Screening Effects of Methanol Extracts of *Diplotaxis tenuifolia* and *Reseda lutea* on Enzymatic Antioxidant Defense Systems and Aldose Reductase Activity

*Diplotaxis tenuifolia* ve *Reseda lutea* Metanol Özütünün Antioksidan Savunma Sistemi Enzimleri ve Aldoz Redüktaz Aktivitesi Üzerinde Olan Etkisinin İncelenmesi

**ABSTRACT**

**Objectives:** The aim of the study was to investigate the effects of methanol extracts from the flowers and leaves of *Diplotaxis tenuifolia* and *Reseda lutea* on the activity of AR, CAT, GST, and GPx.

**Materials and Methods:** Total phenolic and flavonoid contents of the plant samples were evaluated using Folin-Ciocalteu reagent and aluminum chloride colorimetric methods. Also, the effects of extracts on CAT, GST, GPx, and AR enzyme activities were investigated using kinetic assays.

**Results:** The highest phenolic and flavonoid contents were detected in the methanol extract of *D. tenuifolia* leaves with 144.49±0.29 mg gallic acid equivalent/L and 250.485±0.002 quercetin equivalent/L, respectively. The best activity profile for GST and GPx were observed in the extract of leaves belonging to *D. tenuifolia* with IC<sub>50</sub> values of 121±0.05 and 140±0.001 ng/mL, respectively. According to the results, methanol extracts from leaves of *R. lutea* and *D. tenuifolia* showed no significant activity potential on AR. Moreover, none of the studied extracts demonstrated any reasonable CAT activation potential.

**Conclusion:** The results indicated that leaves of *D. tenuifolia* had good effect on the antioxidant enzymatic defense system, which it makes it a good constituent of the daily diet.

**Key words:** *Diplotaxis tenuifolia*, *Reseda lutea*, antioxidant enzymes, aldose reductase
INTRODUCTION

Reactive oxygen species (ROS) is a term used to describe a number of reactive molecules and free radicals derived from molecular oxygen, which are generated by all aerobic species. These molecules are generated as by-products during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation. In normal physiologic conditions, a number of defense mechanisms have evolved to provide a balance between the production and removal of ROS, but alterations of the balance between ROS production and the capacity to detoxify reactive intermediates lead to oxidative stress. It has been caused to a wide variety of states, processes and metabolic diseases such as heart disease, severe neural disorders such as Alzheimer’s and Parkinson’s, and some cancers.1,2 Under oxidative stress, an organism has a variety of defense mechanisms to prevent or neutralize negative ROS effects. These are mainly based on enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) or non-enzymatic components such as vitamin E, vitamin C, glutathione, and flavonoids.3 GST is one of the phase II enzymes and plays a critical role in the detoxification and metabolism of many xenobiotic compounds.4 GPx has an important role as a catalyst in the reduction of hydro peroxides, including hydrogen peroxide (H₂O₂), by using GSH. GPx also functions to protect the cell from oxidative damage. Several studies related dysfunctional GPx with cancer.5 CAT is a very important enzyme of living organisms, which catalyzes the decomposition of H₂O₂ to water and oxygen. Aldose reductase (AR) is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme and it has been implicated in the formation of cancer and diabetic complications such as retinopathy, neuropathy, nephropathy, and cardiovascular disorders.6

Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites. Primary metabolites are compounds that have essential roles associated with photosynthesis, respiration, growth, and development. Other phytochemicals that accumulate in high concentrations in some species are known as secondary metabolites, which possess antioxidant activity. Antioxidant compounds found in different parts of plants involve phenolics, flavonoids, alkaloids, glycosides, tocopherols, carotenoids, and ascorbic acid. These are structurally diverse and many are distributed among a very limited number of species within the plant kingdom.7 Secondary metabolite compounds have played an important role in treating and preventing human diseases. They are important sources for new drugs and are also suitable lead compounds for further modification during drug development.8

*Diplotaxis tenuifolia* (L.) DC., commonly known as ‘wild rocket’, belongs to the *Brassicaceae* family. It was originally found as a crop in Mediterranean and Middle Eastern countries and became popular largely due its pungent aromas and tastes.9 In Turkish folk medicine, *D. tenuifolia* is known as “Yabani Roka” and widely distributed in North and West parts of Turkey. Phytochemical studies show that the aerial parts of *D. tenuifolia* contain significantly high concentration of flavonoids, tannins, glucosinolates, sterols, and vitamin C.9

The genus Reseda is one of the herbs in the *Resedaceae* family. In Turkey, this genus is represented by 15 species including *Reseda lutea* L. and *Reseda luteola* L. It is known as yellow mignonette or wild mignonette and has economic importance. It is widely used in the carpet and rug industry as a source of natural dye due to its high luteolin content. In addition to its staining properties, luteolin has attracted great scientific interest because of its pharmacologic activities. Luteolin displays numerous anti-inflammatory effects at micromolar concentrations, which cannot be completely explained by its antioxidant capacities. In addition, phytochemical analysis of aerial parts of *R. lutea* has shown the presence of flavonoid, anthocyanin, and glucosides.10

The aim of the present study was to evaluate the total amount of the phenolic and flavonoid contents of methanol extract obtained from the flowers and leaves of *D. tenuifolia* and *R. lutea* and to determine their effects on the activity of AR, CAT, GST, and GPx. These enzymes play critical roles in the antioxidant defense system.

EXPERIMENTAL

Chemicals materials

In this study, 4-aminoantipyrine, H₂O₂ and sodium azide (NaN₃) were provided by Acros, USA. Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, reduced glutathione (GSH), GR, horseradish peroxidase, CAT, gallic acid (GA), and quercetin hydrate were supplied by Sigma Aldrich, Germany. Lithium sulphate (Li₂SO₄) and NADPH were purchased from Gerbu, Germany. All other chemicals used were analytical grade and provided by Sigma Aldrich, Germany.

Plant materials

Plant samples of *D. tenuifolia* and *R. lutea* were harvested in July 2010 from Ankara, Turkey, and were authenticated by Prof. Dr. Fatmagül Geven, in the Department of Biology, Ankara University. The plant specimens with their localities and the necessary field records were recorded and numerated as voucher specimen numbers. The voucher numbers of *D. tenuifolia* and *R. lutea* were FG-2010-10 and FG-2010-13, respectively. They were deposited in the herbarium department at Ankara University.

Extraction of plant

Different parts of fresh plant samples (flowers and leaves) were washed with tap water and dried at room temperature before analysis. For methanol extraction, 2 g of dried samples were weighed and ground into a fine powder with liquid nitrogen, then mixed with 20 mL methyl alcohol at room temperature in 160 rpm for 24 h. The obtained extract was filtered over Whatman No. 1 paper and the filtrate was collected. Methanol was then removed using a rotary evaporator at 40°C to obtain a dry extract. The obtained product was dissolved in DMSO and kept in the dark (4°C) to be prevent oxidative damage until analysis.11
**Total phenolics determination**
The total concentration of phenolics in the extracts was determined using the method of Slinkard and Singleton.13 Each plant extract solution (0.1 mL) was mixed with 2 mL of a 2% (w/v) sodium carbonate solution and vortexed strongly. After 5 min, 0.1 mL of 50% Folin-Ciocalteu’s reagent (w/v) was added and vortexed, then incubated for 1 hr at room temperature. Afterwards, the absorbance of each mixture was measured at 750 nm using an ultraviolet (UV) spectrophotometer (HP 8453 A, USA). Results were evaluated using 50, 100, 200 and 400 mg/L of GA as a standard curve and recorded as milligrams (mg) GA equivalent/L of extract.

**Total flavonoid determination**
The total concentration of flavonoids in the extracts was determined using aluminum chloride colorimetry, which was previously described13: 0.1 mL of each plant extract was separately mixed with 0.15 mL of 95% ethanol, 0.01 mL of 10% aluminum chloride, 0.01 mL of 1 M sodium acetate, and 0.25 mL of DMSO. The mixture was incubated at room temperature for 30 min and the absorbance of the reaction was measured at 415 nm with the UV spectrophotometer (HP 8453 A, USA). A standard curve was calculated by preparing quercetin solutions at different concentrations for 25, 50, 100, 150, and 200 mg/L. The total flavonoid content of the extract was expressed as milligrams (mg) quercetin equivalent/L of extract.

**Isolation of cytosol from bovine liver**
Bovine liver was obtained from a slaughterhouse in Kazan, Ankara, Turkey. The liver samples were homogenized in 10 mM potassium phosphate buffer (pH 7.0), containing 0.15 M KCl, 1.0 mM EDTA, and 1.0 mM of DTT, using a glass Teflon homogenizer and then centrifuged at 10,000 g for 20 min. The supernatant was filtered through cheesecloth and the filtrate was centrifuged at 30,000 g for 60 min. The collected supernatants were filtered again and the resultant filtrate was considered as cytosol.14 The prepared homogenates, containing 46.41 mg protein/mL, were kept in ultra-low freezer (-80°C) for future use. The total protein content was determined using the Lowry method.15

**Isolation of aldose reductase from bovine liver**
Bovine liver was obtained from a slaughterhouse in Kazan, Ankara, Turkey. The liver samples were cut into small pieces and washed with 1.0 mM EDTA. It was then weighed and homogenized with threefold 1.0 mM EDTA 50 μM PMSF and centrifuged at +4°C, 10,000 rpm for 30 min. To obtain a 40% saturation, 22.6 g ammonium sulfate was added to every 100 mL supernatant solution and mixed for 5 min on a magnetic stirrer and then centrifuged at +4°C, 10,000 rpm for 25 min. To obtain 50% and 75% saturations, the previous method was repeated adding 5.8 g and 15.9 g of ammonium sulfate to the 100 mL supernatant solution, respectively. The obtained pellets were dissolved with 50 mM sodium chloride and kept in a deepfreeze at -80°C.16

**Assay of glutathione-S-transferase**
GSTs activity was determined against the substrate 1-chloro-2, 4-dinitrobenzene (CDNB), by monitoring thiocether formation at 340 nm.17 Briefly described, the assay mixture containing plant extracts solution (final concentration in the range of 7-476 ng/mL), 200 mM potassium phosphate buffer (pH 6.5) with 50 mM CDNB and 3.2 mM GSH, and bovine liver cytosolic fractions (0.782 mg protein/mL) was prepared and used as the enzyme source to measure GST activity. GSH-CDNB conjugate formation was followed in a 250-μL total volume assay using a multimode microplate reader (Spectra Max M2e, USA) at 340 nm for 240 seconds. The initial rates of enzymatic reactions were determined as nanomoles of the conjugation product of GSH and reported as nmol/min/mg protein.

**Assay of aldose reductase**
AR activity was determined against the substrate, DL-Glyceraldehyde, by monitoring the oxidation of NADPH to NADP+ at 340 nm.18 In brief, the assay mixture consisting of plant extract (5 μL) solution (final concentration in the range of 7-476 ng/mL), AR (4.54 mg/mL) Li2SO4 (320 mM-400 mM), NADPH (9×10^-8 M) KP buffer (50 mM, pH 6.2), DL-GA (6×10^4 M) was prepared and used as the enzyme source to measure AR activity. NADP oxidation was followed in 0.25 mL total volume assay using a multimode microplate reader at 340 nm for 4 min. The initial rates of enzymatic reactions were determined and reported as nmol/min/mg protein.

**Assay of glutathione peroxidase**
GPx activity was measured using a previously reported method.19,20 Also, GPx activity was measured against the substrate, tertiary butyl hydro-peroxide (t-BuOOH), and the decrease in NADPH was monitored at 340 nm. GPx activity changes were measured using purified GPx (37.5×10^-4 U/mL) and plant extracts (7-476 ng/mL) or control (DMSO alone), with 2.0 mM GSH, 0.25 mM NADPH, GSH-reductase (GR, 0.5 unit/mL) and 0.3 mM t-BuOOH, in 50 mM Tris-HCl (pH=8.0). The reaction was initiated by adding GPx and the change in absorbance was recorded at 340 nm for 5 min using a multimode microplate reader.

**Assay of catalase**
CAT inhibition was determined by monitoring a red quinoneimine dye remaining H2O2.21,22 The assay was miniaturized for microplate application and contained plant extraction solutions with a final concentration in the range of 7-476 ng/mL, 50 mM phosphate buffer (pH 7.0), 20 U/mL purified bovine liver CAT, and 0.0961 mM H2O2. The reaction was stopped using NaN3 and incubated at room temperature for 5 min, followed by incubation with chromogen at room temperature for 40 min and then the absorbance was read at 520 nm. The enzyme activity was calculated with respect to the H2O2 remnant, which was determined using a calibration curve constructed in the range of 9.61-307.6 μM H2O2.

**Data analysis**
The data analysis was performed using the Graphpad Prism 6.0 software. The activity of extracts against enzyme targets was calculated as 50% inhibitory concentration (IC50) values obtained from dose-response curves. The enzyme calibration
and the dose-response curve construction were accomplished using 2-3 independent experiments, each in duplicate or triplicate using a multimode microplate reader, in 96-well microplates.

RESULTS

Each extract was prepared by dissolving 2 g of dry samples in 20 mL of methanol. The extraction yields for *D. tenuifolia* leaf samples was 13.02%, and 10.15% and 6.02% for *R. lutea* flower and leaf samples, respectively (Table 1).

The total phenolic contents of extracts were determined by using Folin-Ciocalteu’s method. Additionally, the total amount of flavonoids in extracts were determined using aluminum chloride colorimetry. According to the results, the methanol extract of *D. tenuifolia* leaves has a high amount of total phenolic and flavonoid contents. The results of total phenolic and flavonoid contents of the plant extracts of the plant samples are listed in Table 1.

The activation percent profile of GST, GPx, CAT, and AR enzymes and IC$_{50}$ values of the methanol extracts of plant samples are presented in Table 2. GST activity was determined against the substrate, CDNB, by monitoring the thioether formation at 340 nm. In order to calculate the percentage of GST activity and IC$_{50}$ values, the utilized final concentration of plant extracts in the assay were taken between 7-476 ng/mL. According to the results, which are presented in Table 2, the best activity effect was exhibited in the crude methanol extract of *D. tenuifolia* leaves with IC$_{50}$ value of 121±0.05 ng/mL.

The activity of GPx was determined as the amount of enzyme that converted 1 μM of NADPH per min in 1 mL which is expressed as U/mg of total protein. The final concentration of plant extracts within concentration range of 7-476 ng/mL were used in the assay to calculate the percentage of GPx activity and IC$_{50}$ values. The best activity profile for GPx was observed in the extract of leaves belonging to *D. tenuifolia* with an IC$_{50}$ value of 140±0.001 ng/mL.

AR activity was determined using the substrate DL-Glyceraldehyde, by monitoring the oxidation of NADPH to NADP$^+$ at 340 nm. The methanol extracts from leaves of *R. lutea* and *D. tenuifolia* showed no significant activity with AR (Table 2). In addition, none of the studied extracts showed reasonable CAT activity potential.

DISCUSSION

The aim of the present study was to evaluate the total amount of the phenolic and flavonoid contents of methanol extract obtained from the flowers and leaves of *D. tenuifolia* and *R. lutea*. Furthermore, it was aimed to determine the effects of the extract on the activity of AR, CAT, GST and GPx. Phenolic compounds have at least one or more aromatic rings with one or more hydroxyl groups attached.$^{23}$ Many phenolic compounds and flavonoids have been reported to have potential for antioxidant, anticancer, anti-atherosclerotic, antibacterial, antiviral, and anti-inflammatory activities.$^{24}$ Flavonoids are phenolic compounds found throughout the plant kingdom. They have been shown to possess a variety of biologic activities in organisms. Many flavonoids possess antimutagen, anti-proliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidant and reversal of multidrug resistance activities.$^{25-27}$ Different studies have shown that plant extracts with high polyphenol contents are known as a good source of antioxidant activity.$^{28-30}$

In this study, for the first time, it was shown that the methanol extract from leaves of *D. tenuifolia* contains a high amount of total phenolic and flavonoid compounds. The results indicated that the methanol extract from the leaves of *D. tenuifolia* had a significant effect on GST and GPx activities. Therefore, it can be said that the leaves of *D. tenuifolia* have a good effect on the antioxidant enzymatic defense system. However, it is found that the leaf extracts of *D. tenuifolia* had no effect on AR and CAT activities. It was also demonstrated that the methanol extract from leaves of *R. lutea* contained more phenolic and flavonoid

| Table 1. The percentage yield of dry products obtained from methanol extraction procedure with total polyphenol and flavonoid contents of each plant sample |
| --- |
| **Family** | **Species** | **Parts of plant** | **% yield** | **TPC mg GAE/L** | **Flavonoid mgQE/L** |
| **Brassicaceae** | *Diplotaxis tenuifolia* | Leaves | 13.02 | 144.49±0.29 | 250.48±0.002 |
| **Resedaceae** | *Reseda lutea* | Flowers | 10.15 | 109.01±0.03 | 78.72±0.03 |
| | | Leaves | 6.02 | 133.52±0.02 | 196.80±0.01 |

| Table 2. Glutathione-S-transferase, glutathione peroxidase, catalase, and aldose reductase percentage activities |
| --- |
| **Family** | **Species** | **Parts of plant** | **GST** | **GPx** | **CAT** | **AR** |
| | | | **%** | **IC$_{50}$ ng/mL** | **%** | **IC$_{50}$ ng/mL** | **%** | **IC$_{50}$ ng/mL** | **%** | **IC$_{50}$ ng/mL** |
| **Brassicaceae** | *Diplotaxis tenuifolia* | Leaves | 72 | 121±0.05 | 84 | 140±0.001 | ND | ND | 5 | 231±0.0 |
| **Resedaceae** | *Reseda lutea* | Flowers | 36 | 149±0.004 | 84 | 490±0.05 | ND | ND | ND | ND |
| | | Leaves | 70 | 403±0.015 | ND | ND | ND | ND | 20 | 601±0.002 |

ND: Not determined, GST: Glutathione-S-transferase, GPx: Glutathione peroxidase, CAT: Catalase, AR: Aldose reductase
was analyzed for active

D. tenuifolia was a good source of carotenoids, phenolics, and glucosinolate compounds. It also has antitumor activities on colorectal cancer.\textsuperscript{23} Marrelli et al.\textsuperscript{23} evaluated thirteen hydro alcoholic extracts of edible plants from Southern Italy for their \textit{in vitro} antioxidant and antiproliferative activity on breast cancer MCF-7, hepatic cancer HepG2, and colorectal cancer LoVo. They showed that the lowest antioxidant activity was exhibited by \textit{D. tenuifolia} (DT) extract. In addition, the authors reported that \textit{D. tenuifolia} extract was able to induce an inhibitory activity of cell proliferation of more than 40%.

In another study, the polyphenol content and biologic activities of the main component of \textit{D. simplex} extract was investigated. The analyzed extracts showed that flower extracts exhibited a potent \textit{in vitro} antioxidant capacity using oxygen radical absorbance capacity and displayed a strong anti-inflammatory activity and inhibited nitric oxide release. The findings suggested that the \textit{Diplotaxis} flower was a valuable source of antioxidants and anti-inflammatory agents.\textsuperscript{24} Durazzo et al.\textsuperscript{24} studied the nutritional and antioxidant properties of wild rocket [\textit{D. tenuifolia} (L.) DC.]. The authors determined the bioactive molecular content (vitamin C, quercetin, lutein) and showed bioactivity of polyphenolic extracts from the edible part of rocket in Caco-2 cells. Atta et al.\textsuperscript{25} isolated five main flavonoid glycosides from the ethanol extract of \textit{D. harra} and identified them as quercetin, isorhamnetin 3-rhamnoside, isorhamnetin 3-\textit{o-rutinoside}, isorhamnetin 3-glucosyl-4-rhamnoside and isorhamnetin 3-\textit{o-\beta-glucoside}. They also evaluated the alcoholic extract of plants against some bacterial strains that showed moderate antibacterial activity. Martínez-Sánchez et al.\textsuperscript{26} studied antioxidant compounds, flavonoids, and vitamin C, and also the antioxidant activity of four species from \textit{Brassicaceae} vegetables used for salads such as watercress (\textit{Nasturtium officinale}), mizuna (\textit{Brassica rapa}), wild rocket (\textit{D. tenuifolia}), and salad rocket (\textit{Eruca sativa}). They analyzed the characterization of phenolic compounds and they showed that the leaves of watercress, mizuna, wild rocket, and salad rocket, presented high contents of antioxidant compounds such as flavonoids and vitamin C. Therefore, they are good dietary sources of antioxidants with an important variability of bioactive compounds.

However, no pharmacologic studies have been performed with \textit{R. lutea} extracts to date, but \textit{Reseda} species have been reported to possess various pharmacologic properties such as anti-inflammatory, antioxidant, antibacterial, and antimicrobial effects. For the first time, Benmerache et al.\textsuperscript{27} isolated six flavonoids from the aerial parts of \textit{R. phyteuma}. They also found that the butanolic extract exhibited good antioxidant and antimicrobial activities. \textit{R. luteola} L. has been used as a dye due to its high luteolin content since ancient times. Woelfl et al.\textsuperscript{28} determined anti-proliferative and apoptosis-inducing effects of the \textit{R. luteola} extract RF-40. They found that it contained 40\% flavonoids, primarily luteolin, luteolin-7-\textit{O-glucoside}, and apigenin. Further, it was observed that the isolated flavonoids dose-dependently inhibited cell proliferation and induced apoptotic oligonucleosomes in PHA-stimulated peripheral blood mononuclear cells. Moreover, they showed that \textit{Reseda} extract was an interesting raw material dyeing purposes and for further pharmacologic investigation. In another study, Berrehal et al.\textsuperscript{29} investigated the methanolic and n-butanolic extracts of \textit{R. duriaeana} and \textit{R. villosa} for their antioxidant activity. The authors indicated that the methanolic and n-butanolic extracts of \textit{R. duriaeana} exhibited better antioxidant activity than the respective extracts of \textit{R. villosa}. This may be explained by the presence of more quercetin derivatives in \textit{R. duriaeana}.

From a consideration of ethnobotanical information, seeds of 45 Scottish plant species were obtained from authentic seed suppliers. The n-hexane, dichloromethane (DCM), and methanol (MeOH) extracts were assessed for free radical scavenging activity in a DPPH assay. The results showed that the methanol extract of \textit{R. lutea} seeds exhibited moderate levels of free radical scavenging activity. Also, the n-hexane extract was much less active than the MeOH and DCM extracts.\textsuperscript{29} Tawaha et al.\textsuperscript{30} determined the relative levels of antioxidant activity and the total phenolic content of aqueous and methanolic extracts of a total of 51 Jordanian plant species. They indicated that the aqueous and methanolic extracts of \textit{R. lutea} had remarkably high total phenolic contents and showed good levels of antioxidant activity.

CONCLUSION

In conclusion, the biologic potential of \textit{D. tenuifolia} and \textit{R. lutea} on the antioxidant defense system such as GST, GPx, CAT, and AR were considered in this research. It was shown that the methanol extract of \textit{D. tenuifolia} leaves had a high amount of phenolic and flavonoid compounds. Also, it is indicated that it has good activity potential on GPx and GST. These results might be related to the high content of phenolics and flavonoids found in the species. This work highlights the importance of \textit{D. tenuifolia} as a part of the daily diet.

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