancer effect mechanism of legumes protein and peptides hold a higher expression of anionic molecules in cancer cells membrane than normal cell membrane [29]. So, cancer cell membranes usually carry a net negative charge, while normal cell membranes are neutral. The electrostatic interactions between cationic anticancer peptides and negatively charged membranes can kill cancer cells. This property may provide an alternative for the new drug and the treatment of cancer.

5. CONCLUSION

Chickpea seed proteins and peptides are considered as a source of bio- active components. The results demonstrated that chickpea seed extract can be considered as the proliferation inhibitor on cancer cell lines and this effects lower in normal cells. So, it is proposed that chickpea can also be regarded as an alternative source of a new anticancer drug. Also, chickpea can be consumed as dietary.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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