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Antioxidant and Anticancer Activities of Walnut (*Juglans regia* L.) Protein Hydrolysates Using Different Proteases

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Abstract

Walnut (*Juglans regia* L.) contains approximately 20–25 % protein with abundant essential amino acids. The enzymatic hydrolysate of Persian walnut (Chandler) seed proteins was prepared by incubation with three different proteases, including pancreatic chymotrypsin and trypsin, and a microbial enzyme proteinase K. The hydrolysates were found to possess excellent antioxidant capacities. The peptide fractions scavenged the 2, 2′-anizo-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) free radicals and inhibited the activity of reactive oxygen species. Walnut protein hydrolysates were also tested, for the first time, against the viability of human breast (MDA-MB231) and colon (HT-29) cancer cell lines. MTT, [3–(4, 5dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide], assay was used to assess *in vitro* cancer cell viability upon treatment with the peptide fractions. The peptide fractions showed cell growth inhibition of 63 ± 1.73 % for breast cancer and 51 ± 1.45 % for colon cancer cells. Thus, a direct correlation between antioxidant and anticancer activities of walnut peptide fractions exists and supports their potential therapeutic benefit.

Keywords

Walnut proteins; Bioactive peptides; Antioxidant; Anticancer; Protease

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Compliance with Ethical Standards

Human and Animal Rights This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare that there are no conflicts of interest.

Introduction

The free radical oxidations in humans, which can be a consequence of aging or environmental factors [1], lead to several diseases such as cancer [2]. Cancer treatments are generally costly and involve drugs with adverse side effects. However, diets rich in antioxidants have been reported to reduce the risk of developing several diseases including many kinds of cancer, and scavenge many free radicals [3]. Several studies have reported that bioactive peptides resulting from various food sources exhibit remarkable properties [4–6]. Bioactive peptides are protein fragments with potential biological activities that have important roles in health promotion [7]. Walnut has received significant attentions for its proteins and protein hydrolysates activities including anti-atherogenic, anti-mutagenic and antioxidant activities [8, 9].

Breast cancer is the most common malignancy in females [10], and colon carcinoma is the second most common cause of death from cancer [11]. These tumors are comprised of phenotypically diverse populations of cells with a wide range of incidence and mortality. MDA-MB231 is an invasive ductal carcinoma and metastatic cell line, and HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. The availability of better and more effective therapies for these cancers will have significant clinical implications [11].

The purpose of the present work was to evaluate the potential antioxidant and anticancer activity of the peptides extracted from walnut seeds. We determined the potential protective effects of the walnut proteins and peptide fractions, generated from their enzymatic hydrolysis, against H_2O_2 induced oxidative damage. In addition, the anticancer activity of the walnut peptides generated from chymotrypsin hydrolysis was evaluated using breast (MDA-MB231) and colon (HT-29) cancer cell lines.

Materials and Methods

Materials

Persian walnut, the Chandler variety, was supplied by Center of Excellence for Walnut Improvement and Technology of Iran at the College of Aburaihan, University of Tehran. Chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), proteinase K (EC 3.4.21.64), luminol (5-amino 2,3-dihydro 1,4-phthalazinedione), H_2O_2 (30 % solution), and gold (III) chloride hydrate ($HAuCl_4$), were purchased from Sigma-Aldrich (Germany).

Extraction of Walnut Proteins

The walnuts were manually cracked, shelled, and their kernels were chopped. The isolation of walnut proteins was carried out using an alkali extraction method [12]. The flour was dispersed in 50 mM Tris-base solution(1:15, w:v) and stirred for 2 h at 4 °C. The mixture was then centrifuged at 13,000 ×*g*, and the top phase (oil) was discarded. This step was repeated until a transparent solution was obtained. After adjustment of pH to 4, the solution was again centrifuged (4000 ×*g* for 30 min) to collect the precipitate. The latter was then lyophilized and stored in plastic tubes at −20 °C for further analysis.

Hydrolysis of Walnut Proteins

Enzymatic hydrolysis was performed on fat-free extracts obtained as described above. Proteases used in this study were chymotrypsin, trypsin, and proteinase K, and utilized separately at 37 °C, pH 8. Trypsin and chymotrypsin are digestive enzymes and were selected in order to have the most similarity with physiological conditions. Proteinase K was selected based on our previous work with camel milk casein, where we reported that the peptide fractions resulted from proteinase K digestion had the best antioxidant properties. [13]. Thus, proteinase K was used in the current study to evaluate its effect on walnut proteins.

The walnut protein extracts (50 mg) were dispersed in phosphate buffer (50 mL). The protease was added to the walnut protein mixtures [substrate/enzyme (100/1, w:w)], and it was shaken for certain time intervals. The sampling was done at indicated times, and the fractions were heated at 80 °C for 15 min. The obtained suspensions were centrifuged at 10,000 ×*g* for 10min, and the supernatants were lyophilized and stored at −20 °C.

In Vitro Digestibility Assessment

For determination of protein hydrolysis, we used the o-Phthaldialdehyde assay. In this assay o-phthaldialdehyde (OPA) and 2-mercaptoethanol react with the amino groups resulting from protein hydrolysis. The adduct has a strong absorbance at 340 nm [14]. A fresh OPA solution was prepared daily using 20 % SDS (w:w), 100 mM sodium tetra hydroborate and 100 μl β-mercaptoethanol. OPA was dissolved in 1 mL of methanol. To assay proteolysis of walnut proteins, an aliquot (10–50 μL) was added directly to 1 mL of OPA reagent in a 1.5 mL quartz cuvette. The solutions were shaken manually and incubated for 2 min at room temperature. Immediately after incubation, the absorbance was recorded using a spectrophotometer at 340 nm (Shimadzu, model UV-3100, Kyoto, Japan).

Determination of Antioxidant Activity

The $ABTS^+$ solution [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] was prepared as described by Re et al. [15]. The solution absorbance was adjusted to 0.70 ± 0.03 at 734 nm by deionized water. The hydrolysate (5 μL) at the concentration of 0.50 mg/mL was mixed with $ABTS^+$ solution (200 μl). The mixture was incubated at 25 °C for 6 min and the absorbance was measured at 734 nm using a plate reader Expert 96. All tests were performed in triplicates. On the basis of the results obtained from these experiments, the hydrolysates for 1 h using chymotrypsin were selected for further studies.

In order to isolate the peptides on the basis of molecular weight, the fractions obtained from 1 h treatment with chymotrypsin, which showed the highest antioxidant activity, were centrifuged at 3000 ×*g* for 10 min, and the supernatant was passed through the membrane filters of Amicon ultrafiltration system with smaller molecular weight cut-off sizes of 10, 5 and 3 kDa. Permeates from each filtration cycle were collected as <10 kDa, 5–10 kDa, 3–5 kDa and <3 kDa fractions. All the fractions were freeze dried and stored at −20 °C for further analysis.

Determination of ROS Scavenging Activity

Reactive oxygen species (ROS) activity was measured using a chemiluminescence (CL) method. Based on the CL of luminol, increasing the oxidizing agents, as for instance hydrogen peroxide and ROS, causes increased concentration of 3-aminophthlate, which is an excited state molecule. Briefly, in order to perform the experiments, CL signal of luminol was obtained after injecting 5 μL of gold (III) chloride hydrate ($HAuCl_4$) to the sample containing H_2O_2 using a fluorescence spectrophotometer at 425 nm (Synergy H4 Hybrid Reader; BioTek, USA). Luminol was dissolved in a sodium carbonate buffer (100 mM, pH 11). The stock solution of luminol (10 mM) was stored at 4 °C in the dark [16].

Determination of Anticancer Activity

Determination of anticancer activity was conducted as described by Mosmann [17]. The effect of walnut peptides on HT-29 and MDA-MB231 tumor cells, and normal human umbilical vein endothelial cells (HUVEC) viability was determined using MTT [3-(4, 5-dimethylthiazolyl)-2,5-diphenyltetrazoliumbromide] assay [18]. In this assay, MTT is reduced to blue formazan product that indicates the normal function of mitochondria, and hence reflect cell viability [18]. These tests were performed in triplicates. The rate of growth inhibition was determined using the following formula:

$$\text{Growth inhibition}(\%) = 1 - (A_{\text{samples}}/A_{\text{control}}) \times 100$$

Data Analysis

All data represent the mean value ± S.E. of three independent measurements. Comparison between groups was made using one-way analysis of variance (ANOVA) followed by a specific *post-hoc* test and *t*-test. To analyze the differences SPSS software version 22 was used. The statistical significance was achieved when $P < 0.05$.

Results and Discussion

In Vitro Digestibility Assessment of Walnut Proteins

The proteolysis of walnut proteins was characterized using the *o*-phthaldialdehyde (OPA) test. This method is related to the OPA mechanism of action. During this assay, OPA reacts with the amino terminal of protein and peptide chains. With increased time of hydrolysis, the break in the protein chains as well as the number of free amino termini increases. Thus, this assay can be used as a parameter to determine walnut protein hydrolysis. The extent of proteolysis based on OPA values was determined during the 4 h of proteolysis in the presence of three different proteases (Fig. 1). The analysis of degradation of walnut proteins revealed that the hydrolysis by chymotrypsin was faster than that of trypsin and proteinase K. These results indicated that the maximum level of proteolysis was achieved after approximately 60 min of incubation with these proteases. Thus, the majority of walnut protein degradation occurred during the first hour of hydrolysis.

Antioxidant Activity

The antioxidant potential of walnut peptide samples was determined by scavenging activity of ABTS radicals. A higher absorbance represents a higher ABTS scavenging activity. Figure 2 shows the time of hydrolysis, as well as the type of protease used, both of which affected the antioxidant effects. A significantly higher antioxidant activity was observed with chymotrypsin at nearly all time intervals. All samples showed a significant antioxidant activity ($p < 0.001$) compared to protein sample (time 0). Thus, walnut peptides have substantially higher antioxidant activity than intact proteins. However, since hydrolyzing with chymotrypsin for 1 h showed the highest antioxidant activity among other fractions, chymotrypsin was selected as the protease of choice for further experiments.

Following partial purification of peptide fractions based on molecular weight by ultrafiltration, we determined the antioxidant activity of <10, 5–10, 3–5 and <3 kDa fractions. These results demonstrated that the antioxidant activity of the peptide fractions was significantly decreased compared with the whole hydrolysate. However, the differences among the fractions were not significant, highlighting the synergistic effect among these peptides (Fig. 3).

Chemiluminescence Spectroscopy

Figure 4 shows CL intensity of luminol in the presence or absence of peptide fractions (5 μL of 750 μg/mL). The different concentration of the H_2O_2 was tested as blank sample (no peptide fractions). In comparison with blank, CL intensity in the wells containing the peptide fractions significantly decreased, especially at the concentration of 8 μM H_2O_2. Indeed, this concentration was the lowest concentration with $p < 0.01$, and thus it was considered for evaluating the effect of peptide concentration on the CL intensity. For optimization of the peptide concentration as a scavenging activity, CL intensity of the different peptide concentrations was measured. Figure 5 shows that when the CL intensity reached near constant level, CL emission was decreased along with increasing concentration of the peptide fraction up to 750 μg/mL. Significant differences were observed in comparison with no added peptides.

It has been suggested that the ROS scavenging activity is related to hydrophobic amino acids [19]. The presence of hydrophobic amino acids allows direct electron transferring to reactive oxygen species (ROS) [20]. Kumar et al. [21] and Chen et al. [22] found that hydrophobic amino acids play important roles in the antioxidant activity of peptides. Tang et al. [23] also reported that the antioxidant activity of zein peptides was related to the molecular mass and the hydrophobicity of these peptides. Furthermore, there are indications that an increase in hydrophobicity correlates with an increase in antioxidant potential [24]. In the active site of chymotrypsin, there are several reactive groups near the binding sites for the hydrophobic amino acid side chains. These binding sites prefer large hydrophobic residues [25]. Thus, we propose that the chymotrypsin fractions of walnut protein hydrolysates contain a significant amount of hydrophobic amino acids that are responsible for their high antioxidant activity.

Anticancer Activity

Figure 6 shows that peptide fractions obtained from chymotrypsin hydrolysis exhibit inhibitory activity on survival of the MDA-MB231 and HT-29 cancer cells. The majority of the peptide fractions exhibited strong anticancer activity towards these cells, and among those, the fraction obtained by chymotrypsin hydrolysis in 1 h had the best inhibitory activity. According to antioxidant data, it can be concluded that the antioxidant activity of the peptide fractions and anticancer activity are in line (Fig. 6). The relationship between antioxidant and anticancer activity has been previously reported [26, 27]. In our study the Pearson's correlation coefficient for antioxidant activity and anticancer activity against MDA-MB231 and HT-29 cells was calculated as 0.93 and 0.85, respectively, demonstrating a high relationship. Since the peptide fractions affected MDA-MB231 more significantly than HT-29 cells, the breast cancer cells were selected as a target for further analysis.

Our results indicated that anticancer activity increased significantly along with increased antioxidant activities. To determine the IC_{50} of fractions obtained by chymotrypsin hydrolysis in 1 h, a dose dependent test was carried out with MDA-MB231 cells using fractions that exhibited the highest anticancer activity (IC_{50}: 650 μg/mL) (Fig. 7). The normal primary HUVEC was also incubated with the peptide fractions and no toxicity was observed (not shown).

Several efforts have been made during the last decade to advance novel broad-spectrum anticancer peptides into clinical use. The proteins extracted from the walnut and their hydrolysates had cancer cell inhibitory activity (63 ± 1.73 % inhibition using 1 h chymotrypsin hydrolysis). Other fractions may also contribute to inhibition of cancer cell proliferation, but not to the extent observed using fractions obtained from 1 h chymotrypsin hydrolysis. The varying results in inhibition of cancer cell growth may be attributed to the variations of the amino acid sequences of these peptides.

Several studies on the antioxidant and anti-cancer activity of peptide fractions have been previously reported [28, 29]. In some cases, the peptides have been purified and sequenced. According to the majority of these studies the purified peptides are less efficacious than crude fractions [23, 30]. Thus, because of higher efficiency of crude fractions compared with partially purified peptides (Fig. 3), the crude peptides were used in this study.

Conclusion

In the present study, walnut proteins were hydrolyzed by various proteases to extract antioxidant peptides. Among three proteolytic enzymes, chymotrypsin resulted in the production of the peptides with the highest antioxidant and anticancer activity. These crude peptides obtained after 1 h chymotrypsin hydrolysis was used as the optimum natural antioxidant fraction to enhance antioxidant and anticancer properties of functional foods compared with other proteases. Our data in this study showed a significant correlation between antioxidant and anticancer activity of walnut hydrolysate fractions. Ultrafiltration could partially purify the antioxidant peptides. However, the antioxidant activity was significantly decreased by partial size fractionation. Thus, we concluded that the mixtures of peptides are more effective than the purified peptides, and bioactive peptides from walnut

proteins can be considered a suitable candidate for a new era of nutraceutical products and cancer therapeutics, with limited side effects, and health-promoting benefits as natural products.

Acknowledgments

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Abbreviations

ABTS	2,2′-anizo-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
CL	Chemiluminescence
HUVEC	Human umbilical vein endothelial cells
MTT	3-(4, 5dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide
OPA	o-Phthaldialdehyde
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate

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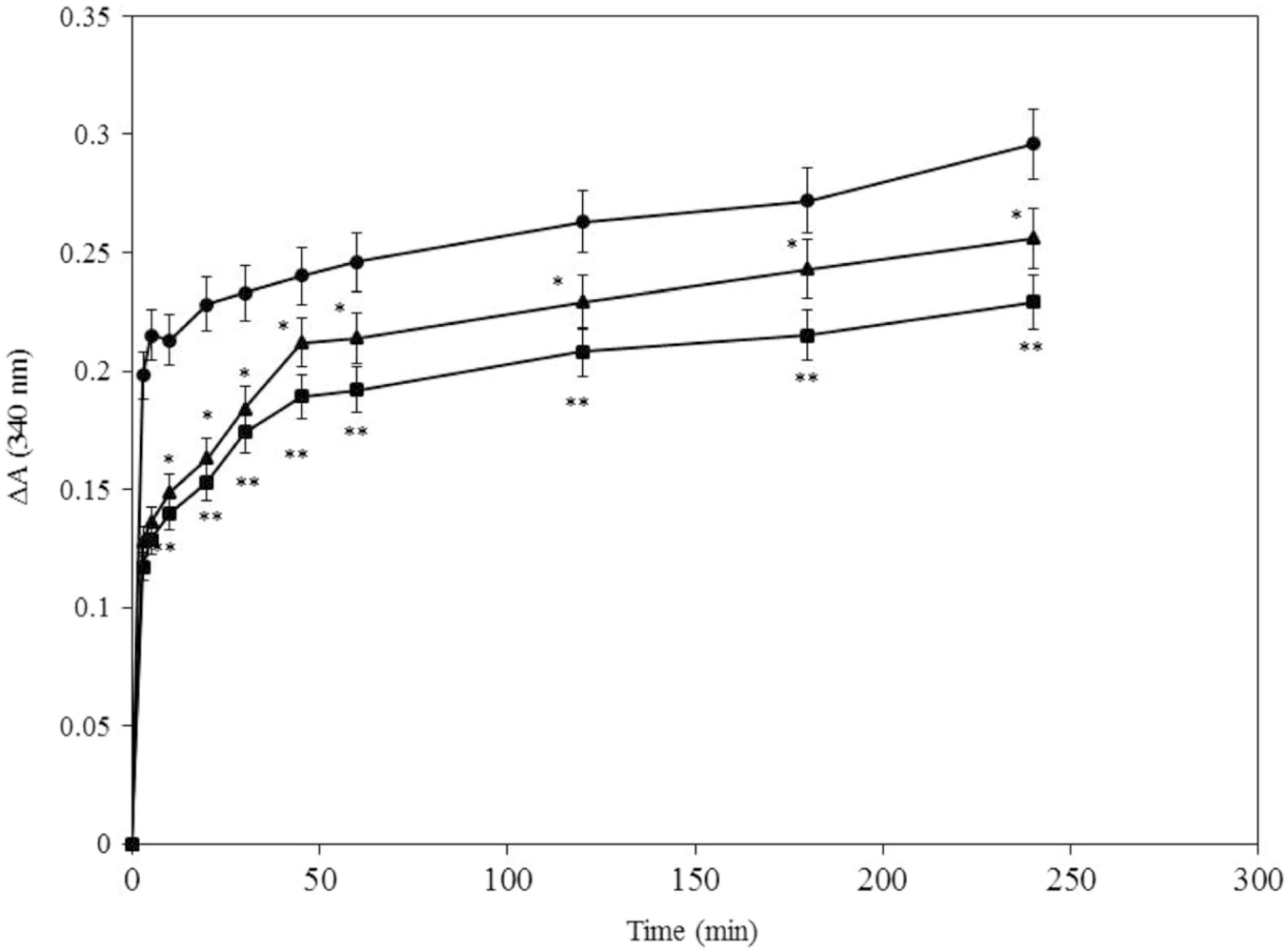

Fig. 1.

O-phthaldialdehyde spectrophotometric assay to measure proteolysis of walnut proteins by chymotrypsin (●), trypsin (■), and proteinase K (▲) for 4 h. Results are the average of three independent measurements. *$P < 0.05$ and **$P < 0.01$ compared with chymotrypsin group (●)

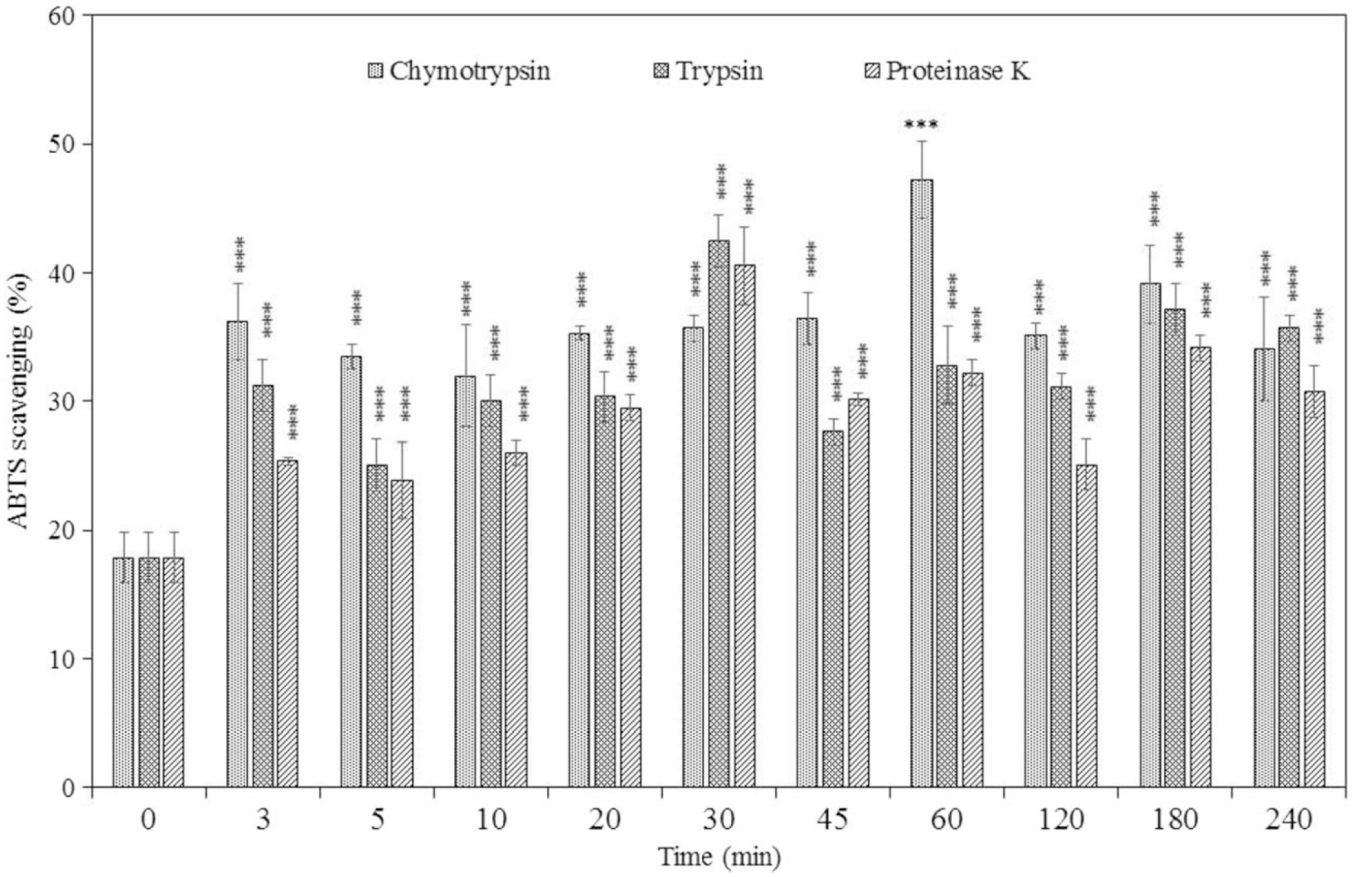

Fig. 2.

Measurement of ABTS radical scavenging for 4 h of hydrolysis by chymotrypsin, trypsin and proteinase K. Results are the average of three independent measurements. A significant difference from 0 h treated cells was observed (***$P < 0.001$)

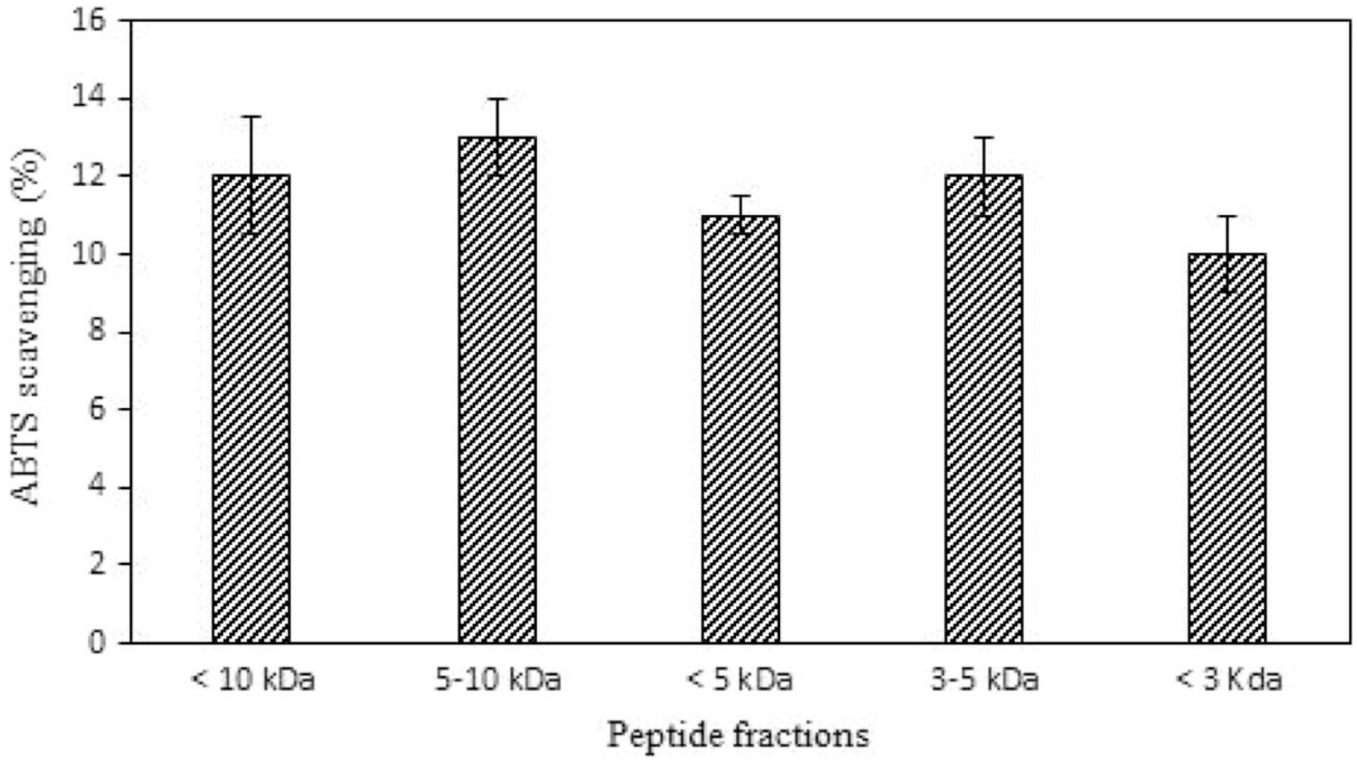

Fig. 3.

Measuring ABTS radical scavenging for peptide fractions resulting from ultrafiltration of 1 h chymotrypsin hydrolysate. Results are the average of three independent measurements

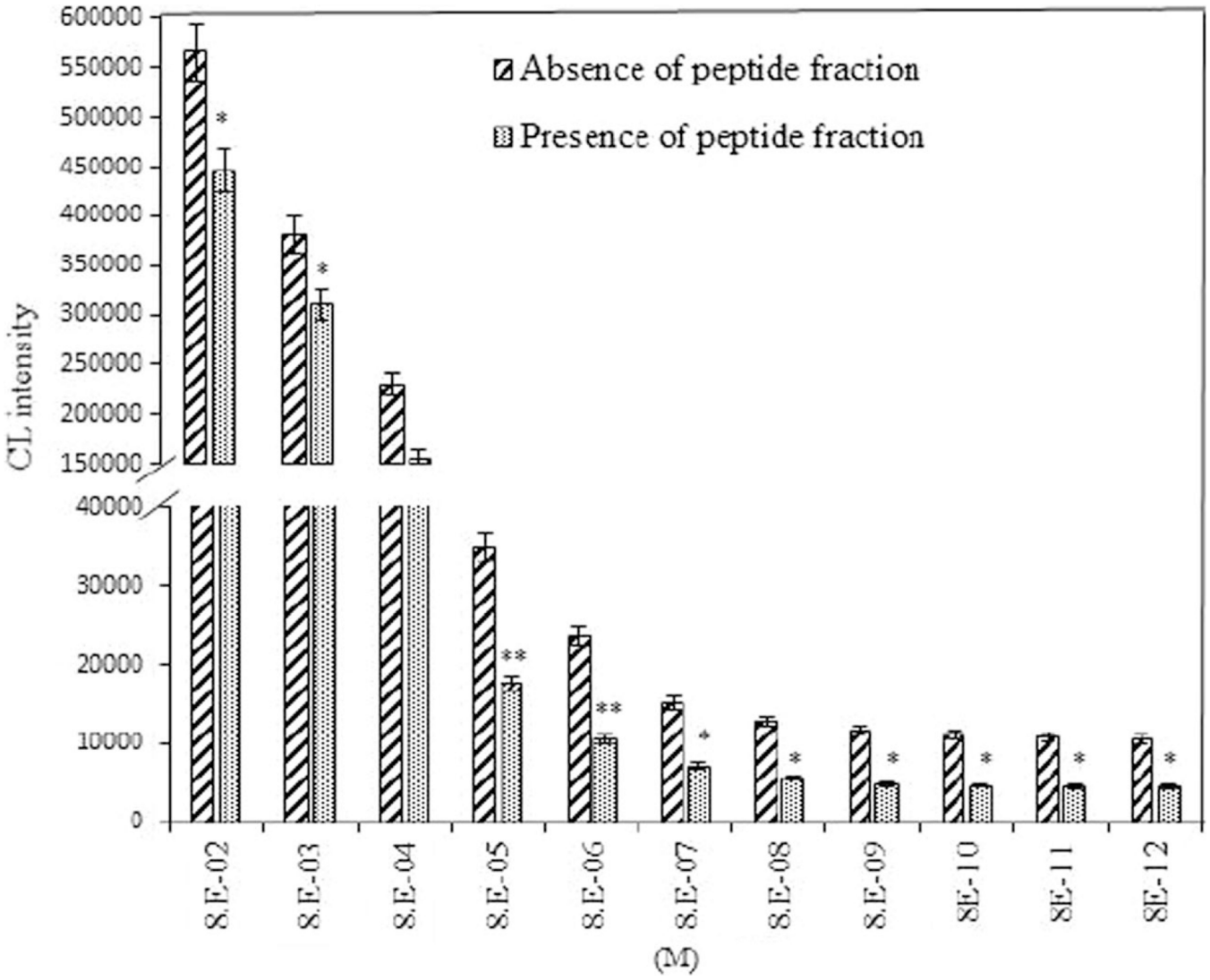

Fig. 4.

CL intensity of different hydrogen peroxide concentrations in the absence or presence of peptide fractions. The concentrations of luminol and peptide fraction were 100 μM and 750 μg/mL, respectively. Results represent the average of three independent measurements. A significant difference between two group (absence and presence of peptide) in each concentration of H_2O_2 was observed (*$P < 0.05$, and **$P < 0.01$ and)

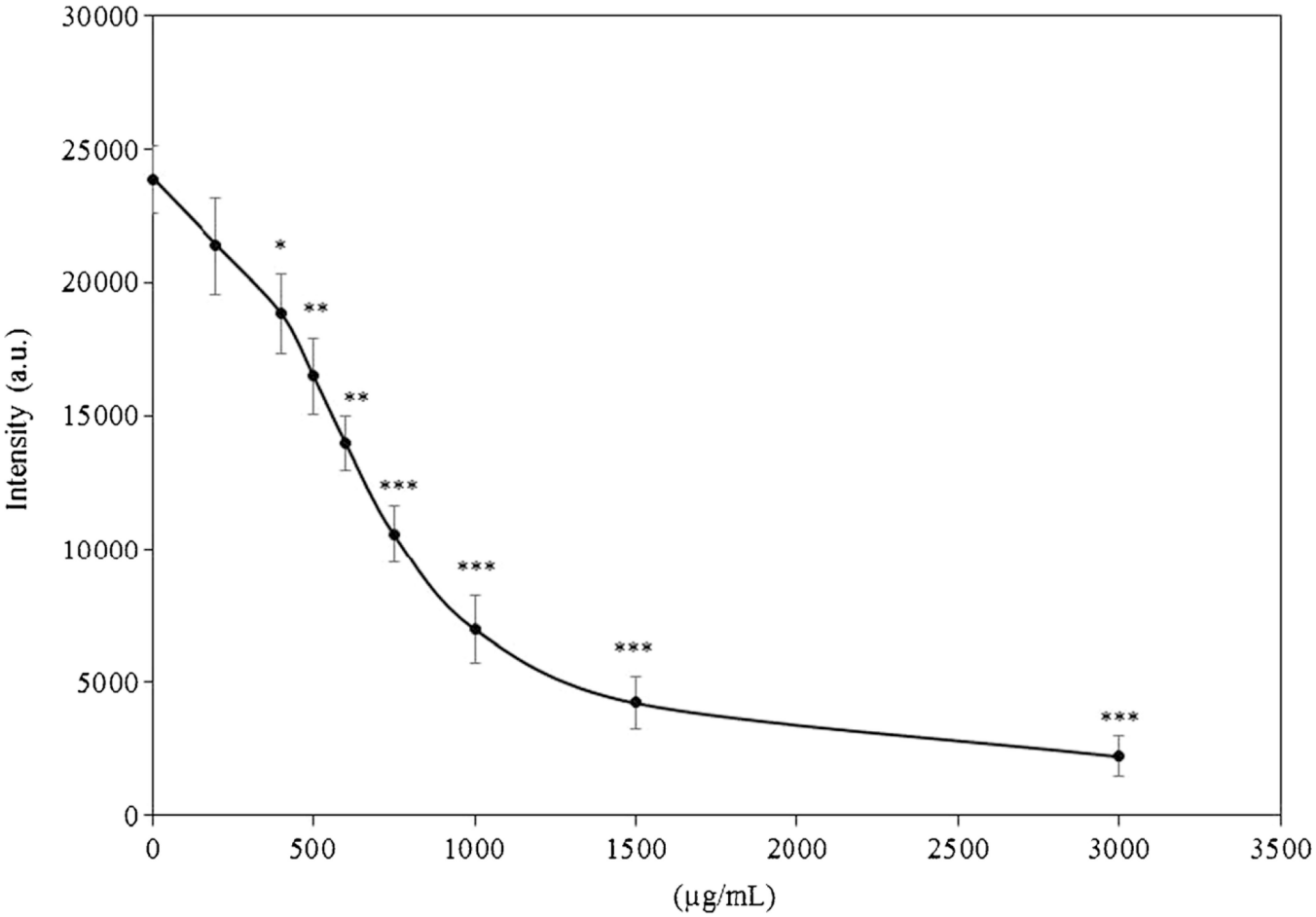

Fig. 5.

Optimization of peptide concentration (obtained from chymotrypsin) based on CL intensity of luminol at 8 μM H_2O_2. The concentrations of luminol and H_2O_2 were 100 and 8 μM, respectively. Results represent the average of three independent measurements. A significant difference from concentration 0 of peptide was observed (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$)

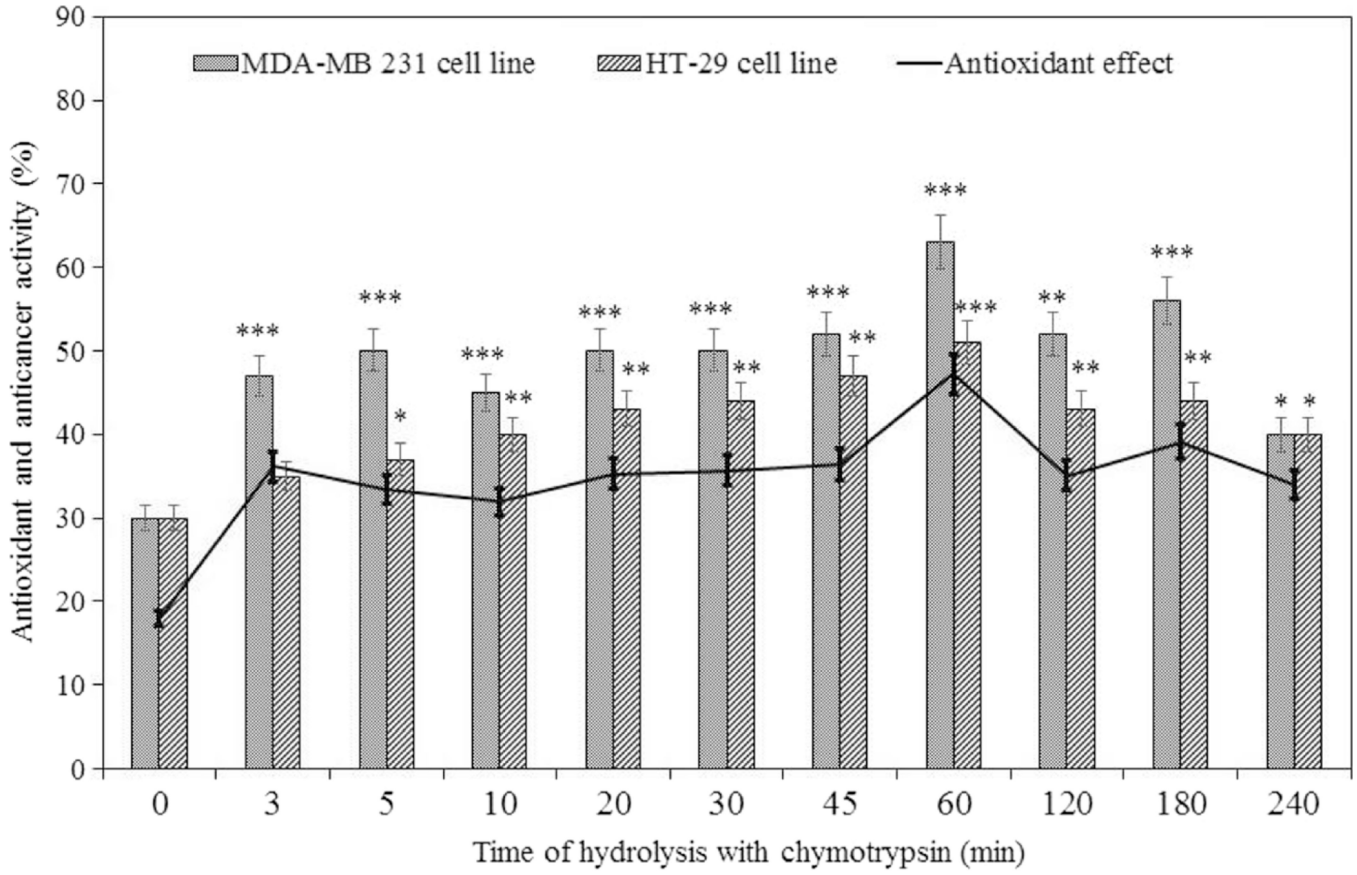

Fig. 6.

MDA-MB231 breast and HT-29 colon cancer cell lines growth inhibition by peptides from walnut chymotrypsin hydrolysate. Control sample is culture medium only. The line chart shows the correlation between inhibition of MDA-NB231 and HT-29 colon cancer cells and antioxidant activity of peptide fractions resulting from chymotrypsin treatment. A significant difference from 0 h treated cells was observed (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$)

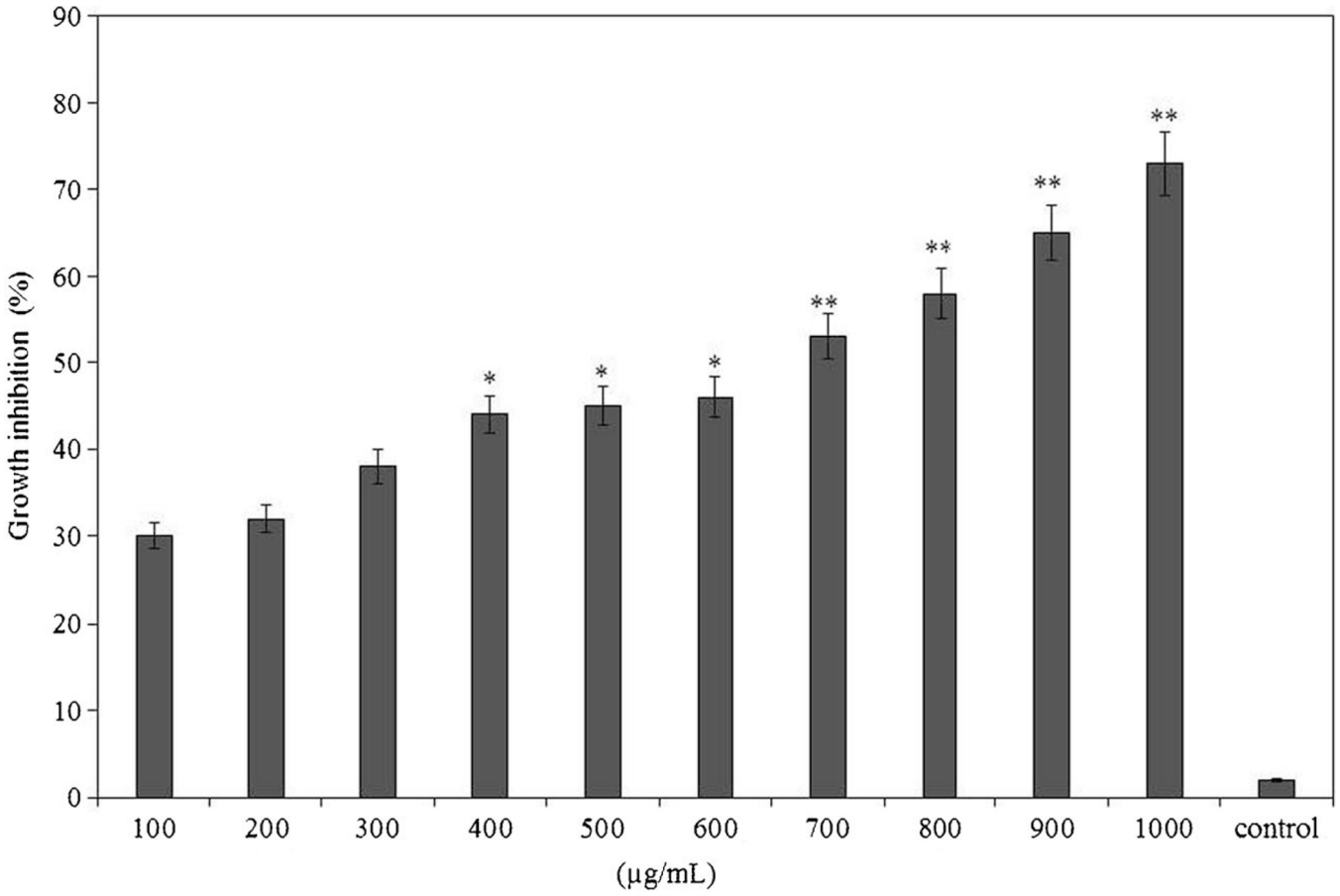

Fig. 7.

Dose response study (100 to 1000 μg/mL) of walnut protein peptide fractions of after 1 h hydrolysis with chymotrypsin on MDA-MB231 breast cancer cells. Control sample is culture medium only. *Significantly different from 0 h treated cells (*$P < 0.05$, **$P < 0.01$)