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EFFECT OF *Rhabdosciadium anatolyi* AND CHLOROGENIC ACID ON SERUM RETINOL, CHOLECALCIFEROL AND PHYLLOQUINONE LEVELS IN EXPERIMENTAL CYCLOPHOSPHAMIDE TREATED RATS

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ABSTRACT

In this study, the effects of chlorogenic acid (*CA*), and ethanol extract of *Rhabdosciadium anatolyi* (*R. anatolyi*) flowers on the levels of vitamins (retinol, cholecalciferol and phylloquinone) in the serum of experimentally cyclophosphamide (*CP*)-induced rats were investigated. Wistar-albino female rats (N=48) of 8 weeks old (200-250 g) were divided into six groups of 8 animals each (control (0.9% NaCl), 200 mg/kg *CP*, 100 mg/kg *CA*, 300 mg/kg *R.anatolyi* flowers extract, 200 mg/kg *CP* + 300 mg/kg *R.anatolyi* flowers extract and 200 mg/kg *CP* + 100 mg/kg *CA)*. At the end of the 7-day study, blood samples were collected from the rats' heart and serum was extracted. Retinol, cholecalciferol and phylloquinone levels in the groups were determined simultaneously using simple reversed phase HPLC method. According to the results of statistical analysis, a significant difference ($p<0.01$, $p<0.05$) was observed between the control group and the groups treated with 200 mg/kg *CP* and 200 mg/kg *CP* + 300 mg/kg *R. anatolyi* flower ethanol extract, respectively. In addition, a significant difference ($p<0.01$) in cholecalciferol levels between control and 200 mg/kg *CP* groups was detected. However, no statistically significant difference ($p>0.05$) was observed between the groups in phylloquinone levels. It is concluded that *CP* causes free radical damage by causing oxidative stress, and retinol, is more effective and more resistant in the antioxidant defense system compared to cholecalciferol and phylloquinone.

Keywords: *Rhabdosciadium anatolyi,* chlorogenic acid, cyclophosphamide, retinol, cholecalciferol, phylloquinone.

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INTRODUCTION

Cyclophosphamide (*CP*) and its structural analog, ifosfamide (holoxan), are one of the most frequently used alkylating drugs in chemotherapy (Abraham *et al*., 2007; Ibrahim *et al.*, 2024). Although it is a drug used in the treatment of tumors in oncology, it is also used in autoimmune diseases such as behçet's disease, rheumatoid arthritis, vasculitides, lupus, and scleroderma (Coggins *et al.,* 1960; Saadoun *et al*., 2024). The toxic status of the chemical *CP* is linked to its active metabolites acrolein and phosphoramide. Acrolein interferes with the tissue antioxidant defense system (by binding to reduced glutathione -GSH-), leads to the formation of highly reactive oxygen (ROS) derivatives and exerts mutagenic effects for mammals (Ayhancı *et al*., 2009; Alkış *et al*., 2020) In particular, anti-cancer effect of *CP* is due to its use in high doses. This dose and thus toxicity profile varies widely depending on their clinical efficacy (Tripathi and Jena, 2009).

Studies are carried out to determine how effective the antioxidant phenolic compounds in the structure of fruits, vegetables and natural antioxidant-rich plant foods are in various diseases (Okcu and Keleş, 2009). Natural phenolic compounds such as chlorogenic acid (*CA*) are known as powerful antioxidants. *CA* is an ester family and a natural phenolic compound, which is among the quinic acids, an important dietary phenol group, found in the content of various fruits and vegetables, especially in coffee, which is an important source in human nutrition (Higdon and Frei, 2006; Bossoli *et al*., 2008; Nguyen *et al*., 2024). *CA* has a variety of pharmacological activities, including antimicrobial, liver protection, anti lipid peroxidation, antiviral activity and spasmolysis. On the other hand, it is a phenolic compound known for its antioxidant activity, anti-inflammatory and anti-cancer effects (Sun *et al*., 2017).

Determining the active substances in the content of plants and their role in protecting our health have been the subject of experimental studies for a long time. The supplementation of these active substances not only has a nutritional value for the living organism, but their effects are also known to reduce the undesirable effects of certain diseases (Ivanova *et al.,* 2024). We used within the scope of the study *R. anatolyi* is an important plant belonging to the Apiaceae (parsley) family. The Apiaceae family has long been used as medicine, food, spice and animal feed (Burnie, 1996; Hançer and Uruşak, 2017). Aromatic herbs such as parsley and dill, which belong to this family and have a high polyphenol content, are part of the daily diet and are also known as powerful antioxidants (Ciz *et al.,* 2010). Dietary vitamin A is available in animal (retinyl esters and retinol) and plant-based (ß-carotene) forms. ß-carotenes and other carotenoids contain a pigment that suppresses singlet oxygen ($^1O_2$) formation and lipid peroxidation thanks to its antioxidant activity (Liu *et al*., 2009; Ferrier, 2019). Since it can be synthesized in the body, vitamin D, which acts like a hormone, controls calcium and phosphate levels (Akkoyun *et al*., 2014). 1,25-diOH-D3 is the physiologically active form of vitamin D and, like vitamin A, this form interacts with the cell's nuclear DNA (Ferrier, 2019). Vitamin K; In plants, phylloquinone (vitamin K_1) is found in different forms in intestinal bacteria flora, in the form of menaquinone (vitamin K_2). Apart from its role in prothrombin and blood coagulation balance, vitamin K is also known to be important in photosynthesis in plants during the phosphorylation stage (Önaldı, 1980). These antioxidant vitamins play an important role in protecting cells from damage caused by unstable molecules known as free radicals, thus reducing exposure to oxidative stress and reducing the likelihood of disease (Khadim and Al-Fartusie, 2021). Due to all this, the cell, which is able to receive the appropriate components, including vitamins, has the capacity to repair itself and cope with stress.

As a consequence, this study aimed to determine the protective effect of *CA*, a powerful antioxidant, together with *R.anatolyi* flower, and the effects of retinol, cholecalciferol and phylloquinone vitamins in the serum of rats exposed to oxidative stress with *CP*, which is frequently used in chemotherapy and whose side effects have been investigated recently, and to serve as a reference for subsequent studies.

MATERIALS AND METHODS

Chemical Materials Used: Ethanol, methanol, n-hexane, tetrahydrofuran (THF), cyclophosphamide monohydrate (AB348271, abcr-Germany), chlorogenic acid (AB348271, abcr-Germany), retinol (purity≥95%, sigma-Aldrich), cholecalciferol (C9756, sigma-Aldrich) and phylloquinone (purity≥ 99%, sigma-Aldrich), ketamine/xylazine.

This study started in 2018 with the collection of *R. anatolyi* plants. Experimental studies (between 2018 and 2022) were carried out at Van Yüzüncü Yıl University, Faculty of Science, Chemistry-Biochemistry laboratory. At the end of the experimental period, rat serum was prepared and vitamin analyses (retinol, cholecalciferol and phylloquinone) were performed by HPLC-UV method in the Science Application and Research Central Laboratory of the same university.

Plant Material: *R. anatolyi* flowers were collected from Sat Mountains region of Yüksekova District of Hakkari Province at 37º 22' 41" N - 44º 10' 08" D coordinates (Figure 1). Species identification of the plant was made by Dr. Mehmet FIRAT and specimen vouchers were deposited to the herbarium with the code number 34041 (VANF).

Preparation of *R. anatolyi* flower extract: *R. anatolyi* plant used in the study were collected during flowering season and dried in the shade. 1 L EtOH (75%) was added to 100 g dry plant flower sample. Then the solution was kept at room temperature (24°C) for 48 hours on a magnetic stirrer. This process was repeated until sufficient extraction was achieved and the filtrates were combined. The dried extract was obtained after removal of ethanol using a rotary evaporator (40°C/160 mbar), it was then stored in the refrigerator (+4°C) until the start of the study. For the preparation of the plant extract, the work of Cai *et al.* (2004) was adapted to this study with minor modifications.

Animal material: Forty-eight Wistar Albino female rats were used for the study. Animals with a live weight of 200-250 g were kept at room temperature of 21 ± 3°C in a 11:13 hour light/dark cycle. The rats, which were adapted to the environment for one week, were fed with standard feed and tap water throughout the study. The experimental protocol was approved by Van Yuzuncu Yil University Animal Experiments Local Ethics Committee through the ethics committee letter No. 2018/05 dated 31/05/2018.

Establishment of Experimental Animal Groups: Six different groups were assigned, with 8 rats randomly selected in each group. A total of 48 female Wistar albino rats were used in our study. According to the weight of the rats, 95% purity of *CA* and ethanol extract from the flower of *R. anatolyi* were administered by gavage. The study continued for 7 days and at the end of the 7th day, the rats were sacrificed under anesthesia. The groups were observed according to the following situation;

1- Control group: The rats in this group were administered 0.9% NaCl solution every day.
2- *CP* group: A single dose of *CP* (200 mg/kg, i.p) was administered to the rats on the 7th day of the study.

3- *CA* group: *CA* dissolved in 0.9% NaCl solution was administered to rats every day (100 mg/kg/day by oral gavage).
4- *R. anatolyi* group: *R. anatolyi* flowers extracted with ethanol (300 mg/kg/day by oral gavage) was administered daily until the end of the study.
5- *CP* + *R. anatolyi* group: A single dose of *CP* (200 mg/kg, i.p) was applied to the rats on the 7th day of the study, and the *R. anatolyi* (300 mg/kg/day by oral gavage) flowers extracted with ethanol was administered every day until the end of the study.
6- *CP* + *CA* group: Rats were administered a single dose of *CP* (200 mg/kg, i.p) on the 7th day of the study, and also *CA* (100 mg/kg/day by oral gavage) dissolved in 0.9% NaCl was administered every day until the end of the study.

Figure 1. View of *Rhabdosciadium anatolyi* plant flower (Hakkari, Turkey).

Preparation and Administration of Cyclophosphamide (*CP*): 98% purity of *CP* administered intraperitoneally (i.p) is commercially available. *CP* was dissolved in 25 ml of bidistilled water and made ready for injection. With the help of sterile disposable injectors, a single dose of 200 mg/kg *CP* was administered according to the weight of the rats on the last day of the study (Mansour *et al.,* 2017).

Collecting Blood Samples: At the end of the seven-day study and under anaesthesia (ketamine/xylazine), blood samples were taken from the hearts of the rats and stored in glass tubes until further study. The blood taken into the biochemistry tube was centrifuged at 4500 rpm for 15 minutes. The obtained serums were taken into 1.5 ml deionized tubes for the analysis of vitamins and stored at -20°C.

Vitamin (retinol, cholecalciferol, and phylloquinone) analyzes

Standard Solution and Calibration: Retinol, cholecalciferol and phylloquinone stock solutions were prepared at 500 µg/mL. To prepare the standard solution, the stock solutions were diluted with methanol accordingly. Linear regression analysis of the peak area versus standard solution concentrations was used to calculate the calibration.

Extraction Process: The determination of retinol, cholecalciferol and phylloquinone in serum was determined by modifying the method of Su *et al.,* (2002). 100 µL of ethanol (prepared with 0.025% BHT) was added to 100 µL of serum and vortexed for 1 minute. The obtained samples were extracted twice with 800 µL of hexane. After vortexing, it was centrifuged at 6000 rpm for 15 minutes. A total of 500 µL of hexane layer was extracted and evaporated to dryness under a nitrogen stream at 36°C. The remaining residue was dissolved in 50 µL of tetrahydrofuran and 150 µL of methanol was added. After vortexing for 2 minute, 150 µL samples were prepared in glass bottles.

Chromatographic Conditions: Vitamin retinol, cholecalciferol and phylloquinone analyses were performed on a Gl Science C_{18} reversed phase high performance liquid chromatography column (250 x 4.6 mm ID), MeOH (80 ml) + tetrahydrofuran (20 ml) mobile phase, 1500 µL min^{-1} flow rate at 24˚C. HPLC - applications were performed for retinol (325 nm),

cholecalciferol (265 nm) and phylloquinone (248 nm) in 0.1 mL volumes in dark coloured vials in a tray autosampler (-10˚C) using a PDA array detector. Chromatographic analysis measurements were performed by isocratic elution (40˚C), a separation technique of HPLC.

Statistical analyses: Experimental results are presented as mean ± standard error of the mean (X±SEM). The variance analysis (ANOVA) or Kruskal-Wallis (Data were not normally-distributed) test was used to analyze these data, and in cases where differences were significant, Tukey test was carried out for *post-hoc* comparison of mean values. All these analyses were performed using SPSS®, version 23.0 statistical software package.

RESULTS AND DISCUSSION

In Table 1 and Figure 2, serum antioxidant retinol values of rats administered *CP* and levels between groups are shown. When retinol data between the groups were analysed, a statistically significant difference ($p<0.01$, $p<0.05$) was observed between the control and *CP* (200 mg/kg *CP* and 200 mg/kg *CP* + 300 mg/kg *R. anatolyi*) groups. The levels of cholecalciferol and phylloquinone vitamins measured in serum within the scope of the study are shown in Figure 3 and Figure 4, and the data are shown in Table 1.

Table 1. Effect of chlorogenic acid (*CA*) and *R. anatolyi* flowers ethanol extract on retinol, cholecalciferol and phylloquinone levels in rat serum in cyclophosphamide (*CP*)-induced experimental application.

Groups	Retinol (X±SEM)	Cholecalciferol (X±SEM)	Phylloquinone (X±SEM)
Control	$0.47 \pm 0.05^{c,b}$	0.30 ± 0.04^{b}	0.31 ± 0.02
CP	0.27 ± 0.04^{b}	0.14 ± 0.01^{b}	0.26 ± 0.02
CA	0.41 ± 0.04	0.24 ± 0.03	0.31 ± 0.03
R. anatolyi	0.40 ± 0.03	0.23 ± 0.03	0.31 ± 0.02
CP+ *R. anatolyi*	0.29 ± 0.04^{c}	0.22 ± 0.03	0.29 ± 0.03
CP + CA	0.34 ± 0.03	0.23 ± 0.03	0.28 ± 0.02

[b]Difference between the control group and the group administered 200 mg/kg *CP* was significant ($p<0.01$).
[c] Difference between the control group and the group administered 200 mg/kg *CP* + 300 mg/kg *R. anatolyi* was significant ($p< 0.05$).

The superscripts shown in the table represent statistically significant differences between the groups within the scope of the study. Retinol, cholecalciferol and phylloquinone; µmol/L. Each value represents mean ± SEM. *CP*; 200 mg/kg cyclophosphamide, *R.anatolyi*; 300 mg/kg *R. anatolyi* flowers ethanol extract, *CA*; 100 mg/kg chlorogenic acid.

7 Considering the cholecalciferol level, a important relationship was found between the control group and the *CP* group ($p<0.01$). On the other side, it was determined that 100 mg/kg *CA* and 300 mg/kg *R. anatolyi* flowers ethanol extract applied together with *CP* were more effective in coping with oxidative stress. (Figure 3). However, when the phylloquinone level was examined, it was seen that although there was no statistically significant relationship between the groups ($p>0.05$), the application of 100 mg/kg *CA* and 300 mg/kg *R. anatolyi* flowers ethanol extract applied together with *CP* reached the level of the control group (Figure 4).

Discovered in 1958, *CP* has been used in the treatment of various types of cancer in a short time and is still widely used for blood and marrow transplantation as a chemotherapeutic agent (Emadi *et al*., 2009). Although *CP* is one of the alkylating drugs used for treatment purposes in chemotherapy for a long time, its risks are still to be updated (Ahlmann and Hempel, 2016; El-serafi and Steele, 2024). These risks may depend on the dose given, the duration and intensity of treatment, or other factors (including radiotherapy) (Coggins *et al*., 1960). *CP* is called cytotoxic cancer drug as well as well known immunosuppressant in mammals (Kumari and Sahoo, 2015). Since its discovery, *CP* has been found to cause damage to tissues such as the liver, lungs, kidneys, and brain by causing free radical production and disruption of the balance of the antioxidative defense system (Coggins *et al*., 1960; Hao *et al*., 2024; Yadav *et al*., 2024). The toxic effect of *CP* was in parallel with our findings, as it decreased the serum vitamin (retinol, cholecalciferol and phylloquinone) levels in *CP* treated rats (200 mg/kg *CP*) (Table 1). *CP* induced oxidative stress in rats as evidenced by the percentage decrease in retinol levels (Table 1 and Figure 2). In connection with this, the undesirable changes detected in retinol, cholecalciferol and phylloquinone vitamins among the experimental rat groups we created likely results from free radical attacks induced by *CP*.

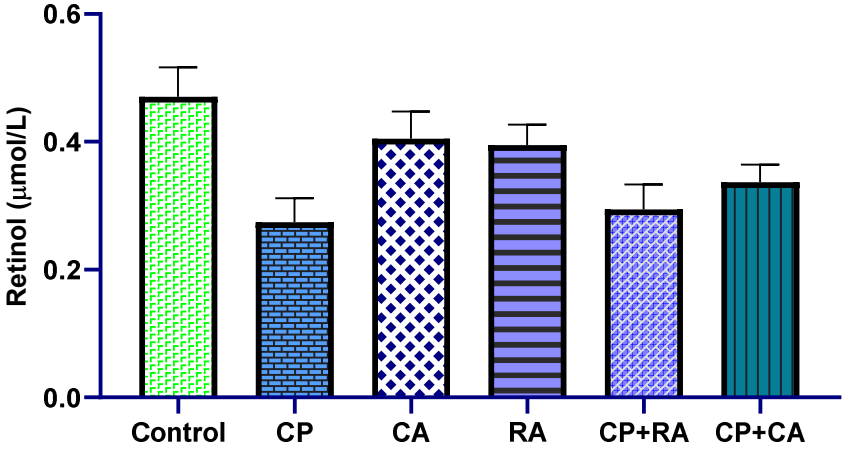

Figure 2. Retinol levels between groups in rat serum of chlorogenic acid (*CA*) and *R. anatolyi* (*RA*) flowers ethanol extract in cyclophosphamide *(CP*)-induced experimental application.

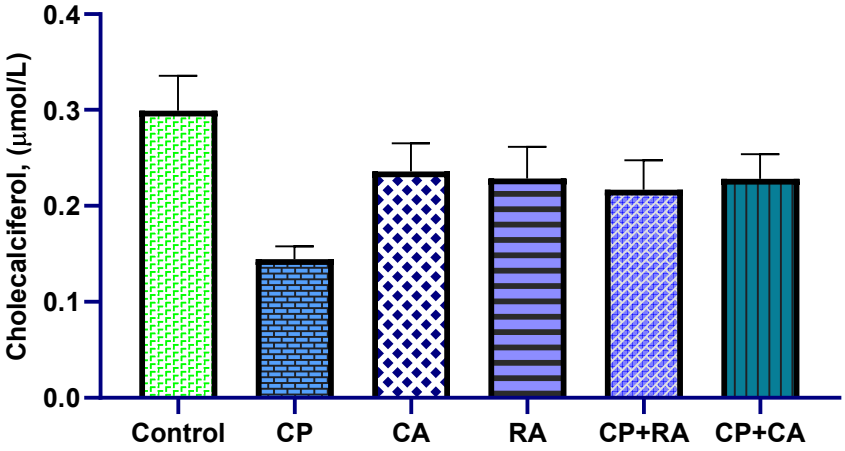

Figure 3. Cholecalciferol levels between groups in rat serum of chlorogenic acid (*CA*) and *R. anatolyi (RA)* flowers ethanol extract in cyclophosphamide (*CP*)-induced experimental application.

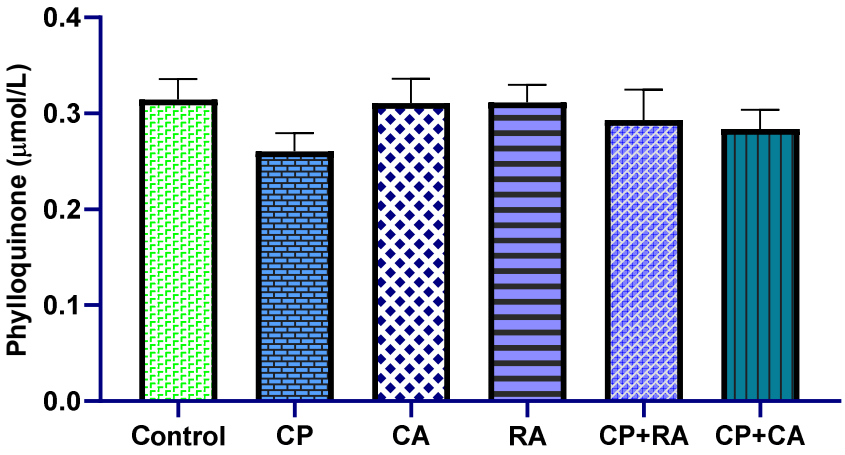

Figure 4. Phylloquinone levels between groups in rat serum of chlorogenic acid (*CA*) and *R. anatolyi (RA)* flowers ethanol extract in cyclophosphamide (*CP*)-induced experimental application.

As studies progress, the relationship between oxidative stress and diseases such as Alzheimer's, Parkinson's, cardiovascular, inflammatory problems, skin, kidney, hypertension, heart, malignant tumours is

coming to the fore. Although the functions, deficiencies, active forms and functions of fat-soluble vitamins retinol, cholecalciferol and phylloquinone in the body are well defined, their roles on oxidative stress have not been well evaluated (Sinbad *et al*., 2019). Although these vitamins are stored in the body, it is important that they are sufficient in any oxidative stress situation and respond to metabolic destruction. Since these vitamins are stored in the body, they do not need to be taken frequently, but when dietary intake is insufficient, this may mean that these stores will be depleted and may be inadequate for coping with stress. As a result, foods poor in antioxidants and a lack of antioxidant vitamins can increase oxidative stress and cause cell death (Khadim and Al-Fartusie, 2021).

Many of the phenolics, both chlorogenic and caffeic acid, have been shown to act as antioxidants in vivo, with multiple mechanisms involving free radical scavenging and metal ion chelation (Azuma *et al*., 2000; Lafay *et al*., 2006). In addition, phenolic compounds, flavonoids and vitamins, which are found in significant amounts in plants, exhibit pharmacological activities such as anti-inflammatory through some mechanisms because of their antioxidant properties (Park *et al*., 2023). Within the scope of our study, it was determined that the application of *CP* (200 mg/kg) together with *R.anatolyi* (300 mg/kg) extract and *CA* (100 mg/kg), which is a strong antioxidant, showed parallelism in serum cholecalciferol and phylloquinone vitamin groups, but when the retinol group was examined, there was a partial decrease in *CP+R.anatolyi* values. This can be said that the *R.anatolyi* plant has a stronger antioxidant property than *CA*.In the literature, no study was found in which the application of *R. anatolyi* flower was tested on serum vitamin levels in rats. The *R. anatolyi* plant has not been the subject of much study since its discovery (Lyskov *et al.,* 2017). Scientists are conducting many in vivo or in vitro studies to determine the active ingredients contained in plants or to see their antioxidant effects. The most important reason for this plant to be the subject of our study is that it is endemic, consumed by local people (non-toxic), and the Apiaceae family to which this species belongs has been the subject of many studies.

The discovery and testing of the effects of functional bioactive substances found in plants is of great interest due to the less side effects not seen in natural medicines. The discovery of their functional properties is gaining popularity due to fewer side effects not seen in natural drugs. In addition, no study has been found in which serum vitamin levels in rats were examined in any plant from the *Rhabdosiadium* species, in which oxidative stress was created by *CP*. However, it has been investigated whether some plants with beneficial effects on the organism and antioxidant properties show benefit against oxidative stress caused by *CP*. For example; Shirani *et al*., (2015) examined nearly 20 plant species in and evaluated the possible toxicity caused by *CP*. In this study, these doses were applied to *picrorhiza kurroa* (200 mg/kg), *Cassia occidentalis L.* (100 mg/kg), *Ficus glomerata roxb*. (250 and 500 mg/kg), *Curculigo orchioides* (50-800 mg/kg), *Leucas aspera* (100-200 mg/kg), *Acacia ferruginea* (10 mg/kg) and *Cardiospermum halicacabus* (25-500 mg/kg) plants. On the other hand, Joshua *et al.,* (2022) observed a significant decrease in vitamin A following *CP*-induced oxidative stress in rats given the aqueous seed extraction of *Datura stramonium L.* plant.

Retinol is an important component in reproduction, growth, protection of epithelial cells and vision. In addition, due to the role of retinol in immune function (Ferrier, 2019), it can be investigated to what extent it has an effect on the immune system against the oxidative damage caused by *CP*. *CA*, a potent compound, has limited studies on the prevention and treatment of diseases (especially eye diseases) (Tang *et al*., 2024). The precursor of retinol, ß-carotene, is also known as an antioxidant substance that repairs free radical damage at the same rate. From this point of view, the significant difference ($p<0.01$) in retinol (µmol/L) levels in the groups evaluated in our study can be explained by the antioxidant properties in neutralising free radicals. The effects of vitamin A on the suppression of *CP*-induced healing as an antineoplastic were investigated by Raju and Kulkarni (1986). They concluded that vitamin A reversed the suppressive effect of *CP* wound healing and also promoted the normally progressive healing of excision wounds.

Vitamin D is critical for the development, growth, and mineralization of the skeleton during its formation years (Holick, 1996). In addition to its well-defined roles in skin, bone and muscle physiology, there is increasing evidence to suggest that vitamin D acts as an inhibitor of the inflammatory response through a variety of pathways (Richards *et al*., 2007). Apart from all these functions of vitamin D, it has been explained in recent studies that antioxidant molecules such as glutathione in nerve cells protect the brain against free radicals by increasing the number of receptors (Sengezer *et al.*, 2016). Decreased concentrations of vitamin D have been associated with an increase in autoimmune diseases such as diabetes type 1, *MS* disease (multiple sclerosis) and rheumatoid arthritis (*RA*), a chronic disease (Richards *et al*., 2007). There are studies on the fact that vitamin D has some physiological benefits (especially on DNA damage) as a supportive treatment for many patients, including cancer patients (Liu *et al.*, 2019). *CP* is metabolised by the cytochrome P450 enzyme system, particularly CYP3A4. Vitamin D may affect these enzymes. However, specific interactions between *CP* and vitamin D are not well established (Robien *et al.,* 2013). When the results of our study were examined, a significant correlation ($p<0.01$) was found in

cholecalciferol (µmol/L) levels between the control and *CP* groups (Table 1). As can be seen in Table 1 and Figure 3, it was concluded that the cholecalciferol (µmol/L) level of *CP*-injected rats decreased and that the dominant view was that *CP* triggered oxidative stress.

Vitamin K is known to act as a cofactor in normal blood clotting by controlling blood viscosity. This is due to post-translational modification of a number of plasma proteins such as prothrombin. On the other hand, this vitamin, which is important in the diet, is mostly found in green leafy vegetables (Gottschlich, 2007). However, although vitamin K is generally recognised as a critical factor in blood clotting, recent research suggests that vitamin K may also be a cofactor in bone metabolism (Plaza and La, 2003). Unlike cholecalciferol and retinol values, no statistically significant difference ($p>0.05$) was detected in phylloquinone levels (Table 1 and Figure 4). This study showed that there was no percentage decrease in phylloquinone (µmol/L) levels in serum values of *CP*-induced rats. There is limited evidence of direct interactions between *CP* and vitamin phylloquinone. Therefore, whether this vitamin has effects on oxidative stress after *CP* administration should be extensively investigated.

Conclusion: It was found that administration of *CA* and *R. anatolyi* flower extract to rats subjected to oxidative stress by *CP* (200 mg/kg) did not result in any significant change in serum phylloquinone levels. However, a statistically significant difference ($p<0.01$, $p<0.01$) was found in retinol and cholecalciferol levels between the *CP* and control groups. It can be suggested that the serum vitamin levels of *CA* that we administered synthetically with the ethanol extract of *R. anatolyi* flowers are almost close to normal, which may be due to the antioxidant property of *R. anatolyi* flowers. In addition, the data of this study can serve as a reference for many future studies.

Conflict of Interest Statement: The authors have no financial support or relationship that could create a potential conflict of interest.

Author's Contributions Statement: AB and SE designed the study, AB and MF collected the plant material. AB provided experimental work, formal analysis, manuscript writing and editing, and SE provided statistical editing. All authors thoroughly reviewed the final version of the manuscript and agreed on the manuscript before publication.

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