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Characterization of Nanoparticles Containing Achillea Phrygia and Their Antioxidant and Antiproliferative Properties

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ABSTRACT

In this study, antioxidant and antiproliferative properties of ''*Achillea phrygia*'' were investigated. Since the antioxidant and antiproliferative activity of B sub-extract from plant's chloroform extract is higher than other extracts, B sub-extract of *Achillea phrygia* was used in the preparation of nanoparticles. Characterization of nanoparticles was made, antiproliferative activities and IC_{50} values of nanoparticles and only chloroform extract was calculated by cell culture studies and XTT cell viability test. According to the results, it was observed that the extract-loaded chitosan nanoparticles had a strong antiproliferative activity in both MCF 7 and HT29 cells. The results of this study support that bioactive extract of the *Achillea phrygia* plant can be prepared as an herbal medicine candidate by preparing formulations with biopolymers and being investigated in cancer studies.

Keywords: Cytotoxic activity, Antioxidant activity, Nanoparticle, Bioactive material, MCF 7 cell line.

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Introduction

Developing effective drug and treatment strategies has excellent importance in maintaining the human healthy. Therefore, significant scientific studies and financial support are used to discover and design new effective substances and treatment methods. In addition, due to the serious side effects of traditional treatment strategies and synthetic chemotherapeutics, it is widely accepted that natural herbal active substances are the leading and key sources for patient prosperity [1]. Angiogenesis plays significant role in the mechanism of many diseases. Therefore, scientists mind to use natural and medicinal herbal extracts to modulate angiogenesis [2].

Bioactive materials have drawn attention for their pharmacological effects, which can be of great interest in treating cancer. Bioactive medicinal plants with anticancer potential not only ensure nutritional benefits, but also prevent the progression of cancer through various mechanisms of action in the human body [3]. Various bioactive substances of plant, animal and microbial sources show recognizable anticancer potential with various mechanisms of action such as antimetastatic, antioxidant, etc. Bioactive extracts have a high efficacy potential in the treatment of cancer, and they can be alternative treatments for the treatment of various carcinogenesis [4]. Natural compounds are very important as interesting repositories of biologically active compounds. Historically, natural products for anticancer studies have led to significant success. More than 60% of the clinical use of antitumor drugs consists of natural products, including marine organisms, plants, and bacteria, while more than 3000 plant species are used to fight neoplasms [5, 6]. Scientific study results provide new hopes for the joint use of natural compounds and chemotherapeutics in tumor treatment [7]. In the light of the evidence obtained from the studies, it is aimed to characterize the interactions and efficacy of natural compounds and classical chemotherapy drug combinations against various types of cancer. The promising results can be shown as a new hope for the combined use of natural compounds and chemotherapeutics in cancer treatment [8, 9].

Significant developments were performed to the cancer treatments. However, undesirable effect of cancer proceeds to rise and is becoming one of the most destructive diseases. Therefore, studies to prevent cancer have become an important way in which the fight against cancer can be possible [10]. To cite a few examples, curcumin, the active ingredient in turmeric, can prevent cancer formation, suppress proliferation, and induce apoptosis in tumor cells. Curcumin shows its anticancer activities via using multiple constituents such as transcription factors, antiapoptotic proteins, protein kinases, and cell cycle proteins [11]. Some studies have been conducted on the ability of the *Achillea sp*., which we used in this study, to show anticancer activity in various types of cancer. However, the studies and the results obtained are not sufficient to elucidate these activities. Many medicinal plant extracts with determined biological activities are widely used in the treatment of

various diseases [12, 13]. Extracts and essential oils, which are thought to be effective in these extracts, are obtained as a result of various extraction and distillation processes. These active substances are used for various purposes such as cancer, cardiovascular diseases, central nervous system diseases, digestive system diseases [13]. In addition to the benefits of biological active substances obtained from plants, there are also various undesirable effects. At the beginning of these effects are the lack of targeted therapy, dosing problem and toxic effects on healthy tissues and cells [14]. Nanoparticle drug delivery systems are used to prevent or correct the undesirable effects of bioactive medicinal plant extracts. Targeting bioactive components used in the treatment of diseases, having the desired drug release profile, reducing the toxic effects of drugs, and preventing multi-drug resistance are the most important advantages of these systems [15, 16, 17]. Biocompatible polymers are widely used in the preparation of nanoparticular systems. The most important advantages of chitosan are that it does not show toxic properties, is biocompatible, and has high encapsulation efficiency [18, 19]. In this study, chitosan nanoparticles containing *A. phrygia* extract were prepared. In addition, characterization studies of nanoparticles were performed, and their anticancer activities were evaluated by cell culture studies.

Materials and Methods

Plant Material and Extraction Procedures

Prof. Dr Turan Arabacı determined the *A. phrygia* specimens acquired during field experiments. For future reference, a voucher specimen (T.Arabacı 2962) was deposited in the herbarium of Inonu University, Faculty of Pharmacy, Department of Pharmaceutical Botany. The plant's aerial parts were dried in the shade (at 25 °C) and ground into a fine powder in a mechanic grinder. The maceration procedure was used to extract the powdered samples with organic solvents (methanol and chloroform) until they were colourless. Under reduced pressure, the organic phase was evaporated to dryness. For future analysis, all extracts were kept at 4°C. On the MCF-7 cell line, the cytotoxic activities of chloroform (IC_{50} = 0.041 mg/mL) and methanol (IC_{50} = 127.65 mg/mL) extracts were tested, and chloroform extract was found to be more potent than methanol extract. Column chromatography was used in this investigation to separate the probable active chemicals found in the effective chloroform extract based on their polarity. The chloroform sub-extract (5 g) was inserted to a silica gel column and eluted with toluene, petroleum ether and methanol mixture of increasing polarity of petroleum ether (100%, 1500 mL), petroleum ether /chloroform (75:25, 1500 mL), petroleum ether /chloroform (1:1, 1500 mL), chloroform (100%, 1500 mL), chloroform/methanol (75:25, 1500 mL), chloroform/ methanol (1:1, 1500 mL), chloroform/methanol (25:75, 1500 mL), and methanol (100%, 1000 mL) to obtain eight (A-H) fractions.

Cell Culture

Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Merck Millipore (United States). Penicillin-Streptomycin-L-glutamine solution was purchased from Sigma-Aldrich (Germany). Chitosan (400 kDa, DD 87) and tripolyphosphate (TPP) were purchased from Fluka (Germany). Colorectal adenocarcinoma cells (HT29) and breast cancer cells (MCF 7) were used in this study. XTT reagent was purchased Roche Diagnostic. Cells were seeded in DMEM including FBS (10 %), penicillin (100 IU/mL), streptomycin (10 mg/mL).

Cytotoxicity Assay

Cytotoxic activity of *A. phrygia* extracts was evaluated using the XTT assay against the MCF7 and HT29 cells. Cells were seeded in 96-well plates with DMEM (100 µL) and incubated overnight. *A. phrygia* extracts were dissolved in DMSO for using cytotoxicity assay. Extracts suspended in DMEM were added to each well (40 µg/mL) and same amount of DMSO was inserted to the control group. The cells were incubated for 24 h. Following each well were washed with PBS, XTT (50 µL) mixture and colorless DMEM (100 µL) were inserted to wells and the cells were incubated for 4 h. Micro plate ELISA reader was used to measure absorbance of XTT-formazan at 450 nm. Antiproliferative activity of extracts were calculated compared to control. According to the assessment of XTT results, nanoparticle which indicate the greatest antiproliferative activity against MCF 7 and HT29 cancer cells, were synthesised. XTT assays of nanoparticles with extract and only extract were repeated to calculate the IC_{50} values.

Antioxidant Assays

DPPH assay

The extracts' DPPH free radical scavenging abilities were assessed using an experimental technique reported in the literature [20]. In a nutshell, 240 microliters of DPPH solution (0.1 mM) were mixed with 10 microliters of compounds made at various doses (0.5-5 mg/mL). After that, the mixture was allowed to rest for 30 minutes at room temperature. Using a microplate reader (AMR-100) at 517 nm, the absorbance of the combination was compared to the reference. The experiment was done three times and the IC_{50} values (mg/mL) are used to express the results.

FRAP assay

The FRAP technique was used to assess the effectiveness of compounds (0.5-5 mg/mL) to reduce ferric. After mixing 190 µL of FRAP reagent with 10 µL of ompound for 4 minutes, the absorbance of the combination was measured against a reference using a microplate reader (AMR-100) set to 593 nm. $FeSO_4.7H_2O$ was used to create the standard curve and the compounds FRAP values were expressed as a mM Fe2+/mg extract.

CUPRAC assay

In CUPRAC method, 60 microliters of copper (II) solution, neocuproin solution, and ammonium acetate buffer (1 M) were mixed in this experiment. Shake the solution after adding 10 µL of ethanol and 60 µL of compound. For 60 minutes, the solutions were maintained at room temperature with their mouths closed. Absorbance values at 450 nm were measured at the conclusion of this period against a reference solution that did not include a sample [21, 22]. The CUPRAC findings were expressed as mMTE/mg extract.

Preparation of Chitosan Nanoparticles

Nanoparticles containing *A. phrygia* were prepared using the ionic gelation method. In order to determine the features and differences of nanoparticles, nanoparticles with and without extract were prepared. Initially, a certain amount of chitosan was dissolved in acetic acid (0.5 %) at 1000 rpm under magnetic stirring. Tripolyphosphate solution (0.5% w/v) containing the extract dropped into chitosan solution (0.5% w/v). Nanoparticle solutions were centrifuged at 10,000 rpm for 30 minutes. The supernatant was removed and the pellet was washed with bidistilled water. This process was performed three times. After lyophilization, nanoparticles were stored at +4 ºC.

Measurement of Particle Size And Zeta (Z) Potential

The size and ζ potential measurements were evaluated via a Zetasizer Nano ZS instrument.

Statistical Analysis

All experiments were performed in triplicate. All data from the study were given as the mean ± SD and analysed using Graphpad Prism 5. Statistical differences between the study groups were analysed using a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Mean values were considered statistically significant if $p<0.05$.

Results and Discussion

Characterization of Chitosan Nanoparticles

Properties of nanoparticles such as particle size and ζ potential are very important in terms of biological activity studies. Therefore, these features should be at desired values. Particles were investigated for this purpose. Results of nanoparticles containing extract and pure nanoparticles were shown in Table 1. According to the results, size of nanoparticle (228.34±1.9 nm) including *A. phrygia* extract had higher size than pure nanoparticle (207.56±2.6 nm). In addition, polydispersity index (PDI) values of chitosan nanoparticle with extract (0.187 ± 0.05) were smaller than pure nanoparticle (0.21± 0.03). ζ potential of extract loaded nanoparticle and non-extract nanoparticle were 8.2±0.06 mV and 7.6±0.03 mV respectively. According to the results size homogeneity and zeta potential values of both nanoparticles (pure and extract loaded) are suitable for use.

Table 1. Particle size, ζ potential, and PDI index values of nanoparticles

Samples	ζ potential (mV) ± SD	Size (nm) ± SD	PDI ± SD
*CNPCE	8.2±0.06	228.34±1.9	0.187±0.05
**CNPE	7.6±0.03	207.26±2.6	0.211±0.03

*CNPCE shows extract containing chitosan nanoparticle,
**CNPE shows pure chitosan nanoparticles.

Results of Antiproliferative Activity

Cancer is called an incurable disease because of late diagnosis of the disease and poor awareness of the disease. In addition to the existence of various treatment mechanisms for cancer treatment, there are also many undesirable effects of these treatments. Using the knowledge and experience gained in these treatments, characterization studies of active substances of biological origin and investigation of their activities in cancer treatment constitute very important fields of study [4, 6]. Antiproliferative effect of *A. phrygia* extracts were investigated on HT29 and MCF7 cell lines. Cytotoxic activity results of extracts were indicated in Figure 1 and Figure 2. On Figure 1 results, it was shown that cell viability ranged from 58.27±0.26 % to 79.10±0.34 % in HT29 cells. The results show that the extracts have significant antiproliferative effects on cancer cells. Especially, B extract decreased the proliferation of HT29 cells about half. According to the result it can be concluded that the extracts of *A. phrygia* have significant cytotoxic activity on HT29 cells.

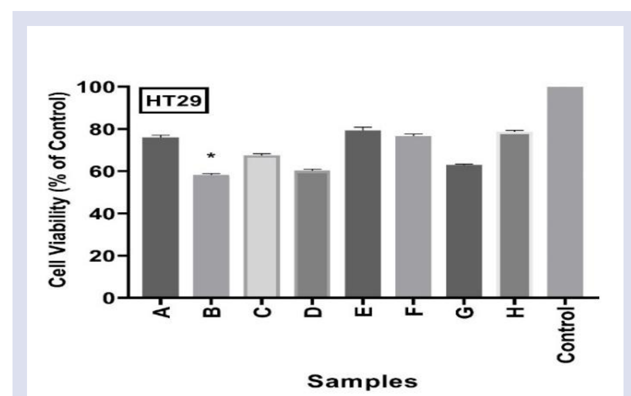

Figure 1. Cytotoxic activities of sub-chloroform extracts of A. phrygia on HT29 cell line. *B extract showed the most antiproliferative activity on HT29 cells.

Figure 2 results showed that cell viability ranged from 56.67 ± 0.32 % to 87.92 ± 0.47 % in MCF 7 cells. It can be said that these extracts have a significant cytotoxic effect on MCF 7 cancer cells, since the cell viability is below 70 % in MCF 7 cells to which A, B, D and G extracts were treated. B extract of *A. phrygia* showed the highest anticancer activity, significantly reducing the viability of cancer cells in both MCF 7 (56.67 ± 0.32 %) and HT29 (58.27 ± 0.26 %) cell lines. In the light of these results, nanoparticles of the B extract were prepared and applied to MCF 7 and HT29 cancer cells and their IC_{50} values were calculated.

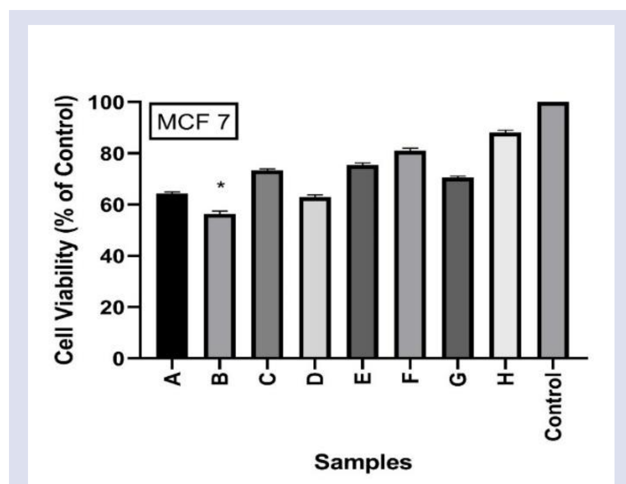

Figure 2. Cytotoxic activities of sub-chloroform extract of *A. phrygia* on MCF 7 cell line. *B extract showed the most antiproliferative activity on MCF7 cells.

IC_{50} values were calculated by applying B extract and nanoparticle containing B extract to HT29 cells at certain concentrations and performing XTT test. Figure 3 results indicated that both the nanoparticle with B extract and only B extract showed significant antiproliferative activity on HT29 cells associated with an increase in concentration. When the B extract and the nanoparticle containing the extract were treated with HT29 cells at a concentration 100 µg/ml, cell viability was observed as 46.87±0.43 % and 45.90±0.23 % respectively. On only B extract, the viability of the HT29 cells was between 46.8± 0.43 % and 71.96±0.34 %. In addition, cell viability of nanoparticle with B extract, ranged from 45.90±0.23 % to 65.48±0.37 %. IC_{50} values of nanoparticle with B extract and only B extract were 58.28 µg/mL and 66.32 µg/mL respectively. This result shows that our samples formed by coating B extract with nanoparticles significantly reduced HT29 cancer cell proliferation and increased the cytotoxic effect compared to the extract alone. In addition, nanoparticle loaded with B extract had higher cytotoxic activity on HT29 cell line than only B extract.

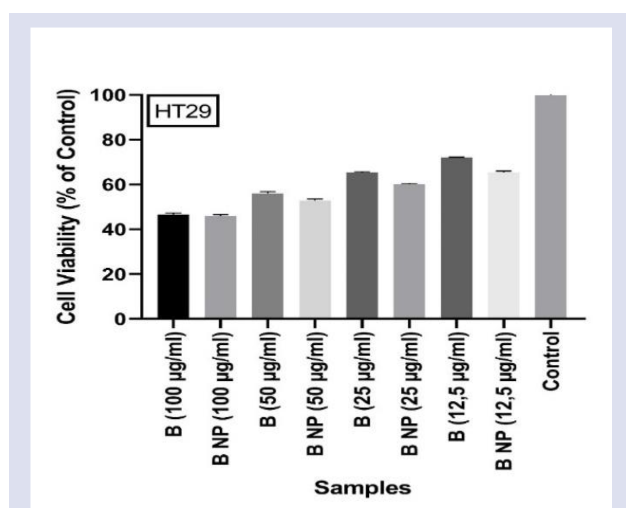

Figure 3. Concentration-related antiproliferative activities of B extract and nanoparticles containing B extract. B NP indicates nanoparticle include B extract.

XTT assay results showed that B extract and B extract loaded nanoparticles had stronger antiproliferative effect. Cell viability of B extract was between 35.29±0.18 % and 54.70±0.26 %. Cell viability of B extract loaded nanoparticle was between 29.47±0.32 % and 51.27±0.18 % depending on the concentration. Taking advantage of these valuable results it can be said that *A. phrygia* extracts have significant cytotoxic and antiproliferative effects on HT29 and MCF 7 cell lines. Especially, D extract loaded with nanoparticle showed the highest antiproliferative effect on both cell lines.

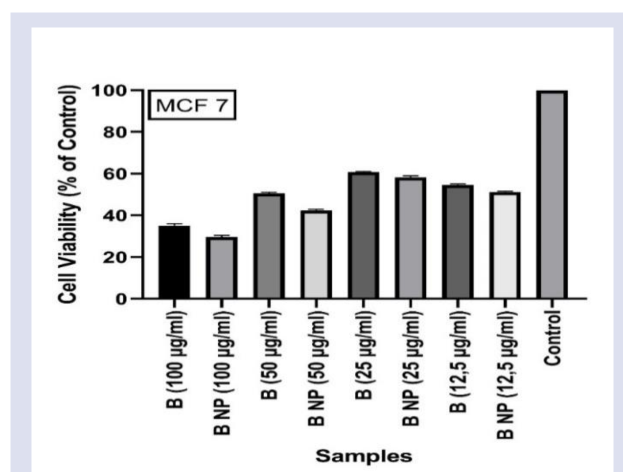

Figure 4. Concentration-related antiproliferative activities of B extract and nanoparticles containing B extract. B NP indicates nanoparticle include B extract.

Results of Antioxidant Activity

Antioxidant activities of 8 different sub-extracts obtained from the plant were evaluated using DPPH, CUPRAC and FRAP methods (Table 2). For DPPH activity, the extracts had IC_{50} values ranging from 0.399 to 1.399 mg/mL. The findings showed that the B extract (IC50:0.399 mg/mL) had higher DPPH radical scavenging activity compared to the other extracts. In addition, in this study, it was determined that the radical scavenging activity of all extracts showed lower radical scavenging activity than the ascorbic acid (IC_{50}:0.0028 mg/mL). When the FRAP values obtained as a result of this study were compared among themselves, it was determined that the B extract (40.984 mM $FeSO_4$/mg extract) of plant showed stronger iron (III) ion reduction potential compared to the other extracts. In addition, it was determined that all extracts had lower FRAP values than the BHT compound (86.004 mM$FeSO_4$/mg). The extracts have CUPRAC values ranging from 0.422 mM to 2.149 mM. The D (2.149 mMTE/mg extract) and B (1.713 mMTE/mg extract) extracts obtained from the plant have higher the potential to reduce Cu(II) to Cu(I) compared to other extracts. In this study, all extracts were found to have a lower CUPRAC value than the ascorbic acid compound (3.213 mMTE/mg). As a result, the findings obtained from the study showed that the B extract had the strongest antioxidant activity potential compared to the other extracts.

Table 2. Antioxidant activities of 8 different sub-extracts obtained from the plant

A. phrygia Chloroform sub-extract	DPPH (IC_{50}: mg/mL)	FRAP (mMFeSO$_4$/mg extract)	CUPRAC (mMTE/mg extract)
A	1.399±0.929	36.307±0.698	0.422±0.008
B	0.399±0.091	40.984±0.201	1.713±0.065
C	0.573±0.094	38.694±1.007	1.681±0.078
D	0.621±0.017	32.501±0.550	2.149±0.108
E	0.861±0.123	36.372±0.806	1.609±0.088
F	0.565±0.095	32.050±0.349	1.351±0.065
G	1.189±0.335	29.018±0.403	0.779±0.025
H	0.650±0.123	28.050±0.201	0.682±0.003
Ascorbic acid	0.0028±0.0004	-	3.213±0.076
BHT	-	86.004± 4.914	-

Conclusions

In this study, antioxidant and antiproliferative properties of sub-chloroform extracts from *Achillea phrygia* were investigated. It was determined that the B extract obtained from the plant had strong antioxidant and antiproliferative effects compared to other extracts. Nanoparticles with B extract, which has the highest antiproliferative activity in HT29 and MCF 7 cancer cells, were prepared by cell culture studies. Appropriate formulations were determined by performing particle size, zeta potential and polydispersity index tests of nanoparticles, and the anticancer activities of these formulations were evaluated with the XTT test. According to the results, it was observed that nanoparticles containing B extract showed significantly higher antiproliferative activity compared to only B extract.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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