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RESEARCH ARTICLE

Determination of Industrially Significant Bioactive in Oleander (*Nerium oleander* L) Flowers with Different Colors Utilized as Traditional Medicine

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HIGHLIGHTS

- The peroxidase enzyme was purified from the oleander (*Nerium oleander* L) plant, which grows in Muğla and blooms between June and September.
- Free radical scavenging activity and reducing power were determined in the plant extract using the CUPRAC method.
- The suitability of the oleander plant for industrial usage was determined by the amount analysis of vitamins A, C, and E and aroma components.

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ABSTRACT

The main purpose of this study was to investigate the industrial usage of oleander (*Nerium oleander* L) plant, growing in Muğla and blossoming from June to September, by means of purifying peroxidase enzyme, determining aroma components and focusing on free radical scavenging activity and reduction force as well in accordance with CUPRAC method. The quantities of vitamins A, C and E in oleander plant were found out additionally. Phenolic compounds and IR spectra were also determined. At the end of the research carried out, the peroxidase enzyme was purified from oleander plant and it was concluded that this flower could be used in industries such as cosmetics, food and medicine.

Contents

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1. Introduction

Enzymes have been the most studied subjects in the field of biochemistry. Enzymes have been widely used in agriculture, food and chemical industries. Cheese, yogurt, wine, bread in making, enzyme-catalyzed reactions are used. In recent years, the importance of enzymes in the diagnosis and treatment of diseases has increased steadily [1].

Peroxidases (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) catalyze the oxidation of various electron donor substrates (e.g., phenols, aromatic amines) by hydrogen peroxide. They are extensively used in clinical biochemistry and immunoassay. Other applications include the synthesis of various aromatic compounds and the removal of peroxide from foodstuff and industrial wastes [2]. Peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, rigidification and eventual lignification of cell walls, in the biosynthesis of H_2O_2, in the protection of plant tissues from damage.

Numerous defense and protection mechanisms exist in plant and animal cells. These mechanisms are necessary for the organism to protect itself against the toxic effects of normal oxygen metabolism [3]. In this respect, studies on the investigation of antioxidant defense mechanisms in biological systems have gained importance. One of the most important factors that causes aging is that oxidative damage has a radical mechanism.

Natural antioxidants are found in cereals and legumes, in vegetables, in fruit with and without crust, in seeds, in leaves, in flowers, in roots, in medicinal plants and in plant-derived beverages [4, 5]. Antioxidants found in these sources; tocopherols, flavonoids, phenolic compounds, nitrogen compounds (such as alkaloids, chlorophylls, proteins, amines), poly-functional organic acids and carotenes [6]. Instead of natural antioxidants, synthetic antioxidants have been used since the beginning of the twentieth century for cost reasons. However, as a result of studies showing that synthetic antioxidants may be toxic and carcinogenic, there are serious limitations or prohibitions on their use in some countries [7]. These suspicions about synthetic antioxidants have increased the tendency to natural antioxidants and the work in this area has concentrated on plant-derived antioxidants. Natural origin vitamins E and C have been used as antioxidants for many years because of their separate, or synergistic effect on nutrients. However, the antioxidant activities of tocopherol and ascorbic acid are relatively lower than those of synthetic antioxidants.

The antioxidant enzymes and the endogenous antioxidants both have the same mechanism of preventing oxidation [8]. It has been reported that antioxidant enzymes inhibit free oxygen radicals and prevent biological oxidative damage. In other words, the reactive oxygen species in biological systems are also eliminated by enzymes such as antioxidant compounds [9].

Ascorbic acid (vitamin C) is an antioxidant that plays a role in preventing oxidation in liquids. It is found in high concentrations in immune cells and is quickly consumed during infection [10]. Ascorbic acid plays a role in improving the oxidative stress of photosynthesis in chloroplasts of plants. In addition, it plays a role in cell division and protein modification [11]. Vitamin E has many biological functions. The antioxidant function is considered to be the most known and most important function of vitamin E. Enzymatic activity regulation is another important function of vitamin E [12]. Vitamin A (Retinol, Retinal, Carotenoid) is a vitamin that is required by the retina to absorb light in the eye, scotopic vision and color vision. Retinoic acid acts as a hormone-like growth factor for epithelial and other cells [13].

Nerium oleander L. is an evergreen shrub belonging to the family Apocynaceae. *Nerium oleander* L. is a medium-sized flowering evergreen tree of 2-5 m in height and is planted throughout the world tropical regions as garden and roadside trees. This plant possesses cardiotonic, antibacterial, anticancer, and anti-platelet aggregation activity and depresses the central nervous system. All its parts are toxic. It is used in the treatment of abscesses, psoriasis, dermatitis, sores, eczema, warts, corns, scabies, skin cancer, ringworm, herpes, epilepsy, dysmenorrheal, asthma malaria, abortifacients, emetics, heart tonics and tumor [14–16].

In this study; The peroxidase enzyme of oleander (*Nerium oleander*) was purified and the plant's vitamins, organic components were analyzed and antioxidant activities were determined. In short, an answer has been sought as to whether this flower can be used in industries such as cosmetics, food and medicine.

2. Materials and Methods

2.1. Collection of Plant Flowers

Oleander flowers were collected from Muğla countryside in June-September and kept in deep freezing at -80 ° C until used in our experiment.

2.2. Preparation of Peroxidase Extract

In the beginning, oleander flowers were taken from the cold room. Approximately 10 g of oleander flowers were weighed and homogenized by using a blender with 100 ml 100 mM phosphate buffer (pH 7). The homogenate was filtered through Whatman filter paper No. 1 and the filtrate was centrifuged at 10,000 xg for 20 minutes. After centrifugation, the supernatant was collected and analyzed for peroxidase activity and protein content.

2.3. Three Phase Partitioning (TPP)

The TPP of the peroxidase extract was carried out in a 50 mL glass reactor using a mechanical stirrer. In a glass beaker, 10 mL of extract was mixed with 4 g of ammonium sulfate (40%

w / v) at room temperature (30 ° C) and then 10 mL of t-butanol was added. This reaction mixture was stirred at 200 rpm for 100 minutes. The mixture was centrifuged at 6000xg for 20 minutes to facilitate separation of the phases. The mixture was, then, allowed to stand for one hour at room temperature until the phases were separated in a separatory funnel. Three different phases were observed after separation in the separation funnel. The protein precipitate in the middle and the lower aqueous phase was carefully separated to work later. The protein precipitate in the middle was dissolved in a minimum amount of 100 mM phosphate buffer (pH: 7) [17]. The middle and lower fractions of TPP were dialyzed against 100 mM phosphate buffer (pH: 7) for 12 hours and analyzed for protein content and enzyme activity.

2.4. Determination of Homogenate T-Butanol Ratio for Peroxidase Enzyme:

The TPP of the peroxidase extract was performed in a 50 mL glass reactor using a mechanical stirrer. Homogenate was studied in: t-butanol (1.0: 0.5, 1.0: 1.0, 1.0: 1.5 and 1.0: 2.0) ratios (v/v). The highest activity rate was determined. 4 g of ammonium sulfate was added to 10 mL of homogenate and t-butanol was added according to proportions. The reaction mixture was stirred at 200 rpm for 100 minutes and then the mixture was centrifuged at 6000 rpm for 20 minutes to the facilitate separation of the phases. After the procedure, the phases were separated carefully. The purification rate of the middle phase and the activity gain were higher when compared to the upper and lower phases [18].

2.5. Determination of Ammonium Sulfate Concentration for Peroxidase Enzyme

After determining the ratio of T-butanol, the appropriate concentration of ammonium sulfate was determined. Determined crude homogenate: $(NH_4)_2SO_4$ was added separately at concentrations of 20, 30, 40 and 50% (w/v) on the t-butanol ratio. The ammonium sulfate concentration demonstrating the highest activity was determined. Between phases, purification coefficient and activity yield were compared [18].

2.6. Measurement of Peroxidase Activity

Peroxidase activity was determined colorimetrically by using a spectrophotometer (Spectroscan UV 2700, Double Beam UV-VIS Spectrophotometer, Chemito, Ind.), followed by the formation of tetra guaiacol (Amax = 470 nm, ε = 26.6 mm -1 cm -1). The final concentration of the reaction mixture in 100 mM phosphate buffer (pH: 7) was 0.05% H_2O_2 and 18 mM guaiacol. To measure peroxidase activity, 10 mL of the enzyme-containing sample was added to 2.9 mL of the substrate solution and the change in absorbance was continuously measured at 470 nm for 5 minutes. One unit of peroxidase activity was calculated as the amount of enzyme catalyzing 1 mmol guaiacol oxidation per minute [19].

2.7. Determination of Phenolic Components

Extraction conditions of plant samples: For plant sample analysis, methanolic extracts were prepared by using a magnetic stirrer at room temperature for 24 hours and from the available solid particles, the solutions were filtered with blue band filter paper in order to get rid of impurities and ensure homogeneity. After determining the final concentration of the extracts obtained, the extracting solvent was removed on a rotary evaporator at 60 °C and the 2 pH residue was dissolved in 10 mL of distilled water. 5 mL of diethyl ether and, then, ethyl acetate extraction was performed three times. Extracts obtained at the end of extraction were taken out of the evaporator balloons and the solvents were removed from a rotary evaporator at 60 °C. Phenolic component analyzes were performed with HPLC-UV extracts of the bubble contents dissolved in 2 mL of methanol.

2.8. Determination of Phenolic Compounds by HPLC-UV

HPLC-UV analysis was performed on a HPLC system (Elite LaChrom Hitachi, Japan) equipped with a UV-Vis detector at a wavelength of 280 nm. The analyzes were carried out using reverse phase C_{18} column (150 mmx4.6 mm, 5 µm; Fortis) and gradient program with acetonitrile, water and acetic acid. A gradient program with 2% acetic acid (in distilled water) in reservoir A and 70-30% acetonitrile-distilled water in reservoir B is given in Table 1. In addition, the sample and standards were optimized for operation by setting the injection volume to 25 µL to get the mobile phase flow rate as 1.2 mL.min^{-1}, and to set up column temperature as 30 ºC [20].

Table 1 RP-HPLC-UV gradient program

Time (min)	A 2 % Acetic Acid (in distilled water)	B 70-30 % Acetonitrile-distilled water
0.01	95.00	5.00
3.00	95.00	5.00
8.00	85.00	15.00
10.00	80.00	20.00
12.00	75.00	25.00
20.00	60.00	40.00
30.00	20.00	80.00
35.00	95.00	5.00
50.00	95.00	5.00

2.9. Antioxidant Activity Determination in Extracts

The activities of hexane and methanol extracts of flowers of oleander plants were determined by DPPH free radical scavenging and CUPRAC methods.

2.9.1. Determination of Total Antioxidant Activity (β-Carotene-Linoleic Acid Method)

The antioxidant activities of the analysis samples were determined according to the β-carotene color removal method. For this procedure, after the stock solutions of the samples were prepared, an equal volume of β-carotene-linoleic acid emulsion was added with the appropriate buffer solution the incubation at 50 ° C. As a control, the mixture of equal volume buffer solution and β-carotene-linoleic acid were used. Every 30 minutes, the absorbance against the blank solution will be read by a spectrophotometer at 470 nm. Incubation was terminated by the control reaching minimum absorbance [21].

2.9.2. Determination of DPPH Free Radical Scavenging Activity

The free radical scavenging activities of the extracts were determined by using 1,1-Diphenyl-2-picrylhydrazyl free radical [22]. 10 mg of the oleander flowers were weighed out of hexane and methanol extracts, dissolved in 10 mL of ethanol and stock solutions were prepared. 2-20 µL of this stock solution was taken. The volume was adjusted to 40 µL with ethanol and 160 µL of 0.1 mM DPPH solution was added to the solution. The prepared solutions were measured for absorbance at 517 nm after incubation for 30 minutes at room temperature in the dark.

%inhibition values were calculated from these absorbance values.

The absorbance values of the samples were evaluated against the control. The free radical scavenging activity (% inhibition) was calculated using the following equation;

$$\% \text{ inhibition} = \left[\frac{A_{control} - A_{exract}}{A_{control}}\right] x100 \quad (1)$$

Three parallel runs were performed on each sample. Butylated hydroxyanisole (BHA) and α-tocopherol were used as standard [17].

2.9.3. Determination of Reduction Force According to CUPRAC Method

The cupric ion (Cu^{2+}) reduction capacities of the hexane and methanol extracts of the flowers of oleander plants were modified by applying the CUPRAC method [23]. 10 mg of the prepared hexane and methanol extracts were dissolved in 10 mL of ethanol to prepare stock solutions. 2.5-25 µL of this stock solution was taken and 67 µL volume filled with ethanol. The solutions were vortexed by adding 61 µL of 10 mM CuCl 2 solution, 61 µL of 7.5 mM of neocuprin, and 61 µL of NH_4Ac buffer (1 M, pH = 7). The solutions were allowed to stand for 30 minutes at room temperature with their caps closed.

At the end of the time period, the absorbance values at 450 nm of the solutions were measured against the reference solution without the sample. Three parallel runs were performed on each sample. BHA and α-Toc were used as standard [17].

2.10. Determination of Vitamin C in Oleander Flowers

The amount of vitamin C in oleander flowers was determined by titration with dichlorophenolindophenol (DCPIP). 0.1% DCPIP solution was added dropwise to the plant homogenate. The titration was terminated by the formation of blue color.

Each 1 cm^3 DCPIP with 0.001 g was calculated to correspond to 0.0015 g ascorbic acid and the amount of vitamin C in the sample was calculated using the following formula.

The amount of Ascorbic acid in mg/100g is:

$$n = V \times F \times 100 / W \quad (2)$$

2.11. Determination of Vitamin E in Oleander Flowers

The ester form of tocopheryl acetate was hydrolyzed by the addition of H_2SO_4 by taking 0.125, 0.25, 0.5 and 1 µg/mL of α-tocopherol standard solution. The medium was, then, added with 0.5 mL of 0.01 M tetrazolium blue, 5 mL of 0.2 M NaOH and methanol. After 10 minutes of incubation at 90°C, 526 absorbances were measured and a standard plot was generated. The amount of vitamin E of oleander flowers was calculated using the standard graphic.

2.12. Determination of Vitamin A in Oleander Flowers

The vitamin A content of oleander flowers was analyzed by Prasad et al. [24]. The method was based on the principle that vitamin A forms a colored compound with 4-hydroxy-3-methyl benzaldehyde and that this compound shows maximum absorbance at 610 nm. Than β- carotene, 0.25; 0.5; 1.0; 2.5 and 5.0 mg / mL were taken and a colored derivative was formed with 4-hydroxy-3-methyl benzaldehyde. The standard graph was drawn. Then, the extract samples from the oleander flowers were subjected to measure the vitamin A content of samples and calculated.

3. Results

The results of the purification of peroxidase by the three-phase separation system prepared from the oleander flowers in optimized conditions are given in Table 2. 40% (w/v) ammonium sulfate saturation and in the 1:1.5 (v/v) homogenate: t-butanol ratio, the enzyme was purified from the mid-phase of the UFA system with an activity yield of 372,09% and purification coefficient of 1.064.

In the TPP system to efficiently collect the enzymes at the interface, studies were carried out in the presence of 40% ammonium sulfate and 1.0: 0.5, 1.0: 1.0, 1.0: 1.5, 1.0: 2.0 homogenate: t-butanol (v / v). After the process, the phases were carefully separated. The amount of protein was most observed in the medium phase [25, 26].

The supernatants were discarded. The homogenate was brought to 40% (w / v) ammonium sulfate saturation and different amounts of the t-butanol (1: 0.5, 1: 1, 1: 1.5, 1: 2, v / v) were added. Phase separation at room temperature was expected to occur. In each medium and sub-phase obtained from the homogenate: t-butanol ratios, peroxidase activity and protein were determined and the phase with the highest yield and purification coefficient was determined. The highest activity was observed in the middle phase. In this TPP system, while the ammonium sulfate saturation was 40% (w / v) and the homogenate: t-butanol ratio was 1: 1.5, the enzyme predominantly preferred to remain in the middle phase. The results are given in Table 3.

The optimum homogenate: t-butanol ratio was found to be 1: 1.5 in ammonium sulfate precipitation studies. Since this ratio was studied in 40% ammonium sulfate saturation, 20%, 30% and 50% ammonium sulfate concentrations were studied separately. For each medium and sub-phase, ammonium sulfate concentrations, peroxidase activity and protein levels were determined. The phase with the highest yield and purification coefficient was determined.

Table 2 Purification results of peroxidase enzyme's with three-phase separation system from oleander flowers (40% (w/v) ammonium sulfate, 1: 1.5 (v/v) homogenate: t-butanol ratio)

Samples	Homogenate	Middle phase
Activity (EU/ml)	0.215	0.800
Total Activity (U)	21.5	80
Protein (mg/ml)	27.25	96
Specific Activity (EU/mg)	0.78	0.83
Purification (fold)	1	1.064
Yield (%)	100	372.09

There was no significant difference between the groups (Table 3).

Table 3 The results of peroxidase enzyme's from oleander flowers, homogenate: t-butanol ratio

Homogenate t-butanol ratio	Purification coefficient	Yield (%)
1-0.5	0.75	55.04
1-1	2.5	113.64
1-1.5	3.52	527.25
1-2	0.72	533.16

As a result of this study, the ammonium sulfate saturation was 40% (w / v) and the homogenate: t-butanol ratio was 1: 1.5, whereas the enzyme preferred to remain in the middle phase (Table 4).

Table 4 The results of ammonium sulfate concentration of peroxidase enzyme in oleander flowers

Homogenate: t-butanol ratio	Purification coefficient	Yield (%)
%20	8.6	225.85
%30	3.53	529.22
%40	5.83	687.51
%50	1.44	422.,3

Standard chromatogram was produced by standard analysis before analyzing phenolic compounds in oleander flowers analysis with HPLC-UV (Figure 1). Then, phenolic components of oleander flowers of different colors were determined by HPLC-UV analysis and results were given in Table 5. For the white oleander flowers, the contents of vanillic acid (180.35%), routine (31.55) and ferulic acid (29,12) were high. These components are abundant in natural food products and have high antioxidant capacity.

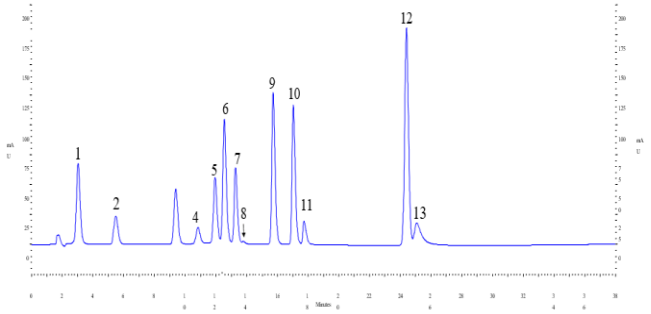

Figure 1 Phenolic acid standard chromotogram 1. Gallic acid, 2. Protocatequic acid, 3. p-OH benzoic acid, 4. Catechin, 5. Vanilic acid, 6. Caffeic acid, 7. Syringic acid, 8. Epikatechin, 9. p-Kumaric asit, 10. Ferulic acid, 11. Routine, 12. t-Cinnamic Acid, 13. Luteolin

Table 5 Phenolic components of oleander flowers of different colors

	Standards	µg extract per g sample		
		White sample	Pink sample	Red sample
1	Gallic acid	n.d.	n.d.	n.d
2	Protocatechuic Acid	12.34±0.01	11.95±0.05	0.40
3	*p*-OH Benzoic Acid	27.01±0.02	26.52±0.08	0.63
4	Catechin	n.d.	n.d.	n.d
5	Vanilic Acid	180.35±0.02	233.55±0.01	6.58
6	Caffeic Acid	2.17±0.02	1.88±0.01	n.d
7	Cyringic Acid	10.17±0.03	18.82±0.05	0.34
8	Epicatechin	9.16±0.01	n.d.	0.12
9	*p*-Kumaric Acid	9.43±0.05	3.95±0.01	0.15
10	Ferulic Acid	29.12±0.06	4.63±0.03	n.d
11	Routine	31.55±0.02	n.d.	n.d
12	Daizein	n.d.	24.73±0.09	0.02
13	*t*-Cinnamic Acid	0.10±0.02	n.d.	n.d
14	Luteolin	5.43±0.09	7.19±0.03	0.79

After the extraction of oleander flowers, IR spectra measurements were taken. It was observed that natural oleander flowers gave the same peaks when the IR results were compared. The results are given in Figure 2, Figure 3, and Figure 4. As a result of the data obtained from the studies, it was concluded that the oleander plant can be a very important raw material source for the cosmetic and pharmaceutical industries.

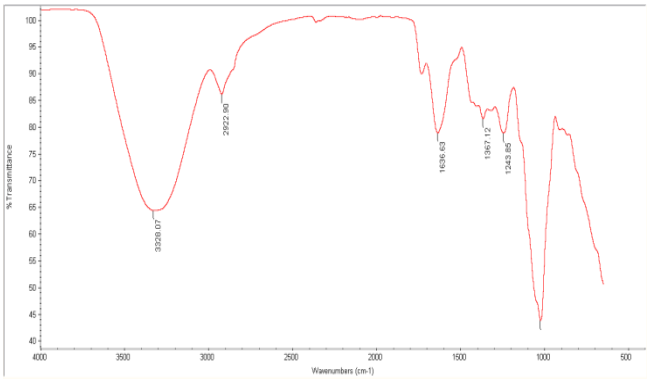

Figure 2 IR spectrum as a result of extraction of white oleander flower

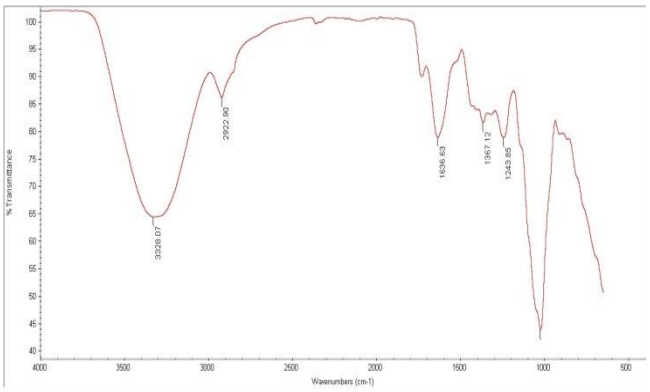

Figure 3 IR spectrum as a result of extraction of pink oleander flower

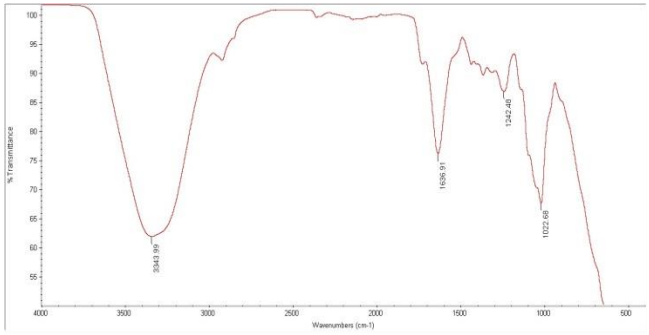

Figure 4 IR spectrum as a result of extraction of red oleander flower

It is known that the methanol extract of oleander contains polar molecules. According to the results, it can be said that the red oleander plant in increasing concentration contains more polar molecules by looking at the higher activity. It can be said that the pink species is rich in phenolic compounds but the white oleander species contains fewer polar molecules.

These data indicate that the oleander plant has a rich content of antioxidant compounds (Figure 5 and Figure 6).

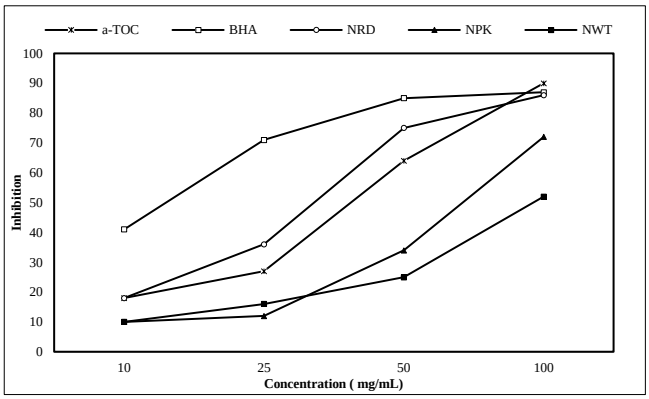

Figure 5 DPPH free radical removal result graph (*NRD: *N. oleander* red, NWT: *N. oleander* white, NPK: *N. oleander* pink)

According to the results, it was observed that the methanol extract prepared from the red flowering species of the oleander plant inhibited DPPH free radicals at the closest level to the BHA synthetic antioxidant standard, followed by pink species. The lowest activity was seen in white species.

When the copper ion reduction potential of the methanol extracts prepared from oleander plants was examined, it was determined that the highest reduction potential was obtained from red species and pink and white species, respectively.

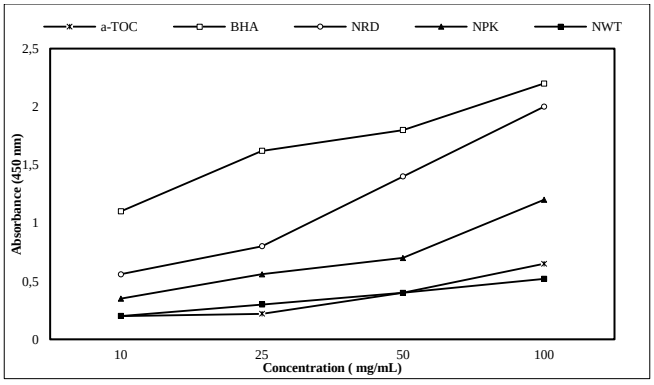

Figure 6 CUPRAC result graph (*NRD: *N. oleander* red, NWT: *N. oleander* white, NPK: *N. oleander* pink)

The amount of vitamin C in oleander flowers was determined by titration with dichlorophenolindophenol (DCPIP).

A standard graph was obtained from the standard solution of α-tocopherol for the determination of vitamin E and, then, the same conditions were measured at 526 nm from oleander flower homogenate. Vitamin E content was determined by using standard graphics (Figure 7.

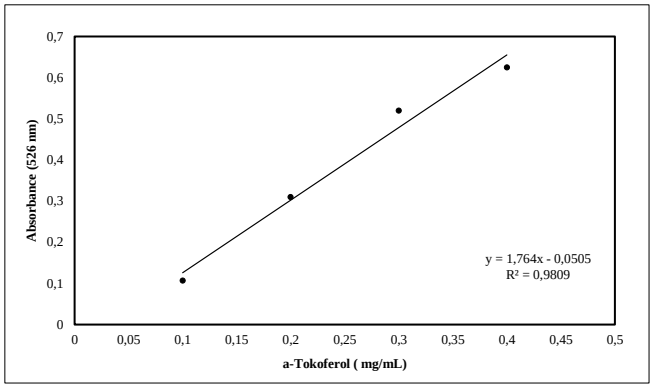

Figure 7 Standard graphic for the determination of vitamin E

Firstly, β-carotene was used to create standard graphics (Figure 8). Then, the specimen obtained from the flowers were measured using the same method and amount of vitamin A were calculated. Vitamin A, C and E contents of oleander flowers are given in Table 6.

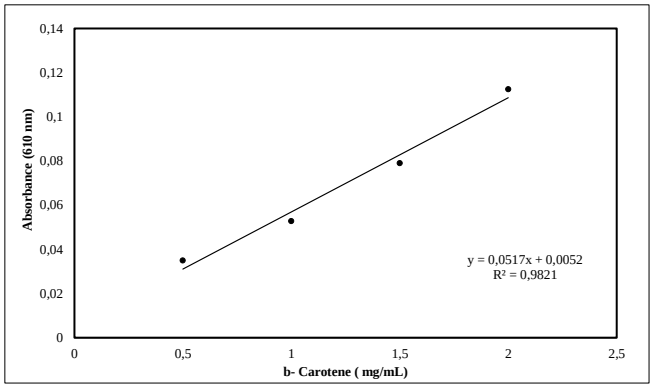

Figure 8 Standard graphic for the determination of vitamin A

Table 6 Vitamin determination results of oleander flowers

Vitamin	Water extract (mg/100 g)	Petroleum ether extract (µg/100 g)
Vitamin A (β-carotene)	-	61±0.26
Vitamin E (α-tocopherol)	-	34±0.17
Vitamin C (Ascorbic acid)	18±0.30	-

4. Discussion

It was determined that the peroxidase enzyme activity and protein amount purified from the oleander flower by the triple phase method were quite high. Peroxidase enzyme was obtained from Oleander (*Nerium oleander* L) flowers with 372% yield by UFA technique. As a result of the literature research, it is stated that the PFA method is a very suitable purification method ([17]).

The phenolic components of oleander flowers were examined and it was observed that the amount of vanillic

(180.35%), rutin (31.55) and ferulic acid (29.12) was high for the white oleander flower. These components are found in large amounts in natural food products and have high antioxidant capacity. IR spectrum measurements were taken as a result of the extraction of oleander flowers. As a result of the comparison of natural oleander flowers, it was observed that the flowers gave the same peaks. As a result of the data obtained from the studies, it has been concluded that the oleander plant can be a very important raw material source for the cosmetics and pharmaceutical industries.

It is known that the methanol extract of the oleander plant contains polar molecules. According to the results, it can be said that the red oleander plant contains more polar molecules by looking at the higher activity of the red oleander plant at increasing concentrations. It can be said that the pink type is also rich in phenolic compounds, but the white oleander type contains fewer polar molecules. These data show that the oleander plant has a rich content of antioxidant compounds [27, 28].

As a result of the high antioxidant activity of the extracts obtained from oleander flowers, there is a possibility that they can be turned into a drug that can be used in the treatment of diseases [29–31]. As a result of this information obtained, it is seen that it will contribute to the pharmaceutical industry in Turkey.

5. Conclusion

Purification of peroxidase enzymes of oleander plants of different colors, that growing in the natural environment of Muğla and blooming between June and September, determination of some vitamins, phenolic compounds and isolation of new compounds with high antioxidant activity makes this study more valuable and interesting.

Declaration of Conflict of Interest

The authors declare no conflict of interest.

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