Istanbul J Pharm 53 (2): 193-198
DOI: 10.26650/IstanbulJPharm.2023.1202165
Original Article

Antioxidant, tyrosinase inhibitor, and cytotoxic effects of *Anthemis aciphylla* Boiss. var. *aciphylla* and *Cota dipsacea* (Bornm.) Oberpr. & Greuter

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Cite this article as: Sumer Tuzun, B., Fafal, T., Ilhan, R., Kivcak, B., & Ballar Kirmizibayrak, P. (2023). Antioxidant, tyrosinase inhibitor, and cytotoxic effects of *Anthemis aciphylla* Boiss. var. *aciphylla* and C*ota dipsacea* (Bornm.) Oberpr. & Greuter. *Istanbul Journal of Pharmacy, 53*(2), 193-198. DOI: 10.26650/IstanbulJPharm.2023.1202165

ABSTRACT

Background and Aims: The study aims to examine the antioxidant , tyrosinase inhibitory, and cytotoxic activities of the methanol and chloroform extracts of *Anthemis aciphylla* Boiss. var. *aciphylla* and *Cota dipsaceae* (Bornm.) Oberpr. & Greuter in order to evaluate the results regarding different polarities.

Methods: The study evaluates the total phenolic and total flavonoid content of the extract sand the *in vitro* antioxidant activities using the DPPH, ABTS, superoxide radical, and nitric oxide scavenging activities. Tyrosinase inhibitor activity was identified by Masuda's method. Cytotoxicity was investigated using the MTT assay.

Results: The *A. aciphylla* var. aciphylla methanol extract (*AAM*) was the most active extract in term of overall antioxidant activity. The IC_{50} values for DPPH, ABTS, and superoxide anion radical activity were found to be 19.57, 46.82, and 5.610 µg/mL, respectively. Nitric oxide scavenging activity percent inhibition of the *AAM* was 46.47%. The tyrosinase inhibitory activity of *AAM*, was also the most effective, thus confirming the study's hypothesis. Moreover, no cytotoxic activity was observed to be present in the extracts with biological activity.

Conclusion: According to the hypothesis, the tyrosinase inhibitor effect should be observed mostly in extracts and compounds that show the high antioxidant activity mostly originating from phenolic substances. *AAM* confirmed this hypothesis as the most active extract regarding both the tyrosinase inhibitor effect as well as antioxidant activities. Furthermore, the MTT assay was used to find *AAM* to be non-cytotoxic at the tested concentrationsThis indicates *AAM* to be able to be used more reliably in medicine and as a food preservative.

Keywords: *Anthemis, Cota*, antioxidant activity, cytotoxic activity, tyrosinase inhibitor activity

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Submitted: 11.11.2022
Revision Requested: 27.01.2023
Last Revision Received: 13.06.2023
Accepted: 14.06.2023
Published Online: 28.08.2023

INTRODUCTION

The use of natural resources, including herbal medicines, has become quite common in the therapeutic field. The secondary metabolites they contain involve important biological activities. Phenolic compounds found in plant extracts are particularly known for being the most important group responsible for biological activities involving antioxidant activity. Oxidative stress plays an essential role in chronic diseases such as cardiovascular disease, diabetes, and cancer. Antioxidants protect the body from oxidative stress by scavenging free radicals (Zengin, Aktümsek, Güler, Çakmak & Yıldıztugay, 2011; Albayrak, Atagün & Aksoy, 2017). Several reports are found describing the involvement of free radicals in carcinogenesis, with a general relationship found between antioxidant defense systems and melanogenesis. The most important point of the relationship is the result of the synergistic effect of tyrosinase inhibitors by increasing their antioxidant effectiveness in scavenging free radicals while working (Wang et al., 2018). UV radiation stimulates reactive oxygen species (ROS) production in the skin, increasing skin pigmentation. ROS are formed as a result of DNA damage, UV radiation, and immune responses to cancerous cells. As mentioned, increased ROS production has long been observed in many types of cancers and different diseases (Witgen and Kempen 2007; Fuchs-Tarlovsky, 2013; Venza et al., 2021).

Skin rejuvenation and hyperpigmentation disorders have become very important in recent years. Nowadays, the focus is on tyrosinase enzyme, which is an essential and rate-limiting enzyme involved in the biosynthesis of melanin, the group of pigments responsible for pigmentation in mammals. Melanin is a copper-containing multifunctional oxidase (Masuda, Yamashita, Takeda & Yonemon, 2005). In the course of melanogenesis, the process for synthesizing melanin, tyrosinase catalyzes the hydroxylation of L-tyrosine to DOPA (β-3,4-dihydroxy phenylalanine), then allowing Dopaquinone to be oxidized. Overproduction of melanin causes hyperpigmentation disorders such as melasma and age spots. Tyrosinase inhibitors show activity through hydroxyl groups that bind copper at the active site of tyrosine. Polyphenols, especially flavonoids, long-chain lipids, and steroids, are the most common enzyme inhibitors. A complex is thought to bind mechanically, the oxygen of phenol to copper, followed by electrophilic monooxygenation of the ring to both copper ions (Zolghadri et al., 2011; Sarıkürkçü, 2020).

The genus *Anthemis* is in the Asteracea family, with 81 taxa and 51 species registered in the flora of Türkiye (Davis, 1984; Güner, Ozhatay, Ekim & Baser 2000). Ethnobotanically, *Anthemis* is used internally for gastrointestinal disorders, abdominal pain, stomach pain, hepatic diseases, coughs, carminative, and kidney stone reduction and externally for burns and skin irritation (Baytop, 1984; Gürhan & Ezer, 2004). Its major secondary metabolites are sesquiterpene lactones, flavonoids, phenolics, acetylene, and essential oils. In addition, the genus is known to have antioxidant, antimicrobial, anticholinesterase, antiproliferative, antispasmodic, and anti-Helicobacter pylori effects (Honda, Yesilada & Tabata, 1996; Kultur, 2007; Uğurlu and Seçmen, 2008).

The plant *Anthemis dipsacea* was included in the *Cota* genus in 2012 with the study of Güner et al. . *Anthemis dipsacea* Boiss. is known as the synonym for the plant *Cota dipsacea* (Bornm.) Oberpr. & Greuter (Güner et al., 2012). While the genus *Cota* had previously been considered a species in the genus *Anthemis*, it is now considered a separate genus consisting of 63 taxa worldwide and being distributed throughout Western Europe, North Africa, and Asia. There are 22 taxa in Türkiye, 9 of which are endemic. The ethnobotanical uses of the *Cota* species indicate that the infusion prepared from their capitulum is beneficial for stomachaches, sore throats, and coughs. Fresh capitulum are crushed and used for gingivitis. In addition, the aerial parts are known to be used for cancer treatment among people. Although not many detailed studies are found on the genus, it is known to have many common points in terms of biological activity and secondary metabolites due to its similarities to the genus *Anthemis*. The *Cota* genus contains polyphenols and sesquiterpene lactones as major secondary metabolites. In addition, anti-inflammatory, antioxidant, and various enzyme inhibitory activities (e.g., cholinesterase, tyrosinase) have been detected in the genus. *Anthemis* and *Cota* are both similar genera from the *Asteracea* family, which contains many phenolic components. Therefore, both can be considered as potential inhibitors due to their rich phenolic content (Karadeniz, Cinbilgel, Gün & Çetin, 2015; Talhouk et al., 2015; Ozek et al., 2019).

This study aims to reveal the antioxidant and tyrosinase inhibitory activities in accordance with the overall phenolic and flavonoid content and assumes that active extracts will show non-cytotoxic properties suitable for consumption. Hence, this study uses the methanol and chloroform extracts of different polarities were used.

MATERIALS AND METHODS

Plant material

The aerial parts of *Anthemis aciphylla* var. *aciphylla* and *Cota dipsaceae* were collected from Bozdağ, İzmir in May 2022 and identified by Prof. Dr. Tuğçe Fafal. Voucher specimens were deposited in the herbarium of Ege University Faculty of Pharmacy, Department of Pharmacognosy and registered under Nos. 1654 and 1655, respectively.

Extraction

After the *Anthemis* and *Cota* species were air-dried and powdered by mill, 10 g of the plants were macerated with 200 mL of methanol at room temperature for 24 hours to prepare the extracts. Plants were extracted three times. After filtering, the extract was evaporated to dryness in a rotavapor. The same procedures were repeated with chloroform and then stored at 4°C for use.

Determining the total phenolic content of the extracts

Total phenolic substance analysis was performed according to the common Folin-Ciocalteu method. The total amounts of the phenolic substances in the extract were calculated with the graph formula prepared from different concentrations of gallic acid. 2.8 ml of deionized water was added to 0.1 ml of the extract diluted at the appropriate rate, and then 2 ml of 2% Na_2CO_3 was added. This was lastly mixed with 0.1 ml of Folin

reagent and left for 30 minutes and measured at 750 nm. Experiments were performed three times with the results averaged (Orlando et al., 2019).

Determining the total flavonoid content of the extracts

The total flavonoid concentration was calculated colorimetrically using a UV spectrophotometer. According to this method, 1.5 ml of ethanol was added to 0.5 ml of the appropriately diluted sample, then 0.1 ml of $AlCl_3$ was added. Lastly, 2.8 ml of deionized water was added and incubated for 40 minutes. Absorbance was measured against ethanol at 415 nm. Experiments were performed three times, and the results were averaged (Orlando et al., 2019).

***In-vitro* antioxidant activities**

DPPH radical scavenging activity

Esmaeili et al.'s (2010) method has been modified to measure this. Accordingly, the DPPH solution prepared in appropriate media was added to the samples within a certain concentration range and incubated for 30 minutes in the dark. The absorbances were read against methanol at 517 nm, with α-Tocopherol being used as normal (Esmaeili and Sonboli, 2010).

ABTS radical scavenging activity

Rer et al.'s1999 modified method was used for checking the ABTS radical scavenging activity. The ABTS radical was prepared from a stock ABTS solution with potassium persulfate, then diluted with ethanol until obtaining an absorbance of 0.7 at 734 nm. After adding 0.1 mL of extract/standard to the 1 mL of diluted ABTS solution, the change in absorbance at 734 nm was incubated for 6 min. at room temperature, with α-Tocopherol being used as normal.

Superoxide anion radical scavenging activity

Checking for this was carried out according to Patel et al.'s research. 10 µL of standard extracts at different concentrations, 15 µL of 12 mM EDTA, 10 µl od 0.1 mg/mL NBT (nitro blue tetrazolium), 5 µL of 0.2 mg/mL riboflavin, and 160 µL of 0.067mM potassium phosphate buffer (pH 7.4) were placed in microplate reader and incubated under fluorescent light for 5 minutes. Ascorbic acid was used as normal. The absorbance at 560 nm was then measured, with the IC_{50} being calculated using GraphPad Prism 5(Patel et al., 2010).

Nitric oxide scavenging activity

The nitric oxide scavenging activity was carried out using the Griess reagent. 50 µl of standard extract at various concentrations were mixed with 100 µL of methanol. Next, 2.0 mL of sodium nitroprusside (10 mM) in a phosphate buffer saline at 7.2 pH was added to each tube. The solutions were incubated at room temperature for 150 minutes to produce nitrite ions. At the end of the incubation, 5.0 mL of the Griess reagent were added to each tube. The absorbance of the chromophore was measured at 546 nm. Ascorbic acid was used as normal (Patel et al., 2010).

***In-vitro* tyrosinase inhibitory activity**

Masuda et al.'s method was modified for this. 110 µL of the phosphate buffer (0.01 M, 6.8 pH), 10 µL of plant extract at different concentrations, and 20 µL of tyrosinase solution (200 unit/mL) were mixed. After 10 minutes of incubation at 37°C, the reaction was initiated by adding 20 µL of L-Dopa and allowed to incubate at 37°C for another 10 min. The absorbance was measured at 475 nm, with Kojic acid being used as normal (Masuda et al., 2005).

Cell culture

B16-F10 melanoma cells were cultured in a Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. Cells were seeded into a 96-well cell culture plate at a density of 8,000 cells. After establishing the morphology of the cells, *AAC* extracts were prepared at doses ranging from 2.5-100 µg/mL and applied to the cells for 48 hours. Dimethyl sulfoxide (DMSO) was used as negative control. Activity at concentrations above 200 was not taken into account.

MTT assay

The MTT assay was carried out after a 48-hour incubation. The MTT-medium mixture was added to 96-well cell culture dishes at a final concentration of 0.5 mg/mL and incubated for 4 hours. After the medium was removed, 200 µL of DMSO was added to dissolve the formazan salts, and the measurement was taken at a wavelength of 570/690 nm with a plate reader (Varioskan, Thermo Fisher Scientific, USA).

Statistical analysis

All results are expressed as mean ± *SD*. Analysis of variance was also performed, and significant differences between means were determined using Duncan's multiple range tests at a level of $p < 0.05$ in IBM SPSS (ver. 25).(Table 3)

RESULTS AND DISCUSSION

Pharmaceutical research has recently focused on plants containing free radical scavengers due to their importance in preventing and treating several disorders. The current study evaluates the antioxidant activities of methanol and chloroform extracts from *A. aciphylla* (*AAM*, *AAC*) and *C. dipsacea* (*ADM*, *ADC*). Total phenolic and flavonoid content and antioxidant assays have been summarized in Table 1. The results for *AAM* indicate it has strong DPPH scavenging activity as well as significant amounts of total phenol and total flavonoid. *AAC* also has moderate DPPH scavenging activity; however, *ADM* and *ADC* were less effective. After searching the literature searching, medicinal plants containing high amounts of total phenol and flavonoid were seen to possess strong antioxidant activity. Given that, the results from this study are seen to be in line with previous studies regarding *A. cretica* L., *A. tinctoria* L., *A. desertii* L., and *A. palestina* L. Belhaoues et al.'s study determined a correlation to be present between phenolic compounds and antioxidant activity, with flavonoids observed to have a dose-dependent relationship with antioxidant activity. The total phenol content of *A. praecox* L. was found to be 39.65 mg GAE/g in the extract, with the total flavonoid amount being 2.28 mg QE/g. The IC_{50} values for the DPPH and ABTS radical scavenging activities were observed to be quite low (i.e., to show strong activity. (Sut et al., 2019; Belhaoues et al., 2020; Bursa, Aras, Kılıç & Buldurun, 2020). The *AAM* result regarding ABTS scavenging activity is 46.82 µg/mL, whereas the positive control (Trolox) is 18.87 µg/mL, showing almost 1/3 the ef-

fect of *AAM*. The literature shows *A. cotula*, *A. praecox*, and *A. chia* to also have significant effects (Sut et al., 2019; Belhaoues et al., 2020; Bursal et al.,2020). Chemsa et al.'s research on *A. stiparum* subsp. *sabulicola* (Pomel) Oberpr. determined the DPPH value as 92.69 µg/ml (IC_{50}) (Chemsa et al., 2018). Emir and Emir's research determined the amount of total phenolic and flavonoid substances as 21.7 mg GAE/g extract and 9.7 mg QE/g extract;(Emir and Emir, 2020) they gave DPPH as mg TE/g extract and hence is not comparable here. study on *A. cotula* L. calculated an IC_{50} value for the DPPH radical scavenging activity of 165.72 µg/ml (Gür et al., 2018).with *A. chia* L.'s total phenolic substance being 42.38 mg GAE/g extract and total flavonoid amount as 28.99 mg QE/g extract. also evaluated DPPH and ABTS radical scavenging activities but provided no trolox equivalent values, thus they could not be compared exactly(Sarıkürkçü, 2020). Total phenolic and flavonoid contents of the *A. tinctoria* L. var. *pallida* methanol extract were determined as 100.09 mg GAE/g extract and 48.54 mg RE/g extract, respectively. In addition, the *A. cretica* L. subsp. *tenuiloba* total phenolic and flavonoid contents were found to be 46.73 and 45.08, respectively. *A. desertii* total flavonoid content was found to be 2.92 mg GA/g extract. However, the DPPH radical scavenging activity again cannot be compared because the percent inhibition was given. However, after performing positive control, it was found to be the second most-effective plant (Orlando et al., 2019).

Moreover, *C. altissima* showed ABTS scavenging activity similar to *ADM* and *ADC* (Göger et al., 2021). In their recent research, the superoxide anion radical scavenging activity of *AAM* was 5.610 µg/mL, which is higher than the positive control ascorbic acid value of 5.992 µg/mL. In contrast, *AAC* was determined as the extract with the lowest activity. *ADM* and *ADC* indicated average effects on superoxide anion radicals. Regarding the percent inhibition of the nitric oxide scavenging activity, ascorbic acid inhibited 60%, while what came from the *AAM* extract had 46.47%. The extract that showed the least effect was again *AAC*.

According to this study's hypothesis, the tyrosinase inhibitor effect should be observed mostly in extracts that show antioxidant activity and should also have a significant amount of total phenol and flavonoid content. The *AAM* extract confirmed the hypothesis as the most active extract regarding both the tyrosinase inhibitor effect as well as antioxidant activity (Table 2). The results regarding antioxidant and tyrosinase inhibitor activity are also seen to be statistically compatible ($p \leq 0.01$) (Table 3). Sarıkürkçü's study identified the phenolic contents of *A. chia* L. and examined their antioxidant and enzyme inhibitory (tyrosinase, α-amylase) effects (Sarıkürkçü, 2020). Sut et al.'s , study investigated antioxidant activities of plant extracts obtained by different extraction methods using the DPPH, ABTS, FRAP, CUPRAC, and metal chelation methods. The highest effect was the CUPRAC reducing power (435.32 ± 9.60 mg TE/g extract) from the extract prepared with the accelerated solvent method. Şener et al. also examined the alpha-amylase, alpha-glucosidase, cholinesterase, and tyrosinase inhibitory activities of the extracts prepared with different techniques and observed the activities (Şener et al., 2017).*Anthemis* species and investigated tyrosinase, alpha-amylase, alpha-glucosidase, and cholinesterase in terms of inhibitory enzyme activities(Orlando et al., 2019).

When examining the studies on the *Cota* genus, performed a detailed phytochemical study on the *Cota fulvida* Grierson to examine antioxidant, antidiabetic, anti-inflammatory, and antime-

Table 2. *In-vitro* tyrosinase inhibitory activity results.

	Tyrosinase inhibitory activity results (IC_{50}) (µg/mL)	Cytotoxic activity results (IC_{50}; µg/mL)
AAM[a]	110.73±0.50	na*
ADM[b]	227.8±0.06	na*
AAC[c]	na*	11.77
ADC[d]	na*	na*
Kojic acid	37.40±0.42	

* not active; [a]*Anthemis aciphylla* var. *aciphylla* methanol extract; [b]*Anthemis dipsaceae* methanol extract; [c]*Anthemis aciphylla* var. *aciphylla* chloroform extract; [d]*Anthemis dipsaceae* chloroform extract.

Table 1. *In-vitro* antioxidant activity results.

	Total phenolic content (mg GAE/g)[e]	Total flavonoid content mg QE/g)[f]	DPPH radical scavenging activity (IC_{50}) (µg/mL)	ABTS radical cation scavenging activity (IC_{50}) (µg/mL)	Superoxide radical scavenging activity (IC_{50}) (µg/mL)	Nitric oxide scavenging activity (% inhibition) (at 324 µg/mL concentration)
AAM[a]	214.81±0.12	36.48±0.22	19.57±0.62	46.82±0.22	5.610±0.26	46.47±0.54
ADM[b]	104.66 ±0 .01	12.055±0.112	110.88±0.28	360.9±0.12	80.15±0.41	21.52±0.46
AAC[c]	1.256 ±0 .3	3.127±0.61	66.48±0.02	465.9±0.22	482.1±0.08	8.72±0.01
ADC[d]	3.425 ±0 .1	6.251±0.61	166.6±0.05	163.4±0.11	332.6±0.05	29.23±1.63
Ascorbic acid			4.474±0.31		5.992±0.01	60.0±0.05
Trolox				18.87±0.01		-

[a]*Anthemis aciphylla* var. *aciphylla* methanol extract; [b]*Anthemis dipsaceae* methanol extract; [c]*Anthemis aciphylla* var. *aciphylla* chloroform extract; [d]*Anthemis dipsaceae* chloroform extract; [e]Gallic acid equivalent; [f]Quercetin equivalent.

Table 3. Statistical analysis.

	Sum of Squares	df	Mean Square	F
Between Groups	1540542.434	24	64189.268	642178.750
Within Groups	4.998	50	.100	
Total	1540547.432	74		

Groups: Biological acitivities.

lanogenesis activities and used LC-MS/MS to identify phenolic acids, phenylpropanoid, and flavonoid-containing substances. They also determined the essential oil content using GC-MS. The methanol extract of the plant showed the highest DPPH scavenging activity (IC_{50}: 0.131 mg/mL)(Ozek et al., 2019). study examined many Turkish plants to investigate the DPPH scavenging activity of *Cota pestalozzae* Boiss and also provided the mean IC_{50} range for all plants, reporting IC_{50} values between 18.67-425.19 µg/mL(Karadeniz et al., 2015) Apart from these, no other study was found regarding the *Cota* genus in terms of genera. When comparing all the results from the current study with the literature, they are seen to be compatible.

In conclusion, this research has examined whether certain plants have a cytotoxic effect on cells and observed those with tyrosinase inhibitory activity to be non-cytotoxic. Only chloroform extract *(AAC)* gave a very low IC_{50} value in contrast to the antioxidant and tyrosinase inhibitor activities. Comparable results are also seen in similar studies on tyrosinase inhibitor activity, antioxidants, and inhibition of melanoma cells (Gomez et al., 2001; Wang et al., 2011; Sun et al., 2017; Wang et al., 2019). The fact that the plant inhibits tyrosinase activity without a cytotoxic effect (Table 2) makes it more suitable for the food and pharmaceutical industry. Further studies can examine its effects on melanogenesis in cells and accordingly provide more detailed information about their use in hyperpigmentation. These results indicate the *AAM* extract to potentially contain bioactive substances and to maybe have the potential for use as a depigmentation agent in skin disorders and as an anti-darkening agent in the food industry.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- B.S.T.; Data Acquisition- B.S.T., R.İ., P.B.K.; Data Analysis/Interpretation- B.S.T., R.İ. T.F.; Drafting Manuscript- B.S.T.; Critical Revision of Manuscript- B.S.T., P.B.K., B.K.; Final Approval and Accountability- B.S.T., T.F., R.İ., B.K., P.B.K.

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

Financial Disclosure: Authors declared no financial support.

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