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RESEARCH ARTICLE

EVALUATION OF TOTAL PHENOLIC, FLAVONOID CONTENT, AND ANTIOXIDANT ACTIVITIES OF *CROCUS CHRYSANTHUS* (HERB.) HERB. WITH CONVENTIONAL SOXHLET EXTRACTION

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Abstract

Species belonging to the genus *Crocus* L. have some traditional uses against some cardiovascular diseases, diabetes, Parkinson's disease, depression, apoptosis, atherosclerosis. In this study, total phenolic, total flavonoid and antioxidant potentials of the plant extract obtained using *Crocus chrysanthus* (Herb.) Herb.were investigated. *C. chrysanthus* was extracted with 70% ethanol using a soxhlet apparatus. While total phenolic content was found to be 127.48±0.79 mg gallic acid equivalent (GAE/g), total flavonoid content was found to be 41.67±0.84 mg quercetin equivalent (QE/g). Radical scavenging (DPPH) and metal chelation (CUPRAC and FRAP) methods were used to determine the free radical scavenging properties. While DPPH radical scavenging was found to be 85.27%, CUPRAC was found to be 56.83±0.17 mg trolox equivalent (TE/g). In the FRAP method, the reducing power was found to be 57.68±0.21 trolox equivalent (TE/g). According to the results obtained, it was determined that the ethanol extract of *C. chrysanthus* exhibited antioxidant properties. In addition, phenolic and flavonoid content was determined. As a result, *C. chrysanthus* extract can be recommended as a source of bioactive components with potential use against chronic diseases caused by oxidative stress. Future in-depth studies are recommended to determine the biological effects of compounds isolated from *C. chrysanthus* to determine the main compounds modulating the observed activities.

Introduction:-

Natural products are an integral part of a healthy and sustainable lifestyle and offer a wide range of benefits to individuals and societies. For example, phytochemicals have a wide range of biological activities, from antioxidants to antiinflammatories. In this way, they can act as a protective shield against degenerative and chronic diseases such as diabetes, cancer, and cardiovascular disease [1, 2, 3]. In this context, there is a growing interest in the scientific community to examine new natural products. Plants tend to develop the ability to produce secondary phenolic metabolites, which are vital parts of their interaction mechanisms with their environment, reproductive strategies, and defensive behaviors. These phenolic compounds also contain natural antioxidants that have beneficial and protective effects on human health. Oxidative stress is thought to be the cause of various disorders in different parts of the human body/organs due to the excessive production of reactive nitrogen and oxygen species [4, 5]. Antioxidants can delay the development of chronic diseases and are also widely used as food additives to protect foods from oxidative degradation [6, 7, 8, 9].

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Medical, aromatic and many other plants contain chemical compounds that exhibit antioxidant properties. However, there is still little scientific information about the antioxidant properties of plants that are less commonly used, especially in the kitchen and medicine [10].

Iridaceae Juss. (Irisaceae) are herbaceous plants with bulbs, tubers or rhizomes. There are approximately 66 genera and 2025 species in the world. A cosmopolitan family, Irisaceae has great economic importance, first of all, in horticulture and as a spice. The genus *Crocus* L., one of the seven genera of the Iridaceae family in Turkey, is represented by 103 species and subspecies taxa, approximately 55 of which are endemic. Crocuses are cormus (stem tuber) plants. They have flower colors in yellow, white, purple, blue, lilac and violet tones. They bloom in spring or autumn [11]. *Crocus chrysanthus* (Herb.) Herb. blooming in spring was collected from Karınca Mountain in Pozantı district of Adana province. Karınca Mountain is located in the north of Pozantı district within the borders of Adana province. The geographical location of the area is 37°29'44" North and 34°52'23" East. The study area, whose highest point is 2206 m, is located 8 km from Pozantı district and 55 km from Niğde province and has an area of approximately 71 km².

The aim of this study was to investigate the antioxidant activity, total phenolic and total flavonoid contents of *C. chrysanthus* aerial parts in order to determine their biological properties.

MaterielandMethods:-

Collection of plant samples

Spring flowering *Crocus chrysanthus* (Herb.) Herb. (Sarıçiğdem) was collected from Karınca Mountain 37°32'54.95"N-34°56'16.46"E approximately 1250-1350 m. The collected samples were pressed and dried according to herbarium rules and made ready for identification. Illustrated Flora of Turkey Volume 3 [12] was used in the identification studies of the samples. During the identification, the "English-Turkish Botanical Guide" [13] and Illustrated Flora of Turkey-Volume 1 [11] were used for Latin terms (Figure 1).

Figure 1:- Identification of *C. chrysanthus* specimen.

Extraction of soxhlet extract

In order to apply the Soxhlet extraction method, the plant sample was dried, broken into small pieces with the help of a shredder and these particles were filled into an extraction cartridge made of cellulose. The extraction cartridge was then subjected to extraction with 70% ethanol solution for 6 hours. The dissolved substance was transferred to the distillation flask. The solvent was evaporated and the extract was obtained [14].

Determination of DPPH radical scavenging activity

The free radical scavenging activities of the solutions were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The method proposed by Dusgun et al. [15] was carried out with some modifications required

by the experimental conditions [15]. 20 µL of plant extract was pipetted into test tubes and 180 µL of 1 mM DPPH solution was pipetted onto it and incubated for 30 minutes in a lightproof place at room temperature. Experiments were performed in 3 parallels. After incubation, absorbance at 517 nm was recorded against a blank consisting of methanol. The decreasing absorbance gave the remaining amount of DPPH solution, i.e. free radical scavenging activity. Percent DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Radical scavenging power (\%)} = \left(\frac{A_0-A_1}{A_0}\right) * 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract sample/standard at 30 min. The analysis of all the samples was done in triplicate.

Copper (II) reducing antioxidant capacity method (CUPRAC)

For this study, 500 µL of Copper (II) Chloride ($CuCl_2$) solution and 500 µL of ammonium acetate (1 M pH:7.0) solution were added to 2.5 mL centrifuge tubes. 500 µL of Neocuproine ($C_{14}H_{12}N_2$) ($7.5x10^{-3}$ M) solution was added to each tube. 100 µL of plant extract was added to the tubes and completed to 550 µL with distilled water. Distilled water was used instead of extract for blank samples. It was incubated for 30 min at room temperature and in a water bath (50ºC). The absorbance at 450 nm was read against the blank sample [16].

Ferric reducing power measurement (FRAP)

Crocus chrysanthus (Herb.) Herb. extracts were prepared at 1 mg/mL concentrations and placed in 5 mL tubes. 100 µL was taken from the samples. Ethanol was placed in the control tube instead of the sample. 2.5 mL of 0.2 M phosphate buffer (pH: 6.6) and 2.5 mL of 1% potassium ferric cyanide were added to each tube and the mixture was heated in a water bath at 50°C for 30 min. It was left to incubate. 2.5 mL of 10% trichloroacetic acid (TCA) was added to the tubes taken from the water bath and the tubes were centrifuged at 6000 rpm for 15 minutes. 2.5 mL of the supernatant separated by centrifugation was taken and 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3.6H_2O$ solution were added. The absorbance of the samples at 700 nm was read against distilled water [3].

Total phenol content

Total phenol content was adapted according to the method of Singleton and Rossi using the Folin–Ciocalteau reagent. The Folin–Ciocalteau method is based on the formation of blue colored compounds by transferring electrons from phenolic compounds to phosphomolybdic and phosphotungstic acids in an alkaline medium, and these compounds are read spectrophotometrically at an average of 760 nanometers (nm). 0.1 mL of the prepared plant extracts were added to 1 mL of Folin–Ciocalteau solution (diluted 10 times) and incubated for 5 min. 1 mL of 7.5% sodium bicarbonate solution was added. After 90 min of incubation, the absorbances of the samples were read at 765 nm. The results were calculated according to gallic acid standardsas milligram gallic acid equivalents (mg GAE/g extract) [17].

Total flavonoid content

Total flavonoid content was determined using the $AlCl_3$ method. Briefly, the plant extract (1 mL) was mixed with the same volume of aluminum trichloride (2%) in methanol. Similarly, a blank was prepared by adding the sample solution (1 mL) to methanol (1 mL) without $AlCl_3$. After 10 min of incubation at room temperature, the absorbances of the sample and blank were read at 415 nm. The absorbance of the blank was subtracted from that of the sample. It was used as a routine (quercetin) reference standard and the total flavonoid content was expressed as milligram quercetin equivalents (mg QE/g extract) [18].

Statistical analysis

The experiments were performed in triplicate, and differences between the extracts were compared using an ANOVA and Tukey's test. Pearson correlation analysis was used to establish the link between total bioactive components and biological activity assays.

Results:-

Antioxidant properties of the ethanolic extract of *Crocus chrysanthus* (Herb.) Herb.were tested using three different tests and the obtained results showed that extract exhibited antioxidant properties (Table 1). These tests (DPPH, CUPRAC and FRAP) showed that the ethanol extract of the plant has free radical neutralizing activity, reduction and chelation abilities. The differences among antioxidant tests were significant in terms of free radical neutralizing activity of the *C. chrysanthus* extracts tested in this study (p<0.05). The DPPH radical scavenging activity was 41.74±0.18%, 59.68±0.21% and 85.27±0.17% at concentration of 0.25, 0.50 and 1 mg/mL, respectively. In addition,

CUPRAC metal chelating activity was 21.14±0.15, 35.57±0.16 and 56.83±0.17 mg TE/g at concentration of 0.25, 0.50 and 1 mg/mL, respectively. Moreover, FRAP chelating activity was 18.67±0.18, 29.78±0.17 and 57.68±0.21 mg TE/g at concentration of 0.25, 0.50 and 1 mg/mL, respectively.

Table 1:- Antioxidant properties of *C. chrysanthus* ethanolic extract.

Sample	Concentration (mg/mL)	DPPH (%)	CUPRAC (mg TE/g)	FRAP (mg TE/g)
Ethanol extract of *C. chrysanthus*	0.25	41.74±0.18[a]	21.14±0.15[a]	18.67±0.18[a]
	0.50	59.68±0.21[b]	35.57±0.16[b]	29.78±0.17[b]
	1.00	85.27±0.17[c]	56.83±0.17[c]	57.68±0.21[c]

[a-c] The same letters within the same coloumn shows no statistical difference at $p<0.05$ level.

The total phenolic and flavonoid contents of the ethanol extract of *C. chrysanthus* were measured and shown in Table 2. The differences were significant in terms of total phenolic and flavonoid contents *C. chrysanthus* extracts tested in this study ($p<0.05$). Total phenolic contents of the ethanol extract of *C. chrysanthus* were 35.72±0.87, 69.85±0.91 and 127.48±0.79 mg GAE/g at concentration of 0.25, 0.50 and 1 mg/mL, respectively. Total phenolic contents of the ethanol extract of *C. chrysanthus* were 15.13±0.82, 27.59±0.78 and 41.67±0.84 mg QE/g at concentration of 0.25, 0.50 and 1 mg/mL, respectively.

Table 2:- Total phenolic and flavonoid content of *C. chrysanthus* ethanolic extract.

Sample	Concentration (mg/mL)	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
Ethanol extract of *C. chrysanthus*	0.25	35.72±0.87[a]	15.13±0.82[a]
	0.50	69.85±0.91[b]	27.59±0.78[b]
	1.00	127.48±0.79[c]	41.67±0.84[c]

[a-c] The same letters within the same coloumn shows no statistical difference at $p<0.05$ level.

Discussion:-

The antioxidant properties of extracts obtained from many plants attract great attention in the food and health industry. *Crocus* species are used intensively by the public for various purposes in Türkiye and especially in Sakarya province. DPPH is a stable free radical that is generally used as a tool to evaluate the free radical scavenging activity of antioxidants [19]. In this research, DPPH free radical scavenging activity was found to be the highest at 1 mg/mL concentration as 85.27±0.17%. In the studies conducted on methanol extract of *C. chrysanthus*, DPPH free radical scavenging activity was reported 46.31±0.89% [8]. This value is weaker than ethanol extract of *C. chrysanthus* of this research. The high DPPH radical scavenging activity obtained in this study may be related to the solvent used. In another study, The DPPH scavenging ability of various extracts obtained from plant roots was tested and the best radical scavenging ability was provided by the ethanol extract, consistent with the results obtained [20]. Moreover, Awah et al. [21] reported significant DPPH scavenging ability for ethanol extract of plant leaves. Therefore, it is in line with the findings obtained here, which show that ethanolic extract is a better solvent for obtaining high phytochemical content and antioxidant potential. The findings on this work show that extracts of *Crocus sativus* L. obtained from the Iranian region possess antioxidantive potential, and are in agreement with data collected by Assimopoulou et al. [22] who reported the antioxidant activity of *C. sativus*. They belived that the antioxidant activity of saffron could be attributed to two bioactive compounds, crocin and safranal, and a DPPH radical scavenging test on crocin and saffranal exhibited antioxidant activities of 65% and 34%, respectively, at 0.5 mg/mL. In this study, the antioxidant activity of the ethanolic extract at 0.5 mg/mL was 59.68±0.21%. However, DPPH free radical scavenging activity was found to be similar even in different plant species. In the current work, the antioxidant activity in *C. chrysanthus* be attributed to the presence and synergistic effects of phenolic and flavonoid compounds, besides any other active compounds present [22]. The ferric reducing power activity (FRAP) assay was used to determine the reduction potential ($Fe^{3+} \rightarrow Fe^{2+}$) of extract of *C. chrysanthus* [23]. Similar to the DPPH results, the reductive potential of *C. chrysanthus* increased in a dose dependent manner. In this study, FRAP assay results were found 57.68±0.21 mg TE/g at the concentration of 1 mg/mL. In the CUPRAC assay, it was found that the metal reduction rate of the ethanolic extract at 1 mg/mL concentration was found to be 56.83±0.17 mg TE/g. Ethanolic extract of *C. chrysanthus* appeared to be active in the reduction of Fe^{3+}, indicating its antioxidant activity.

Phenolic and flavonoid molecules, often present as secondary metabolites in plants, are significant because of their capacity to function as antioxidants [24, 25]. A widerange of phenolic compounds have been shown to exhibit significant antioxidant activity as well as varying degrees of anticancer, anticarcinogenic, antibacterial, antiviral, or anti-inflammatory effects [26, 27, 28, 29]. Flavonoids, which are often present in leaves, blooming tissues, and pollens [22], play a significant role in human nutrition due to their impact on food [30, 31]. Flavonoids are mostly known for their antioxidant activity, which means they are very good in neutralizing different forms of oxidizing molecules, such as singlet oxygen and other free radicals [31, 32, 33, 34]. In the studies conducted on the ethanolic extracts of *C. chrysanthus*, the total phenolic substance amount was studied at concentrations of 0.25-1 mg/mL. The total phenolic substance amount was found to be 127.48±0.79 mg GAE/g at the highest concentration of 1 mg/mL. In the studies conducted on methanol extract of *C. chrysanthus*, the amount of total phenolic substances was reported as 26.21±0.57 mg GAE/g [8]. This value is weaker than ethanol extract of *C. chrysanthus* of this research. Moreover, In the study conducted on methanolic extracts of *Crocus cancellatus* subsp. *lycuis* Herb., the total phenolic substance amount was found to be 2.08±0.06 mg GAE/g. The high total phenolic substances amount obtained in this study may be related to the solvent used [35]. Total phenolic contents were markedly higher in the ethanolic extract; however, the antioxidant activity was affected by the nature of solvent used. In another study, Lachguer et al. [36] studied on different solvents of *C. sativus* and reported a range of 104.82±4.36 to 214.29±12.68 mg GAE/g. While the total phenolic substance amount of the extract obtained with water was found to be the lowest with 104.82±4.36 mg GAE/g, the amount obtained with diethyl ether was found to be the highest with 214.29±12.68 mg GAE/g. The extract obtained with diethyl ether contained more phenolic substances than the amount found in this study. The reason for this result can be attributed to the different solvents and different types of plants. On the other hand, Satybaldiyeva et al. [37] showed that the total phenolic contents of the extracts from aerial and bulb parts of *Crocus alatavicus* L (Kazakhstan) using different solvents (distilled water, 96% ethanol, 99% methanol, and 99% dichloromethane) were ranged from 13.63 to 72.29 mg GAE/g extract and that the ethanolic extract of aerial part contained the highest total phenolic content. Under different brewing conditions, total polyphenol, and flavonoid contents in stamens and tepals of *C. sativus* from Italy were reported to be in the range of 83-186 mg GAE/L and 34-91 mg CE (catechin equivalents)/L, respectively [38]. It was shown that the total phenolic content of the extracts of the different parts of *C. pallasi* was in the range of 13.48-28.92 mg GAE/g, while the total flavonoid content was in the range of 0.50-31.44 mg RE (rutin equivalent)/g [39]. According to these results, the total phenolic and total flavonoid content of the *Crocus* species may vary depending on the plant species, the geographical region where it was collected, the parts of the plant used, and the extraction method. In the studies conducted on the ethanolic extracts of *C. chrysanthus*, the total flavonoid substance amount was studied at concentrations of 0.25-1 mg/mL. The amount of total flavonoids was determined as 41.67±0.84 mg QE/g at the highest concentration of 1 mg/mL. In the studies conducted on methanol extract of *C. chrysanthus*, the amount of total flavonoid substances was reported as 77.58±0.34 mg QE/g [8]. This value is stronger than ethanol extract of *C. chrysanthus* of this research. It can be concluded that methanolic extract is better than ethanolic extract in terms of total flavonoid substances. However, in the study conducted on methanolic extracts of *C. cancellatus* subsp. *lycuis*, the total flavonoid substance amount was found to be 3,63±0,09 mg QE/g [35]. This value is weaker than ethanol extract of *C. chrysanthus* of this research. It is thought that this situation may be caused by the difference between species.

Conclusion:-

It can be suggested that *C. chrysanthus* sample has remarkable biological properties and important polyphenolic compounds. The sample prepared with ethanol was effective antioxidant and also had high total phenolic and flavonoid content. It is suggested that further studies should be designed to determine the biological effects of the compounds isolated from *C. chrysanthus* and to characterize the main phytochemicals responsible for the reported activities.

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