Clinical and Experimental Health Sciences

Evaluation of *in vitro* Antioxidant, Antimicrobial and Cytotoxic Activities of Crude Ethanol Extract and Fractions of *Achillea sintenisii* Hub. Mor.

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Received: 16.01.2022 **Accepted:** 11.02.2023

ABSTRACT

Objective: The *Achillea* species have been used to treat various ailments due to its anti-inflammatory, hemostatic, spasmolytic and cholagogue effects in the Turkish traditional medicine. However, there is no biological activity studies on some *Achillea* species except for the well-knowns. This work aimed to determine the antioxidant, antimicrobial and cytotoxic activities of the crude ethanolic extracts and fractions of *Achillea sintenisii* using in vitro methods.

Methods: The antioxidant activity was investigated by DPPH (1,1´-diphenyl – 2-picrylhydrazyl), ABTS (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging, total phenol and total flavonoid content, and iron chelating methods. Antimicrobial activity evaluated by micro-plate dilution method against five test organisms. Cytotoxicity was determined by MTT method using MCF-7 breast cancer cell line and PC-3 prostate cancer cell line. Apoptosis was also measured by AO/EB staining.

Results: The n-Hexane fractions showed the highest antimicrobial and cytotoxic activities, respectively. Administration of the extracts on the cancer cells showed a concentration dependent inhibition on cell proliferation. The anti-proliferation effect could be via apoptosis and associated with the cell death.

Conclusion: The results showed that the extracts demonstrated antioxidant, antimicrobial, and cytotoxic activity, also supports the claims of traditional usage.

Keywords: *Achillea sintenisii*; antioxidant; microdilution; cytotoxicity

1. INTRODUCTION

The genus of *Achillea* (Asteraceae) is represented in the flora of Turkey by 42 species and 23 of which are endemic in Turkey (1). A list of Anatolia's most important indigenous economic plants includes *Achillea* species. They are frequently used against abdominal pain, and diarrhea as well used as diuretic, emmenagog and wound healing agents in Turkey (2). In phytochemical studies on *Achillea* species, it has been reported that these species are rich in flavonoids, triterpenes, essential oils, fixed oils and sterols (3-12). A wide range of scientific studies on the biological activities of *Achillea* species have been carried out: antioxidant (4,10-11,13-17), antimicrobial (18–22), anti-inflammatory (23-24), wound-healing (25), cytotoxic (15, 26) and insecticidal activities (5).

In recent years, interest in finding antioxidants from natural sources is increasing day by day. The antioxidant-effective phytochemicals such as flavonoids and other polyphenols obtained from plants have been reported to protect the human body from the disease by inhibiting lipid peroxidation and preventing the spread of free radical reactions. In addition, some adverse effects on human health resulting from long-term use of synthetic antioxidants have limited their use. For this reason, research efforts on the potential natural antioxidants that could replace these synthetic antioxidants, began to attract much attention in a couple of years. Therefore, we evaluated antioxidant activities of different extracts prepared from *A. sintenisii* herbs using various *in vitro* antioxidant methods.

DOI: 10.33808/clinexphealthsci.1058614

The natural plant origin compounds with antimicrobial activity, have been investigated and used as additives in large quantities in foods (27). Because of their antibacterial effects against a wide spectrum of foodborne pathogens, compounds obtained from natural sources have the potential to be employed for food safety (28). Many antibiotic drugs are showing drug-resistance against human pathogenic bacteria. As a result, more research is needed to evaluate the antibacterial capabilities of plant extracts that can be used in food products without causing harm.

In both industrialized and developing countries, cancer is a major public health issue. In cancer, there is an abnormal growth of cells in the body that could result in death (29). The use of natural sources as anticancer agents is based on very old dates. Nowadays, many drugs used in chemotherapy are of natural origin or derived from natural products, especially from plants (30). Therefore, there is an intense interest in anticancer activity research from natural sources. In recent years, many studies have been carried out on the cytotoxic activity of medicinal plants (31–34).

There was very limited study on the essential oil and extracts of *A. sintenisii* for their antioxidant and antimicrobial activity (35). The antioxidant, antibacterial, and cytotoxic activity of this plant has not been thoroughly investigated. Due to a lack of comprehensive data, the current study was designed to investigate the biological potential of this well-documented plant. Therefore, the aim of the present study was to investigate the quantification of total phenolic and flavonoids as well as *in vitro* antioxidant, antimicrobial and cytotoxic effects of different fractions of the crude hydroalcholic extract of aerial part of *A. sintenisii*. Phytochemical screening was also performed on the extracts to evaluate the presence of phytochemical elements.

2. METHODS

2.1. Plant materials

The plant material was collected during the flowering period in the years of 2016 from Sivas province (Figure 1). Plants were identified by Mehmet Tekin, Ph.D. (Locality, B6 Sivas: Ulaş, Ziyarettepe, N35 33 08,9; E37 01 12,1; 1406 m, 12.06.2016). Voucher specimens were kept for record with the collect number of M. Tekin 1712, in the CUFF Herbarium of Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Turkey.

2.2. Chemicals

DPPH, ABTS, quercetin, Butyrylhydroxy toluene (BHT), ferrous sulphate, ferrozine, EDTA and MTT were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid, Folin-Ciocalteu's reagent and 2,3,5-Triphenyltetrazolium chloride (TTC) were obtained from Merck (Germany). The rest of the chemicals and reagents were of analytical quality.

Figure 1. *Habitat image of Achillea sintenisii Hub. Mor.*

2.3. Preparation of crude ethanolic extract and fractions

The shade dried and coarsely powdered aerial parts of *Achillea sintenisii* (100g) were macerated with 80% alcohol (1000 mL) at a water bath with temperature of 40ºC for 48 h. The residue was then filtered using filter paper and extracted twice with ethanol. Following this procedure, all of the extracts were combined and condensed under vacuum using a rotary-evaporator (Büchi, Switzerland) to afford the alcoholic extract (Yield: 12.44%, w/w), and then 10 g of the alcoholic extract after suspending with 500 mL of distilled water, was extracted successively with n-hexane, chloroform, n-butanol and water by separating funnel (The extract from each agent was then filtered, concentrated under vacuum to generate hexane (10.66 %), chloroform (12.93 %), n-butanol (28.7 %) and the final aqueous fractions (26.3 %).

2.4. Phytochemical screening

The prepared crude ethanol extract and partitioned sub-extracts were evaluated by phytochemical qualitative screening tests for herbal secondary metabolites such as terpenoids, alkaloids, antraquinones, flavonoids, tannins, saponins, coumarins, and phenolic compounds. The formation of specific color or the precipitation was regarded as positive for these tests (36).

2.5. Antioxidant activity

The ABTS assay was based on the method of Re et al. with some modifications (37).

Iron chelating activity is one of the commonly used methods to investigate the antioxidant activity, and the complexity of ferric oxide with bivalent iron ions is based on the reduction of the absorbance at the wavelength of 562 nm. Iron ion chelation method was performed according to the method of Salma et al. (38). The ability of ethanolic crude extracts and fractions of *A. sintenisii* to scavenge 1, 1´-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was measured as previously described (39). The total phenolic content of extracts was evaluated using a method described by Lee et al. (40). The

total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. The flavonoid content of each extract was measured based on methods described by Eruygur et al. (41). The results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g extract). The reducing power of the plant extract was analyzed according to the method of Oyaizu (42).

2.6. Antimicrobial Activity

Microbial strains: Antimicrobial and antifungal activities of the extracts and fractions were evaluated against two Gram$^+$ and two Gram– bacteria and one fungus by microdilution method. Test microorganisms were *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa (*ATCC 27853), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 10231). Geometric dilutions of the extracts were performed in a 96-well microtiter plate. Plates were incubated at 37°C for 24 hours for bacteria and 30°C for 48 hours for yeasts under normal atmospheric conditions. At the end of the incubation period, in order to make breeding visible, each well received 50 µL of 2,3,5-triphenyltetrazolium chloride (Merck, Germany) at a concentration of 2 mg/mL and incubated at 37 °C for 2 hours. The first wells without color change were accepted as MIC values. The test was repeated twice, and the same results were achieved.

2.7. Cytotoxic activity

2.7.1. MTT assay

Cancer cells: Human prostate cancer (PC-3) and human breast adenocarcinoma (MCF-7) cell lines were provided from the American Type Culture Collection (ATCC, USA) were used for the cytotoxicity test for the extracts. The cells were cultivated in RPMI 1640 media containing 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cancer cells were cultured at 37 °C in a humidified environment containing 5% carbon dioxide (43). The cells were treated with different concentrations of different extract of *A. sintenisii*. Then plates were incubated for 24 h, the medium was discharged from the 96-well plate, 10 µL of 3-(4,5 – dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added per well, and the plate kept for 2 h in 5% CO_2 humidified incubator at 37°C to allow reaction of yellow colored MTT reduced by mitochondrial dehydrogenases in viable cells to form pink to purple colored formazan. Excess MTT was sucked off, and the resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Using a microplate reader, the absorbance of purple formazan, which is proportional to the number of live cells, was measured at 560 nm (Epoch, USA). The tests were carried out in triplicate. The percentage of cell viability was calculated the following formulae:

% Cell viability= Absorbance of treated cells / Absorbance of the untreated cells × 100

Table 1. *Phytochemical screening results of ethanolic crude extract and fractions of A. sintenisii*

Phytochemical compounds	Phytochemical screening tests	Different fractions of *Achillea sintenisii*				
		n-Hexane Frc.	Chloroform Frc.	Ethanol Ex.	Butanol Frc.	Water Frc.
Carbonhydrates	Molish test	-	-	+	+	+
	Benedict test	-	-	+	+	+
Alkaloid	Dragendorff	–	–	–	–	–
	Mayer	–	–	–	–	–
	Marquis	–	–	–	–	–
Steroid	Salkowski	+	+	–	–	–
Protein	Biuret test	–	–	–	–	–
	Millon's test	–	–	–	–	–
Glycosides	Börntrager	–	–	–	–	–
	Killer-killani	+	+	+	–	–
	Baljet	–	+	+	–	–
Fatty acids	Filter paper stain test	–	–	–	–	–
Saponins	Forth test	–	–	–	–	–
Tannins and phenolics	%5 $FeCl_3$	+	+	+	+	+
	Lead acetate	+	+	+	+	+
	Salted Gelatin	–	+	+	+	+
Flavonoid	Shinoda	+	+	+	+	–
	NaOH	+	+	+	+	–
Coumarin	$FeCl_3$ +HNO_3	–	+	+	–	–
	NaOH + UV	–	+	+	+	–
Essential oil	Sudan III	+	+	+	–	–

2.7.2. Determination of Apoptosis

MCF-7 and PC-3 cells were treated in triplicate with 10 µg/mL concentration of plant extracts in 12-well plates at density of 2 × 10^4 cells/well for 12 hours. A Zeiss Axio inverted microscope (10X) imaging system was used to capture images of cell growth. Quantification of cell growth was done using Methylene blue staining. Acridine orange and ethidium bromide (AO/EB) were used to evaluate apoptosis in MCF-7 and PC-3 cancer cell lines. After a 24 – hour incubation period, 10 µg/mL concentrations of plant extract were applied in triplicate to 12-well plates at density of 2 × 10^5 cells per well. After that, cells were stained with 1µg/mL AO/EB solutions and the fluorescence intensity was measured using a microscope (Zeiss). Apoptotic cells are grouped with red intensity, while living cells are grouped with green intensity.

3. RESULTS

In this study, the ethanol crude extract and different fractions were investigated regarding their phytochemical composition by different phytochemical screening tests. Phytochemical screening of the tested extracts revealed the presence of flavonoids, phenolics, tannins and reducing sugar. Essential oil was also present in the hexane, chloroform fractions and ethanol extract.

The ethanol crude extract and different fractions of the aerial parts of *A. sintenisii* were screened against antioxidant, antimicrobial and cytotoxic activity. Antioxidant activity of the extracts were determined by DPPH, ABTS radical scavenging assay, ferric-reducing antioxidant power (FRAP), ferric ion-chelating, total phenol and total flavonoid content assays. The antioxidant and cytotoxic activity assays were tested at the concentrations of 0-1000 µg/mL. The detailed information about the results of antioxidant activities were given in Table 2 and Figure 2-3.

The Folin-Ciocalteau method was used to determine the total phenol contents (TPC) of the extracts and the TPC was expressed as Gallic acid equivalents (GAE). The TPC of the extracts ranged from 73.67 ± 3.87 to 192.41 ± 1.60 mg GAE /g extract according to our findings (Table 2). The extracts' total flavonoid content was determined using $AICl_3$ colorimetric method and represenTeded as quercetin equivalents (QE). According to the results, total flavonoid content ranging from 5.09 ± 5.84 to 117.69 ± 6.88 mg QE/g dry extract and the content was found in the decreasing order of n-butanol Frc.>Hexane Frc. >Chloroform Frc. > ethanol extract > aqueous fractions.

As for antimicrobial activity, the n-hexane fractions showed stronger inhibitory activity against tested gram-positive and gram-negative microorganisms and the MIC value ranges from 0.31 to 5 mg/mL, while other fractions showed no activity (Table 3).

The cytotoxic activity of the extracts was evaluated by MTT assay, the observed results strongly profile that there was a concentration dependent cytotoxic effect of the extract against MCF-7 and PC-3 cancer cells (Figure 4-5). The apoptosis of the cancer cells treated with the 100 µg/mL concentration of different extracts was determined staining with acridine orange (AO) and ethidium bromide (EB) dual staining and observed by fluorescence microscope. The fluorescence images of AO/EB staining are given in Figure 6 and the staining ratio of MCF-7 and PC-3 cells are shown in Figure 7.

Table 2. *Extraction yield, total phenolic, flavonoid content and antiradical activities of ethanolic crude extract and fractions of A. sintenisii.*

Extract or fraction	Yield of extract (%, w/w)	Total flavonoid content (mg QE/g)	Total phenol content (mg GAE/g)	DPPH radical scavenging activity IC_{50} (µg/mL)	ABTS radical scavenging activity IC_{50} (µg/mL)
Ethanol extract	12.44	41.46 ± 1.61	118.91 ± 1.91	895.42 ± 0.09[a]	590.26 ± 0.55[a]
Hexane Frc.	10.66	98.92 ± 7.35	73.67 ± 3.87	553.56 ± 0.81[b]	337.92 ± 0.93[b]
Chloroform Frc.	12.93	76.71 ± 3.18	176.91 ± 2.39	585.78 ± 1.48[b]	468.83 ± 0.29[c]
n-butanol Frc.	28.70	117.69 ± 6.88	192.41 ± 1.60	394.39 ± 1.04[c]	398.07 ± 1.25[d]
Water Frc.	26.30	5.09 ± 5.84	76.85 ± 2.92	513.17 ± 1.07[b]	429.53 ± 0.51[c]
Ascorbic acid				106.17 ± 0.97[d]	
Trolox					65.53 ± 0.57[e]

Values are expressed as mean ± SD of triplicate experiments. Different letters in the same column were significantly different (p<0.05) from each other.

Table 3. *Minimum inhibitory concentrations (MIC) of different extracts of A. sintenisii aerial parts*

S/No.	Microorganisms	Ethanol extract and fractions from ethanol extract of *A. sintenisii* (Concentration mg/mL)				
		Hexane frc.	Chloroform frc.	Ethanol ext.	n-Butanol frc.	Water frc.
1	*E. coli*	>5	>5	>5	5	5
2	*S. aureus*	0.31	5	>5	>5	>5
3	*P. aeruginosa*	>5	>5	>5	>5	>5
4	*E. faecalis*	>5	>5	>5	>5	>5
5	*C. albicans*	1.25	5	>5	>5	>5

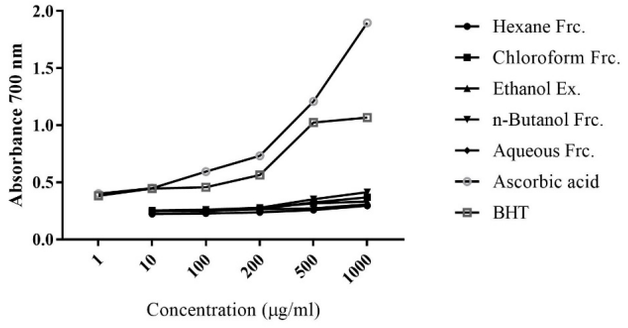

Figure 2. *Ferric reducing power of A. sintenisii extracts*

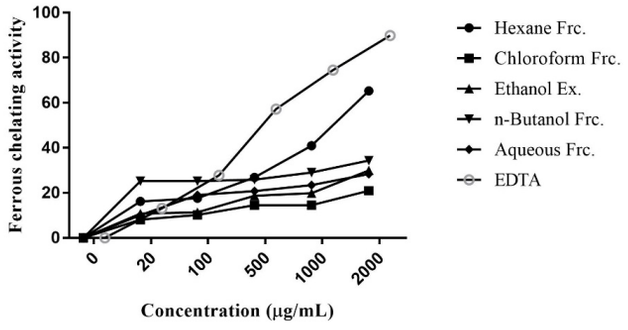

Figure 3. *Iron chelating activity of A. sintenisii extracts*

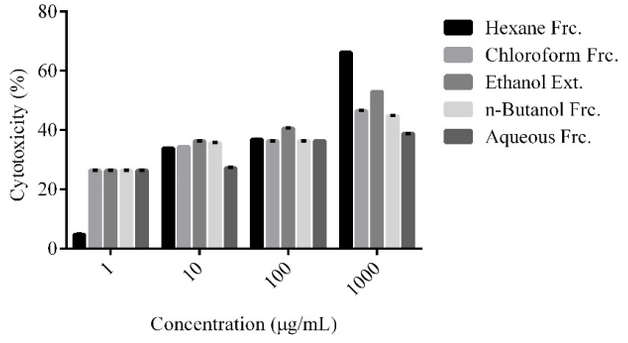

Figure 4. *Cell growth inhibition results of MCF-7 cells treated with A. sintenisii extracts after 24 hours*

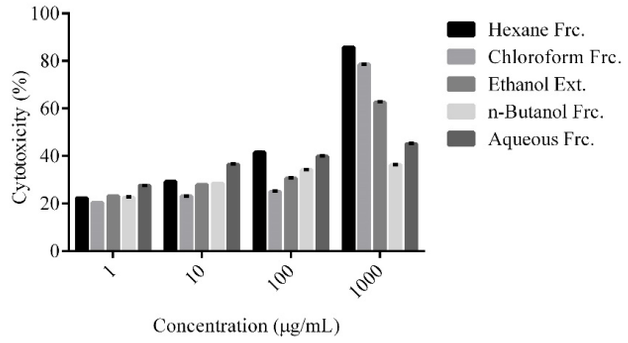

Figure 5. *Cell growth inhibition results of PC-3 cells treated with A. sintenisii extracts after 24 hours*

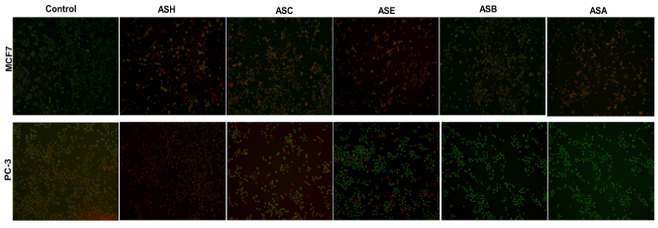

Figure 6. *AO/EB staining fluoresce images of MCF-7 and PC-3 cells treated with different A. sintenisii fractions after 24 hours (100 µg/mL)*

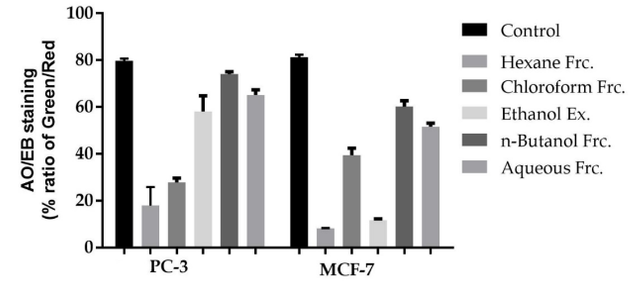

Figure 7*. AO/EB staining ratio of PC-3 and MCF-7 cells treated with A. sintenisii extract (100 µg/mL) for 24 hours*

4. DISCUSSION

Plant extract's antioxidant properties play a vital role in the prevention, treatment, and management of a variety of diseases and disorders associated with inflammation and oxidative stress. The over-releasing of reactive oxygens species in the biological system led to developing of chronic disease. This study showed the *in vitro* antioxidant activity of different extracts obtained from *A. sintenisii* using different antioxidant assays. The radical scavenging activity of different extracts obtained from *A. sintenisii* was determined by DPPH and ABTS radical scavenging assay. The DPPH radical scavenging activity of the different evaluated extracts found to be as n-butanol frc. > aqueous frc. > hexane frc. > chloroform frc. > Ethanol extract. The ABTS radical scavenging activity of the different evaluated extracts found to be as hexane frc. > n-butanol frc. > aqueous frc. > chloroform frc. > ethanol extract. The redox characteristics of flavonoids and phenolics, as well as the structural interactions between different portions of their chemical structure, are primarily responsible for their radical scavenging activity (44). In the Folin-Ciocalteu's assay, the n-butanol fraction was found to be the most polyphenolic enriched extract of TPC (192.41 ± 1.60 mg GAE /g extract) compared to the other fractions. Moreover, there was no data is available on the previous investigations on the TPC of different extracts of *A. sintenisii*. Phenolic contents in extracts obtained from other *Achillea* species were also reported. Agar et al. reported that the total

phenolic content of *A. coarctata, A. kotschyi* and *A. lycaonica* was expressed as mg gallic acid equivalent of 55.16 ± 0.96, 148.00 ± 0.92 and 76.49 ± 1.67 g dry extracts, respectively (26). These results are in agreement with our TPC results found in different extract of *A. sintenisii.* The variance in total phenolic amounts may be related with different parameters such as pretreatment, extraction method, plant species, geographical location and harvesting time etc. In a previous study, phenolic composition of *A. sintenisii* was determined by HPLC method and luteolin, vitexin, and schaftoside were found as the major phenolic compounds in water and aqueous ethanol extracts (45).

Table 3 shows the MIC of different extracts of *A. sintenisii*. All tested bacterial strains were not sensitive to the extracts except for the *S. aureus* and the yeast *C. albicans.* Previously, several *Achillea* essential oils were reported for their antimicrobial activity. The essential oil of *A. teretifolia* and *A. nobilis* were found to be active against the tested human pathogen microorganisms with the MIC value of 0.5-2 mg/mL (18). In another study of *A. teretifolia* essential oil was studied against fourteen microorganisms and showing MIC values of 0.28 to 2.25 mg/mL (46). Karaalp et al. reported antimicrobial properties of thirteen *Achillea* species flower extract and found that hexane extract of *A. coarctata* and *A. setacea* showed antibacterial activity against *E. faecalis* with 31.25 and 62.5 µg/mL MIC values. *A. teretifolia*, *A. multifidi* were found to have antimicrobial activity ranging from 50 to 75 µg/mL against *S. aureus, S. epidermidis* and *S. typhymurium* (47).

Further, we studied the *in vitro* cytotoxicity of the different extracts of *A. sintenisii* against two different human cancer cell lines by commonly used MTT cytotoxicity assay. The extracts all we studied showed cell growth inhibition in a dose-dependent manner up to 1000 µg/mL. In fact, the anticancer activity of *A. sintenisii* has not been shown in the literature. In a previous study, the methanolic extract of *A. odorata* showed strong dose dependent *in vitro* cytotoxicity against MCF-7, HepG2, and WEHI cell lines (48). The ethanol and methanol-chloroform extracts of *A. coarctata* and *A. monocephala* were evaluated for cytotoxic activity against Hela cells and found that the cells highly inactivated over the concentration of 100 µg/mL (49). A recent study has showed that of *A. kotschyi* and *A. lycaonica* have high cytotoxic effect on MCF-7 cancer cells lines (15).

Nevertheless, this is the first report on the *in vitro* antioxidant, antimicrobial and cytotoxic activity of *A. sintenisii* crude ethanol extract and different fractions. Some fractions showed more active than the crude ethanol extract. Further phytochemical investigations are underway to identify the possible active constituents responsible for the biological activity by bioassay-guided isolation techniques.

5. CONCLUSION

The results of the current study reports that all the extracts of *A. sintenisii* have shown significant antioxidant and cytotoxic effects and moderate antimicrobial activity. A significant difference in antimicrobial activity was not observed between the tested extracts except for hexane fraction. The findings of this study point to the possibility of using *A. sintenisii* as a source of antioxidants. Furthermore, the active fraction of *A. sintenisii* may lead to the presence of new cytotoxic active ingredients by phytochemical studies based on its cytotoxic activity.

Funding: *This work was supported by the Sivas Cumhuriyet University Academic Research Grants (Grant number: CÜBAP ECZ-020).*
Conflicts of interest: *The authors declare that they have no conflict of interest.*
Peer-review: *Externally peer-reviewed.*
Author Contributions:
Research idea: NE, MT, ÖÇ
Design of the study: NE, MA, ÖÇ
Acquisition of data for the study: NE, MA, ÖÇ
Analysis of data for the study: NE, ÖÇ
Interpretation of data for the study: NE, MA, MT, ÖÇ
Drafting the manuscript: NE
Revising it critically for important intellectual content: NE, MA, MT
Final approval of the version to be published: NE, MA, MT, ÖÇ

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How to cite this article: Eruygur N, Ataş M, Tekin M, Çevik Ö. Evaluation of *in vitro* Antioxidant, Antimicrobial and Cytotoxic Activities of Crude Ethanol Extract and Fractions of *Achillea sintenisii* Hub. Mor. Clin Exp Health Sci 2023; 13: 517-524. DOI: 10.33808/clinexphealthsci.1058614