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Research Article

Determination of Antioxidant and Anticancer Activities of *C. sativa* Leaf Extracts on MCF-7 Human Breast Cancer Cell Line

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Abstract

Aim: In this study, it was aimed to determine the total phenolic content, flavonoid content, and antioxidant activity of *Castanea sativa Mill*. (chestnut) leaf extract and their anti-proliferative effect on MCF-7 cell line.

Material and Methods: The antioxidant properties of the extract were determined using the total phenolic content, total flavonoid content, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. In addition, the anti-proliferative effect was determined by XTT method in MCF-7 breast cell line. The leaf extract used was applied at different concentrations for 24 hours, 48 hours and 72 hours and the results were evaluated with the Graphpad Prism software program.

Results: In this study, it was revealed that the total phenolic contents of ethanolic extracts of chestnut leaf 58.22 mg GAE/g. Total flavonoid content was 64.62 mg QE/g. The DPPH activity of the leaf extract of chestnut was 80.06%. Moreover, findings showed that Chestnut leaf extract had cytotoxic effect against breast cancer cells depends on concentration and time. The 24h, 48h and 72h most effective IC50 dose were 100.1 µL ,193 µL 15.23 µL, respectively. The study showed that the ethanolic extract of chestnut has anticancer activity by supporting its antiproliferative effect in MCF-7 breast cancer cells.

Conclusion: Findings from this study indicated that ethanol extract of Castanea sativa leaf could potential as medicinal drug in breast cancer treatment.

Keywords: Antioxidant properties, anticancer, chestnut, *C. sativa*, MCF-7

INTRODUCTION

Cancer is a vast group of diseases which begin a tissue or an organ of the body when uncontrollably dividing cells growing and spread another part of body or organs (1). Cancer caused about 9.6 million deaths at the world in 2018 and mortality rate approximately 20.2% (2). Furthermore, it is predicted that 19.3 million new cancer patients could occur annually by 2025 (3). Breast cancer is affecting one in 20 globally and as many as one in eight in high-income countries (4). Surgery, chemotherapy, radiotherapy, hormone therapy is generally used for cancer treatments, but these have more side effects (5). Drug resistance is one of the most important problems for cancer patients. For this reason, several anticancer and anti-infective agents developed from biologically active plants which are widely used to treat a cancer disease (6), because it has minor side-effects, low cost, and high availability. So, it is thought that plants will continue to be the best source to produce drugs used in the treatment of different diseases in the past, present and future (7).

Secondary metabolites produced by plants are generally responsible for the biological properties of plant species used in the world (8). Compounds such as alkaloids, tannins, flavonoids and phenolics found in plants are therapeutic for human health (9). Antioxidants are important for prevent of several diseases like cancer, malaria, neurodegenerative disease (10). The use of antioxidants obtained from low-cost products obtained

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from forest products in the food, medicine and cosmetics sector is very important in terms of the sustainable use of large amounts of waste that cause environmental pollution and the production of valuable products (11).

Castanea sativa (Chestnut) which important trees because of renowned value in forest and agricultural economy naturally occurs in Turkey (12). Chestnut which are rich in carbohydrates and essential unsaturated fatty acids, starch and minerals contain flavonoids and tannins, with gallic acid as the main phenolic representative of hydrolysable tannins (13). The chestnut shells and leaves rich in bioactive compounds (14). Chestnut leaves are used for curing diarrhea and coughs in folk medicine (15), some studies showed the antioxidant potential of phenolic compounds from shells of chestnut (16). Differences among species/cultivars have shown in various studies (13) and it also shown to chestnuts from different geographic locations (17). Different cultivation techniques (soil, nutrients, minerals, irrigation, diseases and pests), climatic, environmental conditions and storage time influence nutritional composition and quality of chestnut (12).

Polyphenols and flavonoids which are found naturally in various medicinal herbs and dietary plants, have been shown to potent antioxidant, anti-inflammatory, biological effects such as anticancer (18) activities. Polyphenols with a lower molecular weight are thought to have more effective biological activity (19). Plant-based antioxidants and anti-inflammatories can prevent inflammatory disease progression or frequency (20). The antioxidant and anti-inflammatory effects of various plants have recently gained the attention (21). Great interest is about more specific information on the different chestnut cultivars because antioxidant and nutraceutical properties of chestnuts and so more studies will be needed to further support their health benefits (22).

In this context, the possible anticancer effect of dry chestnut bark extract, which has a low molecular weight, on six human cancer cell lines (DU 145, PC-3, LNCaP, MDA-MB-231, MCF-7 and Hep G2) was evaluated. It has been determined that it has an inhibitory effect on viability (14). The formation of triterpenoid saponins in the sweet chestnut heartwood of Chestnut and their potential to chemoprevent breast cancer have been reported to be valuable supplements to prevent such diseases (23). Ethanolic extract of the membrane layer of Chestnut, has more antimicrobial activity compared to the water extract (24). Chestnut wood, bark and leaf extracts have photoprotective, neuroprotective, cardioprotective and antioxidant properties for the prevention of chronic and degenerative diseases (25). Therefore, chestnut leaves are used as treatment for curing cough and diarrhea, and these also have antioxidant properties of phenolics from burs (26) natural ingredients such as plant extracts. So, the aim of this study is to determine the antioxidant properties of ethanol extract of Chestnut leaves and to determine the cytotoxic effect of the extract on human breast (MCF-7) cancer cell lines.

MATERIAL AND METHOD

The study protocol was approved by the Ethics Committee of KTO Karatay University Non- Pharmaceutical and Medical Device Research Ethics Committee (Decision No. 2023–045, dated 31.03.2023). .

Plant material

Chestnut leaves were obtained from local chestnut forest in Türkiye in June 2020. After the plant leaves were dried in the shade, they were made ready for extraction.

Plant extraction

The plant extraction was carried out by Downey et al. (2007) method (27). Chestnut leaves were extracted with orbital shaker at room temperature in the dark. The mixture was filtered with Whatman filter paper no1 and then the clear filtrate was removed from ethanol 70% at 40 °C using a rotary evaporator. The crude extracts obtained were weighed to calculate the extraction efficiency. These extracts were lyophilized with lyophilized device. After that, the extracts were weighed in accordance with the doses and prepared by dissolving in sterile water and stored at + 4°C to be applied to the cells.

Total phenolic content

Total phenolics content of Chestnut was determined by using the methods given in the literature (28) involving Folin–Ciocalteu reagent and gallic acid as a standard with some modifications. The solution of extract (0.25 mL) was mixed with diluted Folin-Ciocalteu reagent (1 mL, 1:9) and shake it vigorously then incubate 3 min at room temperature. After incubation, sodium carbonate solution (0.75 mL, 1%) added in the solution and mixed thoroughly. After a 2 h incubation at room temperature, the sample absorbance measured at 760 nm by Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific). The results were expressed as equivalents of mg catechol/100g of fresh weight material (mg GAE/g).

Total flavonoid content

Aluminum chloride colorimetric method was used to determine the total flavonoid content of Chestnut (29). In order to calculate the total flavonoid, the standard calibration curve was made using quercetin. Quercetin stock solution was dissolved in methanol and serial dilutions ranging from 5–200 µg/mL were prepared from this solution. 0.6 mL of diluted standard quercetin solutions or extract were mixed separately with 0.6 mL of 2% aluminum chloride. All mixtures were then incubated at room temperature for 60 minutes and read against the blank with a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific) at a wavelength of 420 nm. The concentration of the total flavonoid content of the sample was calculated from the calibration chart (Y = 0.0162x + 0.0044, $R2$ = 0.999) and expressed as mg quercetin equivalent (QE)/g dried plant material. All determinations were performed in triplicate.

Free radical scavenging activity (DPPH)

Chestnut leaf extract was analyzed using a 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical method according to Cheng et al. (2006) (30). A stock solution of DPPH (200 µM) in ethanol was prepared. The reaction mixture containing 100 µL of DPPH and 100 µL of diluted Chestnut in 96-well plates was incubated at 37°C for 30 minutes. Then, absorbance was measured at 515 nm with a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific). Gallic acid was used as a positive control. The percent DPPH radical scavenging activity was calculated as follows:

$$Percent\ radical\ scavenging\ activity = \left\{1 - \frac{(sample - blank)}{(control - blank)}\right\} \times 100$$

Gallic acid showed 95% radical scavenging activity at 20 µM.

Cell culture

MCF-7 breast cancer cell lines (ATCC® HTB-22™) was obtained from American Type Culture Collection (Rockville, MD, USA). MCF-7 cells were maintained in RPMI 1640 medium which has broad applicability to support the proliferation of MCF-7 cell line. RPMI 1640 medium was prepared by adding 10% Fetal Bovine Serum (FBS) and 0.1% gentamicin. Cells were proliferated at 37°C in a humidified atmosphere of 5% CO2 incubator by changing the medium for every 24 to 48 h. The cells were counted by staining with trypan blue to evaluate whether the cells had grown in sufficient number and cell viability.

Assessment of Cell Viability

The XTT assay was used to measure metabolic activity of chestnut leaf extract exerted inhibitory activity towards the MCF-7 cells. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) is a tetrazolium-based compound which used to determine a colorimetric detection of viable mammalian cells. The assay is supported to extracellular reduction of XTT compound by NADH via by trans-plasma membrane electron transport and an electron mediator. Cells were seeded (1×10^4 cells/mL), in a final volume of 200 µL, in a 96-well microplate and treated with different concentrations of *C. sativa* extracts (1000, 500, 250,125, 62,5, 31,25, 15,62, 7,81, 3,9, 1,95 µM) for 24h, 48h, 72h. 100 µL of XTT solution (0.25 mg/mL) was added to each well according to the manufacturer's instructions and further incubated for 4h at 37 °C. The absorbance was measured UV-Vis Spectrophotometer (Multiskan Sky Microplate, Thermo Fisher Scientific, Waltham, Massachusetts, ABD). IC_{50} values were calculated with linear regression plots as the sample concentration which resulted in 50% reduction of absorbance compared to controlled (untreated) cells by Graphpad Prism 9.2.0 software (La Jolla, San Diego, CA, USA). Each concentration of *C. sativa* extracts was independently assayed with three replicates.

RESULTS

Total phenolic, flavonoid content and DPPH activity

In this study, it was revealed that the total phenolic contents of methanolic extracts of *C. sativa* leaves 58.22 mg GAE/ g. Total flavonoid content was 64.62 mg QE/ g. The DPPH activity of the leaf extract of *C. sativa* was 80.06%.

Morphological analysis of MCF-7 cells

The optical microscope (Nicon E100, Japan) micrographs shows that morphological changes of MCF-7 cells treated with *C. sativa* leaf extracts and the control group without plant extract applied to cells. The trinocular inverted microscope (Nicon TS2, Japan) at 20x magnification was used to acquire morphological images of MCF-7 cells. The image results showed chestnut extract exhibited morphological changes such as cell shrinkage and cell separation in MCF-7 cells, thereby significantly inhibiting cell proliferation (Figure 1).

MCF-7 cells which were treated with chestnut extract were generally reduced in nuclear dimensions, the prominent round cells than control groups. In addition, there was abundant cell debris in the medium of treated groups (Figure 1 b and d).

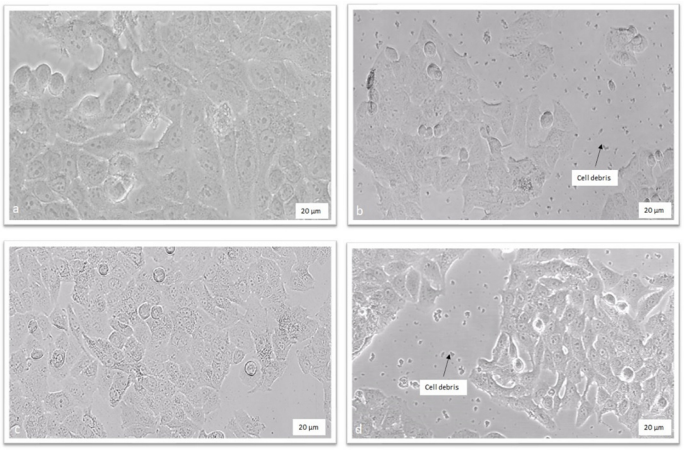

Figure 1. MCF-7 cell line images after treatments with (a) 48h Control group (b) 48h treatment group (c) 72h treatment group (d) 72h control group

Cell viability determination

Cytotoxic effect of *C. sativa* leaf extract on MCF-7 human breast cancer cell line was assessed. Cells were treated with decreasing concentrations of extracts (1000, 500, 250,125, 62,5, 31,25, 15,62, 7,81, 3,9, 1,95 µM) for 24h, 48h, 72h and the cytotoxic effect on cell viability was assessed by XTT assay. Results showed that the extracts possess cytotoxic effect on MCF-7 cell line. Cell viability decreased in a dose and time-dependent manner. The most effective dose in which killed 50% of the cell viability (IC_{50}) was 100.1µL; 193,0 µL; 15,23 µL for 24h, 48h and 72h, respectively.

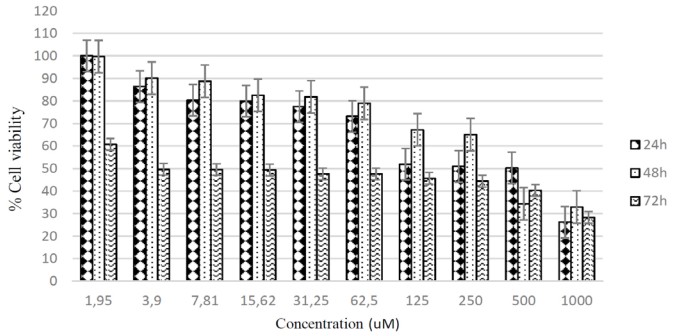

Figure 2. Effect of the *C. sativa* extract on the viability of MCF-7 cells assessed with the XTT assay depend on on 24h, 48h and 72h treatment

MCF-7 cancer cell viability was 50.29 % on 250 µL and 500 µL concentration of chestnut treatment on 24h while it was 26.15% on 1000 µL. Therefore, cell viability was 68.09% on 250 µL concentration and 47.18% on 500 µL concentration on 48h treatment while it was 42.76% on 1000 µL concentration. It was 68.09% on 250 µL; and 47.18% on 500 µL; 42.76% on 1000 µL concentrations of chestnut treatment on 48h. The cell viability was 60.72% on 1,95 µL concentration, 49.66% on 3.9 µL concentration, 49.56% on 81 µL, 49.33% on 15,62 µL conc.47.59% 31.25 µL conc., 47.58% on 62.5 µL concentration, 45.63% on 125 µL concentration, 44.40% on 250µL concentration, 40.34% on 500 µL concentration, and 28.32% on 1000 µL concentration on 72h treatment.

DISCUSSION

Chemotherapy is one of the most common methods for cancer treatment today, but most chemotherapeutic drugs cannot target the cytotoxicity of tumor cells. This situation causes multiple side effects and poor prognosis (31). In order to minimize negative side, studies on natural ingredients such as plant extracts have been used to treat many pathological conditions (6,32,33). The composition of the extracts obtained from plant tissues is related to the extraction procedure and the solvent that was used (34). In many studies, it has been reported that ethanolic extracts are important in revealing compounds with more biologically active compounds than aqueous extracts (35). In this sense several studies supported chestnut has significant antioxidant activity (11,36,37). Therefore, Genovessa et al. (2021) determined that the phenolic content of *C. sativa's* aqueous and ethanolic fruit content of ethanolic extract was 84uM gallic acid and flavonoid content of 16.73 catechin, while the phenolic content of aqueous aqueous extract was 35uM gallic acid and flavonoid content was 5.26uM catechin. The result of the study ethanolic extract of the phenolic and flavonoid compounds are richer than aqueous extract and also the antioxidant activity was that it is high on it. In this study we conducted with chestnut leaf, the total phenolic content was determined as 58.22 mg GAE/g, and the total flavonoid content as 64.62 mg QE/g. These results are in agreement with the study presented above. Polyphenols especially low molecular weight which found in phenolic acid and flavonoid classes (38) are phytochemicals provides powerful biological actions like anticancer activity (19) For this reason, we decided to assess the possible potential anticancer effect of chestnut leaves ethanolic extract on MCF-7 breast cancer cell line. There are various studies in the literature showing the antiproliferative effect of chestnut for different types of cancer (23,25,39). On the other hand, Lenzi et al. (2017), reported that chestnut extract did not have an antiproliferative effect on human T leukemia cells (40). In a study about using the ethanolic extract of chestnut on MCF-7 breast cancer cells, it was suggested that it caused a decrease in the level of ROS in cancer cells and that this extract may have potential anticancer activity (24). Result of the study showed that the ethanolic extract of chestnut has anticancer activity by supporting its antiproliferative effect in MCF-7 breast cancer cells. Besides, studies that chestnut shell extract was able to inhibit cell viability of different cancer cell lines (14).

LIMITATIONS

The limitation of our study is that in vitro studies cannot be predicted based on possible activity in vivo. Due to financial concerns, we could not perform this study in experimental animals with breast cancer. Further research is now required to further understand the full interaction of the relevant signaling pathways.

CONCLUSION

The ethanolic extract of *C. sativa* leaves is already known to be a rich source of phenolic and flavonoid compounds. The antioxidant capacity, total phenolic content, total flavonoid content and data on MCF-7 cells are evaluated as a whole, it is seen that chestnut leaf can be considered as a source of natural antioxidants. In this aspect, the results of the study are a step towards new studies on chestnut. The findings obtained in this study can be concluded that chestnut leaves offer anticancer potential on breast cancer. Based on these findings, chestnut leaves may be helpful against MCF-7 breast cancer cell and further research study may be design for finding the best molecular mechanism behind it.

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Conflict of Interest: *The authors have no conflicts of interest to declare.*

Ethical approval: The study protocol was approved by the Ethics Committee of KTO Karatay University Non-Pharmaceutical and Medical Device Research Ethics Committee (Decision No. 2023–045, dated 31.03.2023).

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