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IN VITRO STUDY ON ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF METHANOL EXTRACT OF *HELICHRYSUM PLICATUM* SUBSP. *POLYPHYLLUM* (ASTERACEAE)

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Abstract

Helichrysum plicatum subsp. *polyphyllum* (*H. plicatum*), is an important species belonging to the family Asteraceae, which is used in alternative medicine. For this reason, it was aimed to determine the antimicrobial and cytotoxic activity of *H. plicatum* extract in this study. Firstly, *H. plicatum* was extracted with methanol for 6 hours using a Soxhlet. Secondly, the antimicrobial activity of the extract was tested against Gram-positive (*Staphylococcus aureus* ATCC®25923, *Bacillus cereus* ATCC®7064, *Bacillus subtilis* ATCC®6633) and Gram-negative (*Escherichia coli* ATCC®25922, *Klebsiella pneumoniae* ATCC®706003, *Salmonella enteritidis* ATCC®13076) bacteria and a yeast (*Candida albicans* ATCC®10231) using well diffusion and microdilution methods. Finally, the cytotoxic activity of *H. plicatum* extract on DLD-1 (human colon adenocarcinoma) and CCD-18Co (normal colon) cell lines was determined by MTT method. According to the results of the antimicrobial effect, *H. plicatum* extract was found to have a stronger antibacterial activity against *Salmonella enteritidis* (24.13±1.15 and 156µg/mL) among Gram-negative bacteria. It was determined to have inhibitory activity against also *Bacillus cereus*

(16.66±1.52 and 312µg/mL) among Gram-positive bacteria. In addition, the extract showed a strong antifungal effect on *Candida albicans* (22.66±0.57 and 312µg/mL). In addition, the *H. plicatum* extract did not show any cytotoxic effect on CCD-18Co cells, while the IC_{50} value on the DLD-1 cell line was determined as 8.17mg/mL. This indicates that the plant extract has a selective toxic effect on cancer cells. These results reveal that *H. plicatum* has the potential to be used for therapeutic purposes after being supported by further studies.

Key Words: Biological activity, *Helichrysum plicatum,* Microdillution, MTT, Well diffusion

Özet

Helichrysum plicatum subsp. *polyphyllum* (*H. plicatum*), alternatif tıpta kullanılan Asteraceae familyasına ait önemli bir türdür. Bu sebeple çalışmada *H. plicatum* özütünün antimikrobiyal ve sitotoksik aktivitesinin belirlenmesi amaçlanmıştır. İlk olarak soksalet cihazı kullanılarak 6 saat boyunca *H. plicatum*'dan metanol ile özüt elde edilmiştir. İkinci olarak, ekstraktın antimikrobiyal aktivitesi Gram-pozitif (*Staphylococcus aureus* ATCC®25923, *Bacillus cereus* ATCC®7064, *Bacillus subtilis* ATCC®6633) ve Gram-negatif (*Escherichia coli* ATCC®25922, *Klebsiella pneumoniae* ATCC®706003, *Salmonella enteritidis* ATCC®13076) bakteri ve mayaya (*Candida albicans* ATCC®10231) karşı kuyu difüzyon ve mikrodilüsyon yöntemleri kullanılarak test edilmiştir. Son olarak *H. plicatum* özütünün DLD-1 (insan kolon adenokarsinomu) ve CCD-18Co (normal kolon) hücre hatları üzerindeki sitotoksik aktivitesi MTT yöntemi ile belirlenmiştir. Antimikrobiyal aktivite sonuçlarına göre, *H. plicatum* özütünün Gram-negatif bakteriler arasında *Salmonella* e*nteritidis*'e (24,13±1,15 ve 156µg/mL), Gram pozitif bakterilerden de *Bacillus cereus*'a (16,66±1,52 ve 312µg/mL) karşı güçlü bir antibakteriyel aktiviteye sahip olduğu bulunmuştur. Ayrıca, özüt *Candida albicans* (22.66±0.57 ve 312µg/mL) üzerinede güçlü bir antifungal etki göstermiştir. Ek olarak, *H. plicatum* özütü CCD-18Co hücreleri üzerinde herhangi bir sitotoksik etki göstermezken DLD-1 hücre hattındaki IC_{50} değeri 8,17mg/mL olarak belirlenmiştir. Bu, bitki özütünün kanser hücreleri üzerinde seçici bir toksik etkiye sahip olduğunu gösterir. Bu sonuçlar *H. plicatum*'un ileri çalışmalarla desteklenerek alternatif amaçlı kullanılma potansiyeline sahip olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Biyolojik aktivite, *Helichrysum plicatum,* kuyu difüzyon, mikrodilüsyon, MTT

1. Introduction

Although the interest in herbal medicines has decreased with the rapid spread of synthetic medicines in the last century, herbal medicines that human beings have used for thousands of years to stay healthy and be treated have become the focus of attention of researchers in recent years. The frequently encountered side effects of synthetic drugs and the damage they cause to organs such as kidney and liver reveal the necessity of minimizing the use of synthetic drugs. It has become essential to investigate the effects of these chemical components, which are generally produced by plants for their own defense mechanisms, on humans and to discover the benefits they will provide. Asteraceae is the first plant family that comes to mind in this sense in terms of the chemical components it contains. The *Helichrysum* genus belonging to the Asteraceae family has about 20 species, 14 of which are endemic, in Turkey. The plant, which is also known as "ölmez çiçek, and mantuvar" among the people, is used primarily to facilitate digestion, as a bile increaser and diuretic, to reduce kidney stones, and to treat wounds and burns (Davis et al., 1988; Erik et al., 2000). In addition, in recent studies, it has been revealed that *Helichrysum* species show many biological activities such as plant extracts, chemical contents and polar extracts, antioxidant, anti-inflammatory, antidiabetic, antiviral, antimicrobial and antimutagenic (Bigovic et al., 2010).

Helichrysum plicatum subsp. *polyphyllum* (*H. plicatum*), flowering in June, July, and August. It is a perennial herbaceous plant. Since its flowers do not fade even when it is completely dry, it is called the immortal flower among the people (Güner et al. 2012). It was determined that the extracts of *H. plicatum* flowers prepared with solvents of different polarity showed significant antioxidant activities. Due to the side effects of synthetic agents used as antioxidants today, it is thought that the plant may be an alternative source of natural bioactive agents in the fields of nutraceutical, pharmaceutical, cosmetics and food processing (Kaya, 2022). It is also known that there are differences in biological activities when suitable extraction methods for standardization of plant products, phytochemical analysis and different solvents are used (Azwanida, 2015). The aim of this study was to investigate the antimicrobial and cytotoxic activity of methanol extract obtained from *H. plicatum.*

2. Material and Methods

2.1. Preparation of extract

H. plicatum was collected during flowering from a natural population in Karaman Sarıveliler village in June-July 2021 (Figure 1). The flowers of plant were dried in the shade, separated from their roots and ground in a grinder. The dried sample weighing about 50 g was extracted in a Soxhlet with methanol for 6 h. The solvents were removed under a rotary vacuum (40°C) until dry (Heidolph Collegiate, USA). The extracts were then stored in a dark bottle at 4°C until testing was performed in the study (Kordalii et al. 2009; Tekin et al. 2021).

Figure.1 *H. plicatum* and the area where it was collected from Sarıveliler village of Karaman

2.2. Antimicrobial activity test

Antimicrobial effect of the *H. plicatum* extract was analysed by the well diffusion and microdillution method (CLSI 2014; Poudineh et al., 2021). The bacteria used as test microorganisms were three Gram-positive (*Staphylococcus aureus* ATCC®25923, *Bacillus cereus* ATCC®7064, *Bacillus subtilis* ATCC®6633), three Gram-negative (*Escherichia coli* ATCC®25922, *Klebsiella pneumoniae* ATCC®700603, *Salmonella* Enteritidis ATCC®13076) bacteria and a yeast (*Candida albicans* ATCC®10231) were obtained from Dr. Ömer Ertürk (Ordu, Turkiye). Gentamicin and ketoconazole (Bioanalyse Ltd., Turkey) were used as positive and dimethyl sulfoxide (DMSO, Sigma) as negative controls.

2.2.1. Well diffusion method

Overnight cultures prepared by inoculation on Tryptic Soy Agar (Merck, Germany) and Sabouraud Dextrose Agar (Biolife, Italiana Srl, Milano), were adjusted to 0.5 McFarland (~1.5 x 10^8 CFU/mL) and 10^7 cells/mL of bacterial and yeast cells in sterile 0.9% saline. The prepared bacteria and yeast solutions were spreaded on to petri dishes containing Mueller-Hinton Agar

(MHA, Biolife, Italiana Srl, Milano) using sterile swab. The extract prepared at a concentration of 10000 ug/mL in DMSO was poured into the wells of 6 mm depth opened with a sterile apparatus on the Müller-Hinton agar plate. Inhibition zone diameters were calculated in mm by measuring with a caliper after the specified incubation time for bacteria (24 hours at 36°C) and yeast (48 hours at 27°C) (CLSI 2014; Poudineh et al., 2021).

2.2.2. Microdillution method

In this method, a 96-Well U-bottom microtiter plate was used to determine the minimum inhibitory concentration (MIC). Mueller-Hinton broth (Cation-adjusted, CAMHB, Biolife, Italiana Srl, Milano) was used as specified in the CLSI criteria. 100 µL of prepared CAMHB was added to the wells in the plate. Extract prepared at different serial dilution concentrations (10000, 5000, 2500, 1250, 625, 312.5, 156.25-78.125 ug/ml) was added to the microplates. Then, 5×10^5 CFUs/mL bacteria were inoculated into each well of 96-well microplates. After incubation, the smallest concentration of the extract that stopped the growth of the microorganism was determined by microdilution method.

2.3. Determining the Cytotoxic Activity of the Extract by MTT test

2.3.1 Cell line and culture

The method proposed by Mosmann (1983) was applied for the MTT test, which is a method based on the measurement of succinate dehydrogenase enzyme activity during the formation of fumarate from succinate. When the density of the cells reached approximately 80% occupancy on the flask surface, they were removed from the surface by treatment with trypsin-EDTA. Human colon adenocarcinoma cells DLD-1 (ATCC CLL-221™) and normal cells CCD-18Co cells (ATCC CRL-1459™) were obtained from the cell stock of Amasya University Central Research and Application Laboratory. DLD-1 and CCD-18Co cells were cultured in Dulbecco's modified eagle medium (DMEM, Sigma) and Eagle's Minimum Essential Medium (EMEM, Sigma), respectively. All cells were incubated at 37°C, 5% CO_2 and 95% humidity.

2.3.2. MTT assay

The cytotoxicity of extract and DMSO (positive control) were performed by MTT assay as previously described (Mosmann, 1983). Cell which was determined as $1x10^4$ cell/100 µL as a result of optimization study for MTT test, was planted in 96-well cell culture plates. After the

period, the medium on the cells was removed by inverting the medium plates, and the media containing the desired concentrations of *H. plicatum* (0.156-10 mg/mL). The absorbance value was analyzed with an ELISA reader at 570 nm wavelength and MTT dye applied after 24 hours of incubation at 37°C.

3. Results

3.1. Antimicrobial effect

The methanol extracts of *H. plicatum* species collected from Sarıveliler village of Karaman were tested on some bacteria and yeast through well diffusion and micro dilution methods. The results are summarized in Table 1. The antimicrobial effect of the extract in the well diffusion method was determined by measuring the inhibition zone diameter in millimeters with a caliper. The minimum inhibition concentration value (MIC value) obtained because of the microdilution test, which is a reference method for antimicrobial activity tests, gives the smallest concentration of the antibacterial agent necessary to inhibit the microorganism causing the infection.

Table.1 Antimicrobial activity of extract, well diffusion method (mm, mean±std) and MIC values (µg/ml)

	Test microorganisms						
	Gram positive bacteria			Gram negative bacteria			Fungus
	S. aureus	*B. cereus*	*B. subtilis*	*K. pneumaniae*	*S.* Enteritidis	*E. coli*	*C. albicans*
H. plicatum DC.	15.66±2.08 625	16.66±1.52 312	- 10000	13.66±1.52 2500	24.13±1.15 156	- 10000	22.66±0.57 312
CN	21.66±0.57 156	15.00±1.00 625	25.66±1.52 ≤ 78.12	25.33±0.57 ≤ 78.12	23.66±0.57 156	23.00±0.00 156	NT
KTC	NT	NT	NT	NT	NT	NT	15.33±0.57 1250
DMSO	- >10000	- >10000	- >10000	- >10000	- >10000	- >10000	- >10000

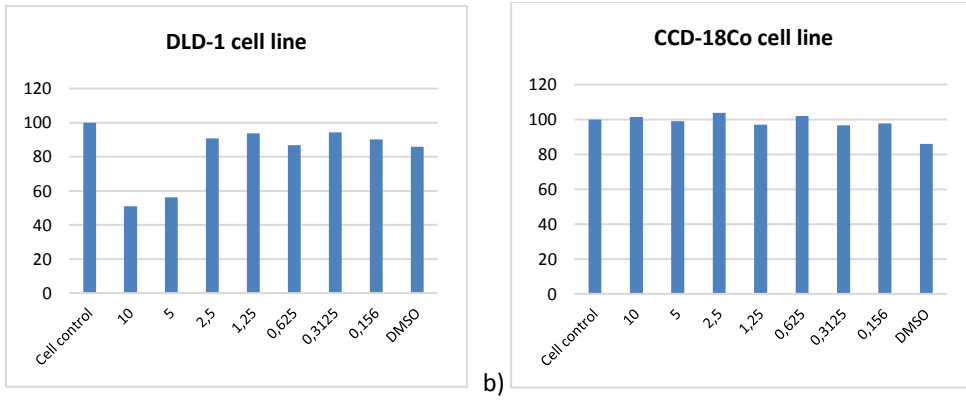
DMSO: Dimethyl sulfoxide
NT: Not tested, (-): Inhibition zone did not occur.
CN: Gentamicin (30 µg), KTC: Ketoconazole (50 µg)

H. plicatum extracts showed various antimicrobial activities against *S*. Enteritidis, *B. cereus*, and *C. albicans*. The maximum effective inhibition of *H. plicatum* methanol extract was found against *S*. Enteritidis (24.13±1.15 / 156 µg/mL) (Figure 2). At the same time, the extracts of *H. plicatum* showed antifungal activity against *C. albicans* (22.66 ±0.57 /312 µg/mL (Table 1).

Figure 2. Well diffusion test. Susceptibility profile of *H. plicatum* extract on *S*. Enteritidis. CN: Gentamicin, H: *H. plicatum* extract

3.2. Cytotoxic activity

The cytotoxic effects of *H. plicatum* extract against DLD-1 cancer and CCD-18Co normal cell lines were analyzed by MTT assay and the inhibitory concentration (IC_{50}) values are specified in Figure 3. For this purpose, cells were exposed to *H. plicatum* extract (0.156-10 mg/mL) prepared at different concentrations at 37°C for 24 hours.



a) b)

Figure 3. Cytotoxic effect of *H. plicatum* extract on CCD-18Co and DLD-1 cells by MTT test

As we can see in Figure 3, it was determined that 50% of the cells died in the DLD-1 cell line and the IC_{50} value was determined as 8.17mg/mL (Figure 3a). In the CCD-18Co cell line, a comparison was made with DMSO used as a solvent control and it was determined that there was

no visible effect on the viability of the cells (Figure 3b). This indicates that the plant extract has a selective toxic effect on cancer cells.

4. Discussion

It is known that extracts of *H. plicatum* have remarkable antioxidant, antimicrobial, and cytotoxic activities (Demir et al. 2009; Tatlı et al. 2009; Apaydın Yıldırım et al. 2017; Taşkın et al. 2020). In the literature, it is reported that the composition of *H. plicatum* is rich in flavonoids (Kulevanova et al. 2000; Acet et al. 2019; Güneş et al. 2019).

In this study, it is prepared an extract of *H. plicatum* in methanol. It has been determined the antimicrobial effect using the disc diffusion method. The cytotoxic activity of the extract has also been researched. Table 1. shows that *H. plicatum* didn't spread the inhibition zone against *B. subtilis* and *E. coli*. However, it was effective against *S. aureus*, *B. cereus*, and *S*. enteritidis. For *S. aureus* and *K. pneumoniae*, although the MIC value of the plant was more than gentamicin, the inhibition zone value of the plant was less than gentamicin. So, it could be said that the plant extract was less effective than gentamicin. On the other hand, for the other bacteria, it is obvious that the plant extract was more effective than gentamicin. In this way, it could be said the extract of *H. plicatum* can be an alternative to gentamicin for *B. cereus* and *S*. Enteritidis. Additionally, according to Table 1, the plant extract spread a wide inhibition zone against *C. albicans*. The MIC value of the extract is less than the value of Ketoconazole, however, it showed a more powerful effect than the value of ketoconazole. It seems that the plant extract has an effective antifungal activity.

The Asteraceae family has been subject to many studies. It has an important place as the beneficial health effects and the contents of the bioactive compound. Some of the Asteraceae family members have shown antibacterial effects against *S. aureus*. Similarly, they pointed to significant bactericidal and bacteriostatic activity against *S*. Typhimurium. The antibacterial effectes of the Asteraceae family could be attributed to their composition of phenolic which have been associated with the control of food-borne pathogens (Petropoilos et al. 2019).

In another study, S*taphylococcus* spp., *E. coli*, and *P. mirabilis* have isolated from urine secretion samples, and some of the Asteraceae family members have been used against these bacteria. Thus, these results suggested that the Asteraceae family could be a therapeutic agent against bacteria (Chiavari-Frederico et al. 2020).

The *Helichrysum* species has attracted attention due to its secondary metabolite content, specifically flavonoids and essential oils. For example, *H. italicum* bioactives are shown to exert a

range of biological activities including anti-inflammatory (Kothavade et al., 2013; Rosa et al., 2017), anti-HIV-1 (Appendino et al., 2007) and cytotoxic (Sala et al., 2002). Then, the *in vitro* cytotoxic effects in human lymphocytes of the *Helichrysum taxa* were investigated. Accordingly, the results suggest a strong interaction between extracts of the *Helichrysum* species and DNA, which could be responsible for cytotoxicity (Eroglu et al. 2010).

In this study, healthy cells and carcinomatous cells were investigated if they would be affected by the extract of *H. plicatum* DC. Figure 2b. shows the CCD-18Co cell line, it is clear that the plant extract didn't affect the healthy cells. Figure 2a. also shows the DLD-1 cell line. So, 5-10 mg/mL of the extract inhibited almost half of the cancer cells. As a result, 8.17 mg/mL is the IC_{50} value. Although the results should be supported by further experiments, *H. plicatum* DC. could potentially be an anticancer agent.

One another research, it is studied the potential of *Helichrysum italicum* extract cytotoxicity activation in different cells. It is reported that the types of extract did not have important differences with respect to the cytotoxicity percentage in either of the investigated cell lines Nevertheless, it can be said that extracts are not toxic under prolonged exposition to the examined cells in the culture (Galovic et al. 2022).

5. Conclusion

H. plicatum extract has an important antimicrobial effect on some Gram-positive (*B. cereus*), Gram-negative (*S. enteriditis*), and *C. albicans*. In addition, it also has cytotoxic activity on colon adenocarcinoma cell line. These results showed that the *H. plicatum* extract is a plant that needs to be clarified by further studies.

Conflicts of interest

All authors declare that there was no conflict of interest in this study.

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