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Research Article
Cellular Defense Enzyme Profile for Non-cytotoxic and Phenol Enriched Extracts of *Heliotropium europaeum*, *Carlina oligocephala* and *Echinops ritro*

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Abstract

Background and Objective: The discovery of traditional plants with some medicinal properties, verifying their biological targets and the bioassay guided standardization of their active components are the particular interest of diverse research groups recently. These efforts may help to revise the therapy modalities with natural product supplements. In this context, the possible biological targets of plants, namely *Heliotropium europaeum*, *Carlina oligocephala* and *Echinops ritro*, with no known medicinal value but recognized for their region specific traditional use, were evaluated. Here, the biological targets were enzymes of the antioxidant and xenobiotic defense mechanisms, with roles on inflammatory response. **Materials and Methods:** Cytotoxicity analyses were performed by using human promyelocytic (HL60) and chronic myelogenous leukemia (K562) cells for circulating models and breast adenocarcinoma (MCF7) cells for epithelial model to evaluate the non-toxic dose range of extracts by virtue of XTT and trypan blue. The target aimed effectiveness of these plants were determined with dose response profiles and IC_{50} values against glutathione peroxidase (GPX), glutathione transferase (GST), catalase (CAT) and superoxide dismutase (SOD) targets, as well as their capacity to reduce free radicals (DPPH) and non-radical hydrogen peroxide (H_2O_2). For standardization purposes, all extract concentrations were brought to 23.81 mg L^{-1} GAE and dilutions were made from these stocks. The IC_{50} values were determined by nonlinear regression analysis, with sigmoidal dose-response 4-parameter logistic equation. **Results:** The results showed that the best DPPH and H_2O_2 scavenging was observed with *E. ritro* extracts. On the contrary, the best enzyme inhibition profile was observed with *H. europaeum* against CAT, SOD, GPX and GST targets. Among the enzymes evaluated, all plants with different fractions also exerted strong GPX and CAT inhibition. **Conclusion:** The enzyme profiling of extracts may reveal the medicinal value of herbal remedies, by identifying their effects on cellular targets. In addition to define how reasonable the use of plants in traditional and complementary medicine (TCM) practices, these efforts may help to improve the standardized supplement preparations to benefit therapeutics with reduced efficiency due to inference with cellular defense and drug resistance enzymes, or both.

Key words: Glutathione transferase, glutathione peroxidase, catalase, superoxide dismutase, cytosolic defense, anti-inflammatory effect

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The world's most commonly known ancient pharmacopeia are based on the well-documented natural plant product recipes of Native Americans, Sumerian, Egyptian, Chinese, Middle Eastern (Mesopotamia) and Indian documents[1-4]. As reviewed recently, the herbal supplements (nutraceuticals) may provide health benefits with proper use[5]. In this context, herbal treatments, as part of traditional and complementary medicine (TCM), are highly recognized practice for primary health care in both developing and developed countries. According to current reports, 20% of the world population rely on herbs, herbal preparations or finished products with standardized herbal materials as plant-based TCM practices[3,6,7].

Moreover, most of the herbal remedies are mainly accepted as antioxidant sources due to their phenolic and flavonoid contents, which are shown to scavenge the radical and non-radical oxygen bearing molecules, known as reactive oxygen species (ROS). Nitrosyl ($NO^{\bullet}$), hydroxyl ($HO^{\bullet}$), hydro peroxide ($HOO^{\bullet}$), alkyl oxide ($RO^{\bullet}$) and alkyl peroxide ($ROO^{\bullet}$) radicals, as well as unstable hydrogen peroxide (H_2O_2) and so forth are commonly known as ROS species. They are mostly the cellular products of proteins, lipids, lipoproteins and nucleic acids. Besides the cellular protection against ROS, known as antioxidant effect, plant polyphenols were also reported to induce toxic damage to cells throughout different mechanisms, the so called pro-oxidant effect[8-10]. Therefore, in relation to their phenolic contents, it is not surprising to evaluate the role of plant extracts on cellular protection mechanism, especially considering that the well-established polyphenols still receive great interest in antioxidant research field[8,11,12].

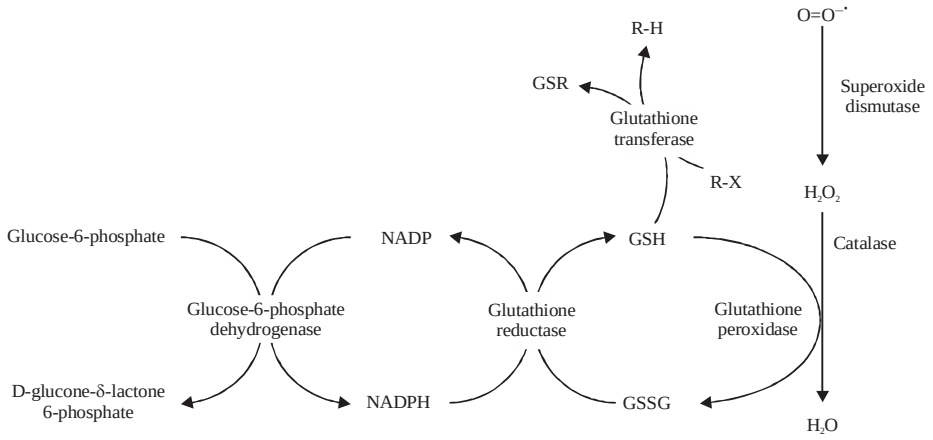
The antioxidant role of the plants may be chemically evaluated through 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay for ROS scavenging capacity and hydrogen peroxide utilization assay for scavenging capacity of unstable hydrogen peroxide (H_2O_2). However, based on recent and growing substantial amount of experimental evidence, the results of these analyses may not be clearly associated with the enzymatic antioxidant role in a biological medium, considering the plant phenols may either induce antioxidant or pro-oxidant effects in relation with enzymes responsible in cellular defense[4,13,14].

In this context, glutathione-s-transferases (GSTs) as major phase II detoxification enzymes, appear to be a good candidate to evaluate medicinal value of the traditional plants. This is because GSTs participate in detoxifying the toxic agents of both internal and external sources by catalyzing the nucleophilic addition of glutathione (GSH) to diverse molecules. This detoxification may occur by interfering with drug transport into the nucleus[15-19], by reducing oxidative stress and free radical related cytotoxicity and by employing peroxidase activity[18]. Another GSH utilizing enzyme with peroxidase activity is glutathione peroxidase (GPX), which reduces the organic hydroperoxides to corresponding alcohols and free hydrogen peroxide to water, while converting GSH excessively to glutathione disulfide (reduced GSH, GS-SG). It may function in either the oxidative or anti-oxidative response of the cell, in such a way that its varying activity levels may protect cells from oxidative damage related to apoptosis on one side and may cause oxidative DNA damage on the other side[20].

Although, they function in different cellular compartments, independent from GPX, catalase (CAT) also has a function in peroxide removal by catalyzing degradation of peroxide to water and oxygen. The change in cellular levels and activity are attributed to various disease pathologies, as seen with GPX and GST. Similarly, CAT is also related to cancer development and associated with drug resistance, with activity alterations depending on disease advancement[21]. Another antioxidant defense enzyme is superoxide dismutase (SOD). It detoxifies the superoxide anion which is an endogenous product and highly toxic to cells upon accumulation. The status of cellular oxidation is maintained with the catalysis of superoxide to hydrogen peroxide by SOD, by decreasing its reactivity. Under normal conditions, the balance between both the activity and the intracellular level of SOD, GPX and CAT has been defined for the survival and healthy functionality of cells[11].

Hence, the screening of extracts through complementary involved enzymes in detoxification and cellular antioxidant defense systems, namely GST, GPX, CAT and SOD, should be considered to analyze the possible biochemical function (scheme 1) of extracts[4,13,22].

The current interest was to understand the biochemical properties of *Heliotropium europaeum, Carlina oligocephala* and *Echinops ritro*, plants consumed as herbal remedies in Eastern Europe and used to reduce inflammatory conditions[23-25]. Of these, *H. europaeum*, as a member of Boraginaceae family, is a well-known traditional plant and has been used in soothing conditions such as warts, insect bites and inflammation of the joints. Moreover, it is also reported as a food supply ingredient for goats. However, it is known as a common poison for cattle and sheep, if fodder or forage is contaminated with this plant, the result is the paralysis of the



Scheme 1: Cytosolic enzymes employed in controlling the oxidative status of cell

animals. On the other hand, the plant is known with dose dependent hepatotoxic effects for humans, upon oral uptake[26]. As a member of the Asteraceae family, *C. oligocephala* is generally used as a natural dye source for yellow coloring in Anatolia. As herbal remedy, it is widely used as diuretic and stomach pain reliever, in addition to relieving inflammation symptoms of eczema, hemorrhoids and acne[27]. Another member of the Asteraceae family is *E. ritro*, a garden plant, also known as a herbal remedy in Eastern Europe. The plant roots are locally known to promote lactation in nursing woman, soothe the breast abscesses and pain and so known as anti-inflammatory for breast tissue. Some of these were found toxic for human consumption and livestock feeding, which was attributed to region specific levels of pyrrolizidine content and the method for extraction of plant[28,29]. As of current literature surveyed, the mechanisms of action by these plants are not clarified so far, there is no study employed to evaluate antioxidant profiling of those to date, but still they are consumed where the plants are part of TCM preparations or traditional herbal remedies. Therefore, in this study, the extracts prepared from locally collected plants were evaluated for their antioxidant effects on cytosolic enzyme targets. These targets have a function on the antioxidant and xenobiotic defense mechanisms, as well as on inflammatory response, with importance in chemotherapeutic resistance in cancer treatment and also in disease development upon accumulation of toxic chemicals in cells.

MATERIALS AND METHODS

This study was conducted at Atilim University Biochemistry Research Laboratory and all facilities available at the Department of Chemical Engineering and Applied Chemistry, Atilim University, Ankara, Turkey.

Chemicals: The 4-Aminoantipyrine (PubChem CID: 2151), was purchased from Acros, USA. Catalase (CAT), 3,5-Dichloro-2-hydroxybenzenesulfonic acid (DHBS), folin-ciocalteu reagent, horseradish peroxidase (HRP), nitro blue tetrazolium (NBT), 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) and other cell culture reagents were supplied from Sigma Aldrich, Germany. Superoxide dismutase (SOD) was purchased from Gerbu, Germany. 1,1-Diphenyl-2-picryl hydrazyl (DPPH, PubChem CID: 2735032) as free radical form, glutathione reductase (GR), xanthine and xanthine oxidase (XOD) was obtained from Calbiochem, Germany. All other chemicals were either molecular biology or analytical grade.

Cell lines: Cell lines HL60 (human promyelocytic leukemia cell, ATCC-CCL-240), K562 (human chronic myelogenous leukemia, ATCC-CCL-243) and MCF7 (human breast adenocarcinoma, ATCC-HTB-22) were previously received as a gift from the Biotechnology Center (Ankara University, Ankara, Turkey) and the Biology Department (Middle East Technical University, Ankara, Turkey) as frozen stocks. Cell lines were cultured and maintained in complete medium (RPMI 1640), supplemented with L-glutamine (2 mM), 5% FBS, streptomycin (100 mg mL^{-1}), penicillin (100 U mL^{-1}), at 37°C in a humidified atmosphere at 37°C and 5% CO_2.

Plant samples and preparation of plant extracts: *Heliotropium europaeum* was collected from the Elvan-Pecenek region, in August 2009, *Echinops ritro* and *Carlina oligocephala* were both collected from the Zir Valley, in July, 2009, Ankara, Turkey. The collected plants were dried in the shade at room temperature, for 15 days. Plants were authenticated by Assoc. Prof. Dr. Fatmagul Geven, Department

of Botany, Ankara University, Ankara, Turkey. Voucher specimens of *H. europaeum*, *E. ritro* and *C. oligocephala* (FG2010-20, FG2010-17 and FG2010-19, respectively) were deposited at the Herbarium, Department of Botany, Ankara University, Ankara, Turkey.

To prepare the plant extracts, first the leaves and flowers of the plants were separated, washed with tap water and then, dried fractions were ground mechanically in liquid nitrogen. The extraction of ground fractions was performed with 10 mL of methanol (99.9%) per gram of ground plant material, for 24 h at 4°C. After the solvent was removed by a rotary evaporator in vacuo (40°C, 337 mbar), the yielded product was weighed and the extraction yield was calculated. The obtained product was dissolved in DMSO for stable storage of extracts at -20°C, unless otherwise stated.

Preparation of bovine liver cytosol: The bovine liver cytosol, or simply the cytosol, was prepared from fresh liver samples from cattle, as described previously[15,16]. Briefly, the liver samples, weighing 20-25 g, were homogenized in 10 mM potassium phosphate buffer (pH 7.00), supplemented with 15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiothreitol (DTT) and centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through cheese cloth and the filtrate was centrifuged at 134,000 rpm for 50 min. The collected supernatants were filtered again and the resultant filtrate was referred to as cytosol. The total protein content was determined by the Lowry method[30].

Preparation of standards, controls and extract dilutions for assays: As standards, ascorbic acid was prepared in distilled water, quercetin in 80% ethanol and gallic acid in DMSO, each from 1 g L^{-1} stock, to afford calibration curves within the final concentration ranges of 0.2-15 mg L^{-1} for ascorbic acid and quercetin and 0.012-1.8 mg L^{-1} for gallic acid. The standard calibration curves prepared with ascorbic acid, quercetin and gallic acid were used to determine the amounts of total phenolic and flavonoid compounds present in extracts. The mother plate of plant extracts, which is the microplate that contains the highest possible sample concentrations, were prepared by dissolving these extracts in the minimum amount of DMSO that completely solubilizes the material. From these plates, the daughter plates of extracts were prepared, where all samples were further diluted with DMSO to the final phenolic concentration of 23.81 mg L^{-1} and stored at -20°C until used in biological assays. Bovine serum albumin (BSA) was prepared in water within the range of 0.10-0.75 g L^{-1} and used to construct a standard calibration curve to determine the protein content by the Lowry Method[30] whenever it is required.

In enzyme assays, the essential experimental controls used to build dose-response curves were negative, vehicle (solvent) or positive controls. Negative control was the suitable buffer, or water that replaces the substrate or enzyme, the vehicle control was the solvent such as DMSO or ethanol, which replaces either extract or standard inhibitor and the positive control, was the standard inhibitor that replaces the extract used in assays. The available standard inhibitors used in assays were ethacrynic acid (EA, 0.1 mM) for GST, sodium azide (NaN_3, 15 Mm) for CAT and glutathione peroxidase (GPX) and copper chloride ($CuCl_2$, 300 Mm) for SOD enzymes.

Methods: Methods were based on chemical, cellular and acellular *in vitro* analysis of extracts to determine the non-cytotoxic dose ranges, the phenolic and flavonoid contents of extracts, the free radical and non-radical peroxide scavenging capacity and the antioxidant potential of extracts via enzymatic assays as explained in following subsections.

Determination of the total phenolics and flavonoids of extracts: Total phenolic contents (TPC) of extracts were measured using the previously described methods[31,32] with slight modifications. Briefly, 0.1 mL of each plant extract with three different dilutions was mixed with 10 fold dilute folin-ciocalteu reagent and incubated for 5 min. Then, 2% Na_2CO_3 (w/v) was added to this mixture and further incubated for 1 h. The samples were transferred to microplates which were scanned at 750 nm for endpoint readings. All incubation steps were accomplished in the dark and at room temperature and all measurements were done in triplicates. By using the gallic acid calibration curve, the phenolic content was expressed as milligram of gallic acid (GAE) per gram of sample.

Total flavonoids content (TFC) was determined by using a method modified by Woisky and Salatino[33]. Briefly, 0.5 mL of plant extracts (sample) was mixed with ethyl alcohol, 10%(w/v), aluminium chloride $AlCl_3$, 1 M sodium acetate and DMSO and this mixture was incubated at room temperature for 30 min in the dark. The samples were transferred to microplates which were scanned at 415 nm against ethyl alcohol for endpoint readings and all measurements were done in triplicates. By using the quercetin calibration curve, the flavonoid content of the samples was expressed as milligram of quercetin per gram of sample.

Cytotoxicity analysis of extracts: Cytotoxicity analyses were performed by using the human cell lines HL60, K562 for circulating model cells and MCF7 for epithelial model cells to evaluate the non-toxic dose range of extracts by virtue of XTT analysis[34]. The growth inhibition of cells was determined by XTT assay and viability by trypan blue dye exclusion methods. The cytotoxicity was determined as growth inhibition exerted by varying concentrations of extracts and reported as the concentration required to induce 50% growth inhibition (GI_{50}) of cells. The method employed was suggested by the assay kit manufacturer (Sigma, Germany). Each analysis was performed twice where each dose was analyzed in triplicates in 6 and 96 well plates for trypan blue and XTT analysis, respectively.

Chemical methods to evaluate radical scavenging activity

DPPH and H_2O_2 scavenging assays: The radical scavenging activity was measured as the inhibition of 1,1-Diphenyl-2-picrylhydrazyl (DPPH free radical) using the previously published method[35] with some modifications to adopt for microplate applications. In the assay, the plant extracts and 50 µM DPPH in methanol, mixed thoroughly in a final volume of 0.2 mL and incubated for 25min at room temperature, in the dark. Then the microplates were scanned at 517 nm. The DPPH radical scavenging activity of each sample was expressed as percentage inhibition of free DPPH radical and IC_{50} values were calculated from the dose-response inhibition curves, where ascorbic acid, quercetin and gallic acid were employed as positive controls, as explained under the following section.

The H_2O_2 scavenging activity was also determined with the use of the previously published method[36] after optimized for micro-scale application. The assay mixture containing 16 mM of phosphate buffer (pH 7.4), 2 mm H_2O_2 (in methanol) and plant extract with varying concentrations within the range of 0.07-2.5 mg L^{-1} incubated at room temperature in the dark for 10 min. The microplates were then scanned at 230 nm and the remaining H_2O_2 amount in the assay medium was determined using a standard curve. The H_2O_2 scavenging activity of each sample was expressed as percentage inhibition of H_2O_2 and IC_{50} values were calculated from the dose-response inhibition curves.

Enzyme assays and data analysis: The inhibitory profiles of extracts were determined by analyzing the data obtained in the presence of extracts and inhibitors or suitable assay controls. The enzyme calibration and the dose response curves of all assays were constructed using two independent experiments in 96 well microplates, each in triplicates, using SpectraMax M2e, Multi-Mode Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The inhibitory activity of each extract was calculated as an IC_{50} value, which is the concentration that exerts 50% inhibition on target enzyme or compound activity. The IC_{50} values were determined from dose-response curves by nonlinear regression analysis. The analysis method used was sigmoidal dose-response 4-parameter logistic equation, via GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA[37]. The inhibitory profiles of extracts were presented as dose-response curves, where, *H. europaeum*, *C. oligocephala* and *E. ritro*, were represented as either short notations (HE, CO and ER) or marks on the curves such as (◊), (♦) and (o), respectively. Also F and L were used as the short notation of flower and leaf extracts, whenever it was required.

Glutathione-S-transferase (GST) assay: The method of Habig and Jakoby[38] was used to perform activity measurements after it was slightly modified and adopted for micro-scale applications, as reported previously[16]. The measurements were performed at 340 nm where the assay mixture contains cytosol (928 mg protein L^{-1}) and varying concentrations of extracts, in 100 mM potassium phosphate buffer (pH 6.5) supplied with 2.4 mM CDNB and 3.2 mM GSH.

Glutathione peroxidase (GPX) assay: The GPX activity was measured as previously reported[39] where the assay mixture contains GPX (37.5 U L^{-1}), varying concentrations of extracts, 2 mM GSH, 0.25 mM NADPH, 0.5 unit mL^{-1} GR and 0.3 mM t-BuOOH, in 50 mM Tris (pH 8.0). The GPX activity was monitored by a decrease in NADPH at 340 nm.

RESULTS

Catalase (CAT) assay: The CAT activity was measured by the previously described protocol[40] after it was adopted for micro-scale applications. In this miniaturized version, the assay mixture contains 20 unit mL^{-1} CAT and 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0). NaN_3 was used to terminate the reaction. After the addition of chromogen solution (0.25 mM 4-AP and 2 mM DHBS supplemented with 0.450 U mL^{-1} HRP) to the assay mixture, the change in CAT activity was monitored at 520 nm.

Table 1: Phenolic and flavonoid content of plant extracts

Plant	Extraction yield (%)	TPC (mg L^{-1})	TFC (mg L^{-1})
Flower			
H. europaeum	9.575±0.016	65.241±0.015	61.002±0.018
C. oligocephala	7.210±0.012	64.873±0.019	74.291±0.017
E. ritro	4.761±0.023	113.042±0.18	107.362±0.031
Leaf			
H. europaeum	8.561±0.009	110.480±0.007	169.210±0.005
C. oligocephala	6.0921±0.035	109.740±0.023	117.062±0.015
E. ritro	8.871±0.003	204.806±0.013	233.113±0.010

*All procedures repeated twice, each in duplicates. Results presented as Mean±SEM

Superoxide dismutase (SOD) assay: The SOD activity was measured by the miniaturized version of the previously described protocol[39], using bovine liver cytosol as enzyme source (928 mg protein L^{-1}), in the presence of 0.2 mM xanthine, 0.05 U mL^{-1} XOD, 0.3 mM NBT in 200 mM sodium carbonate buffer (pH 10.1) supplemented with 0.5 mM EDTA. The change in SOD activity was monitored at 550 nm.

The best extraction method that yields the highest possible polyphenol content of plants was found with methanol at a mild temperature. The methanol extraction of *H. europaeum*, *C. oligocephala* and *E. ritro* were performed within the range of 4-10% yield for the flower and 6-9% yield for the leaf parts (Table 1). Since polyphenols are abundant plant secondary metabolites, the extract concentrations were determined both in terms of phenolic and flavonoid contents.

The highest phenolic and flavonoid contents were found for both fractions of *E. ritro* (Table 1). The final phenolic concentration of extracts was brought to 23.81 mg L^{-1} by further dilutions with DMSO, which is required for standard and extract preparations to be used for both the chemical and biological antioxidant evaluations.

Cytotoxicity analysis was performed with use of XTT assay. The cell viability was determined with basic morphology observed, with use of trypan blue dye exclusion method. Results showed that the final concentration of extracts in the culture medium within the range of 0.001-1 mg L^{-1} have no significant effect on cell viability (Fig. 1), as confirmed with trypan blue dye exclusion analysis. Here, the cell viability was determined as 75-90% for HL60, K562 and MCF7 for 24 h of incubation. Since no growth inhibition below 70% was determined, no IC_{50} or GI_{50} values were available for this assay.

The radical scavenging capacity of the extracts measured as a percentage inhibition of stable DPPH radicals shown in Fig. 2a, where the dark solid line shows the gallic acid standard calibration curve. The IC_{50} values were determined as 0.901, 0.359 and 0.0982 mg L^{-1} for flowers, 2.471, 0.426 and 0.224 mg L^{-1} for leaves of *H. europaeum*, *C. oligocephala* and *E. ritro* (Fig. 2a). Moreover, for gallic acid, ascorbic acid and quercetin standards, the IC_{50} values of DPPH scavenging capacity were determined as 0.2526 mg L^{-1} (14.85 mM), 5.218 g L^{-1} (29.61 mM) and 1.678 g L^{-1} (5.55 mM), respectively. In addition, the unstable hydrogen peroxide scavenging assay was revealed very close scavenging capacity for all extracts (Fig. 2b), where the IC_{50} values were 0.524, 0.604 and 0.563 mg L^{-1} for flowers, 0.538, 0.485 and 0.478 mg L^{-1} for leaves of *H. europaeum*, *C. oligocephala* and *E. ritro*, respectively.

The H_2O_2 and DPPH scavenging profile (% inhibition) for the flower (F) and leaf (L) extracts of plants and the order of curves aligned from top to bottom is: HE (F), HE (L), CO (F), CO (L), ER (F) and ER (L) (Fig. 2a-c).

In terms of the effects on cytosolic enzymes, only flower and leaf fractions of *H. europaeum* induced the reasonable GST inhibition with IC_{50} values of 0.395 mg L^{-1} (Fig. 3a) and 0.0809 mg L^{-1} (Fig. 3b), respectively. *Carlina oligocephala* and *E. ritro* leaf extracts showed mild inhibition of GST. Since the overall inhibitory activity was less than 50% for both leaf and flower extracts, the yielding IC_{50} values were only estimated for both (Fig. 3b). For *E. ritro*, flower extracts showed no effect on GST enzyme activity.

Inhibition of GPX, which is the enzyme both utilizing glutathione and neutralizing peroxides was strongly exerted by the flower of *H. europaeum* with IC_{50} value of 0.0352 mg L^{-1} (Fig. 4a). Whereas, the limited inhibition profiles of *C. oligocephala* and *E. ritro* flower were observed with estimated IC_{50} values of 0.0481 and 0.246 mg L^{-1}, respectively. The leaf fractions of all plants under study showed strong inhibition of GPx (Fig. 4b) with IC_{50} values determined as 0.0245, 0.0455 and 0.0545 mg L^{-1} (Fig. 6), for *H. europaeum*, *C. oligocephala* and *E. ritro*, respectively.

The CAT, another peroxide neutralizing enzyme, was strongly inhibited by the flower extract of *H. europaeum* and *C. oligocephala* (Fig. 5a) with IC_{50} values of 0.00611 and 0.00630 mg L^{-1}, respectively. For leaves (Fig. 5b), with the same order, the IC_{50} values were 0.1273, 0.0633 and 0.0129 mg L^{-1}.

None of the leaf but the flower extracts of the plants showed experimentally produced peroxide radical neutralizing capacity, as determined with inhibition of SOD activity (Fig. 6). Here, *H. europaeum* and *E. ritro* flower extracts exerted strong SOD inhibition with IC_{50} values of 0.2332 and 0.2965 mg L^{-1}, respectively. For *C. oligocephala* flowers, only estimated IC_{50} of 1.232 mg L^{-1} was determined, revealing no actual inhibition.

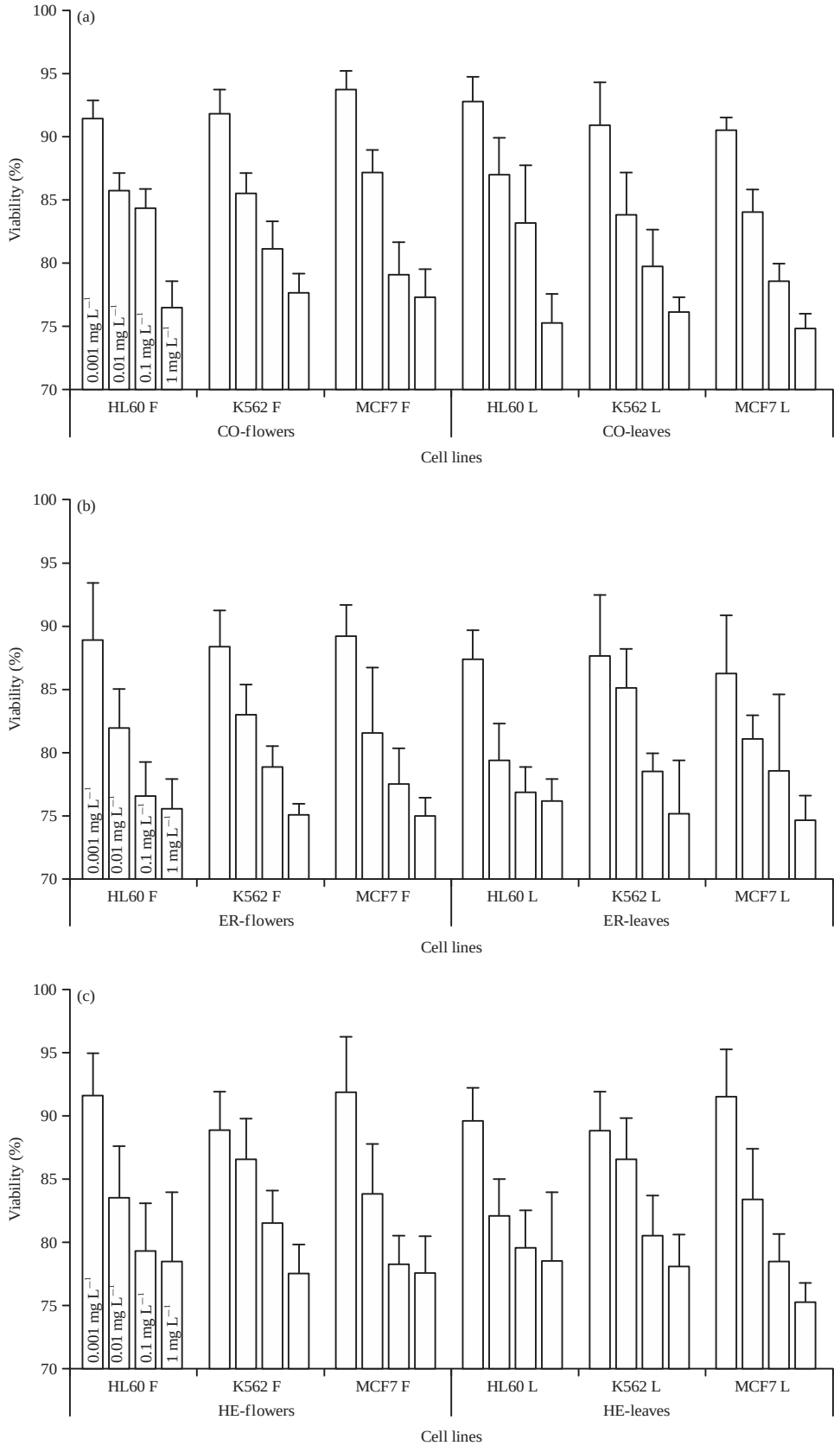


Fig. 1(a-c): Cell viability analysis of extracts with HL60, K562 and MCF7 cell lines

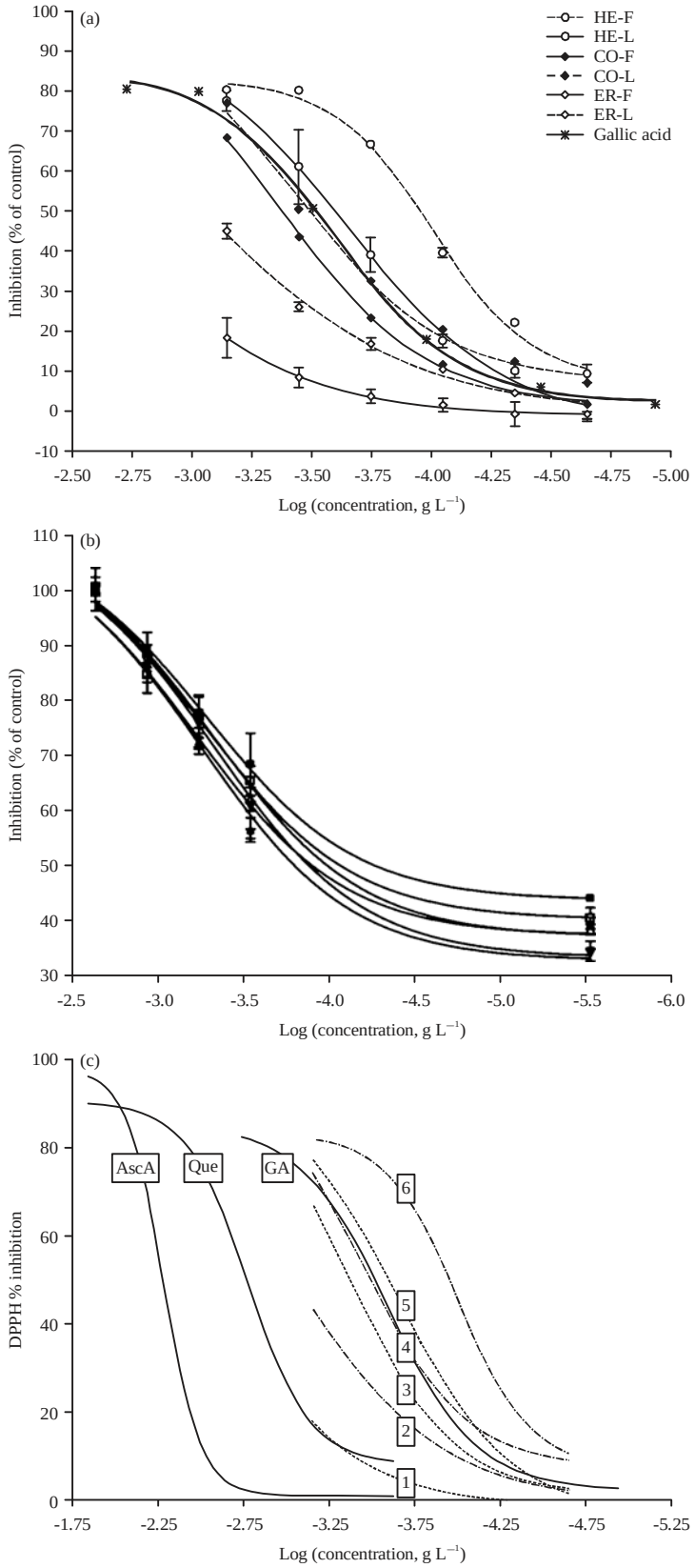


Fig. 2(a-c): (a) DPPH inhibition profile for the flower and leaf extracts, (b) H_2O_2 scavenging profile (% inhibition) for the extracts of plants and (c) DPPH inhibition profile of extract in comparison with ascorbic acid (AscA) and Quercetin (Que) curves

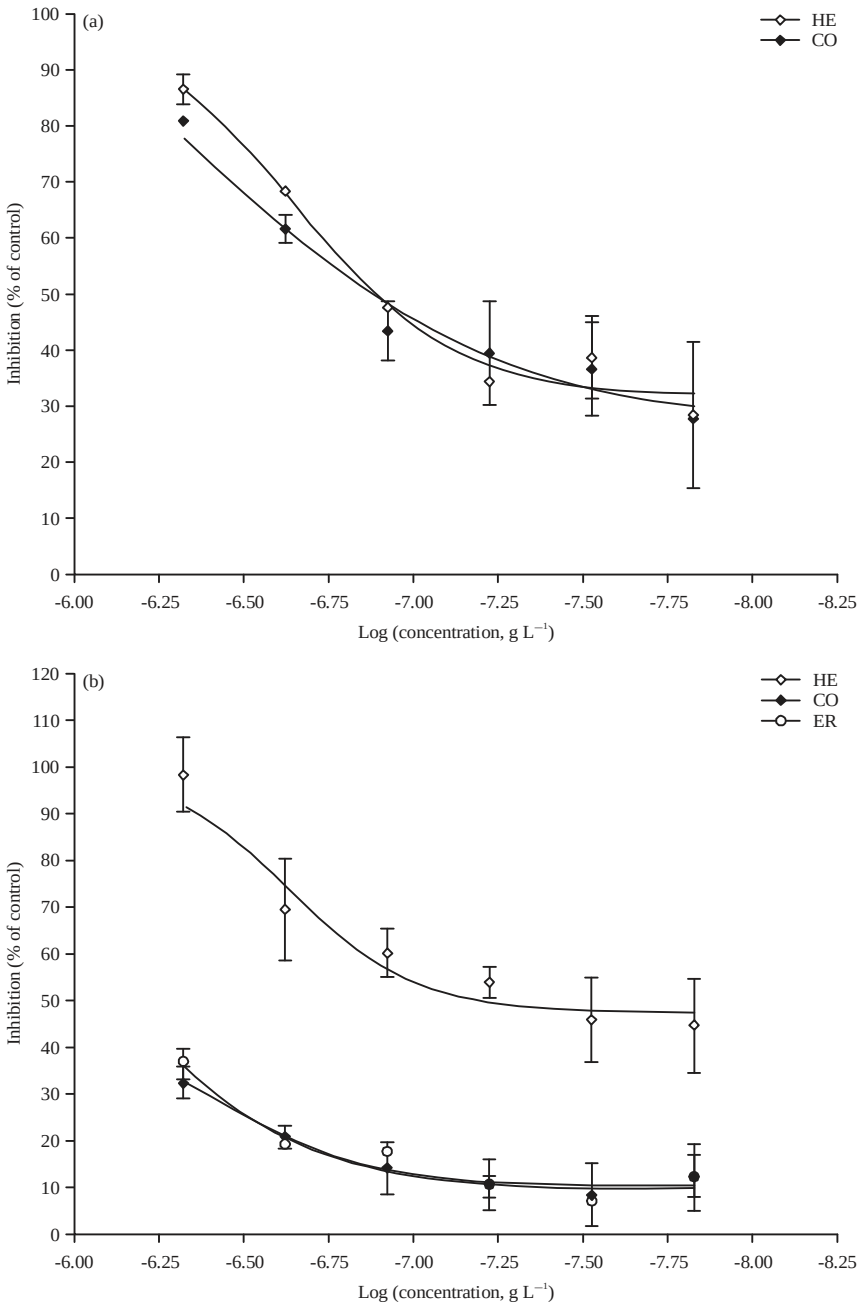


Fig. 3(a-b): (a) GST inhibition exerted by the flower extracts and (b) GST inhibition exerted by the leaf extracts

DISCUSSION

In vitro and *in vivo* studies have shown that plants may provide health benefits with proper use by employing activities such as anti-inflammatory, hepatoprotective, antiviral and anti-cancer. Aromatic plants such as herbs and spices are especially rich in phenolic content and have been widely used in traditional medicine as treatment for many diseases[1,3,6].

These benefits are generally attributed to the antioxidant capacities of the polyphenol content of plants and, hence, found valuable for human use. In this context, it should be recalled that human defense against oxidative stress is functioning by two means: Through non-enzymatic biomolecules and catalytic reactions mediated by enzymes. The former is known for chemical characteristics of molecules to stabilize radical species and unstable peroxide molecules, whereas, the latter is a more sophisticated system, in

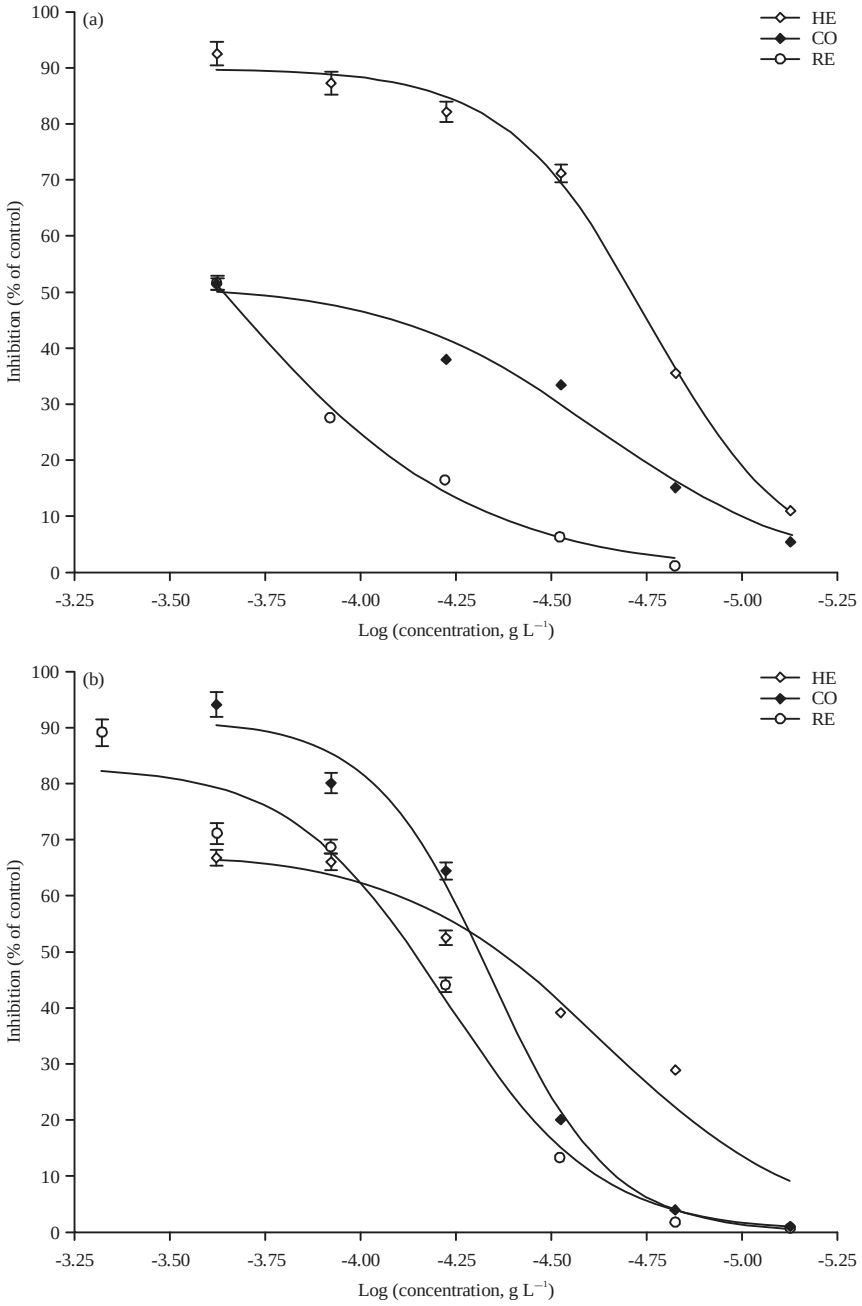


Fig. 4(a-b): (a) GPX inhibition profile for the flower extracts and (b) GPX inhibition profile for the leaf extracts

which enzymes are evolved to catalyze conversion of certain substrates which induce oxidative stress[4,11,22]. Generally, the antioxidant property of plant polyphenols are determined through chemical methods. Among those, stable DPPH free radical is a common method to define the radical scavenging capacity of molecules, both with synthetic and natural origin. As the method depicts, the highest scavenging capacity is reported with the lowest IC_{50} value. In the current study, when DPPH scavenging capacities of plants were compared with respect to IC_{50} values, the highest capacity was observed for both leaf and flower extracts of *E. ritro*. Similar to DPPH scavenging, the best hydrogen peroxide scavenging was observed by *E. ritro* followed by *C. oligocephala* and *H. europaeum* with slightly varying IC_{50} values. Moreover, the extracts of plants showed the scavenging capacities higher than that of the standards, due to the presence of highly complicated ROS scavenging compounds present in extracts. Such behavior is found consistent with other plant extracts analyzed before and attributed in the presence of several polyphenol compounds compared to one isolated phenol or

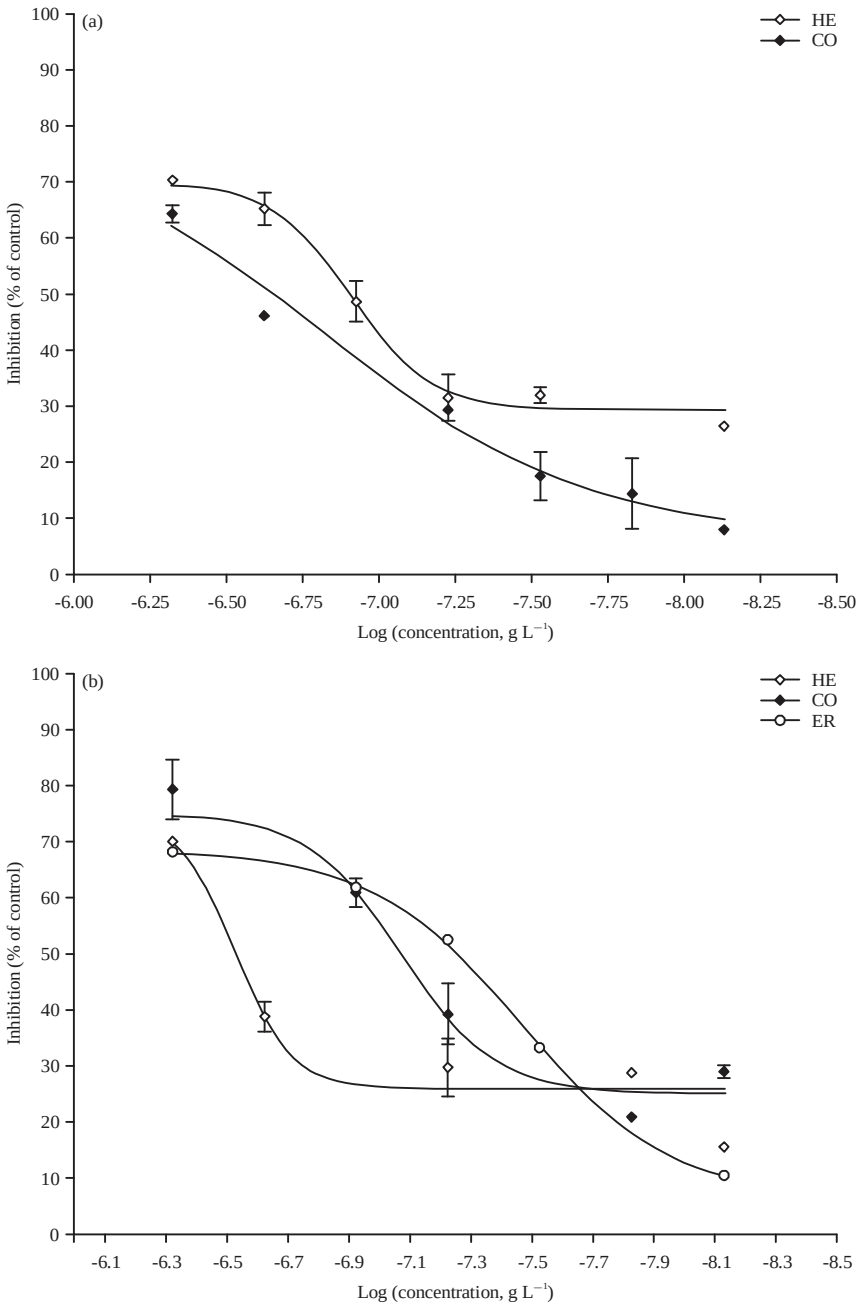


Fig. 5(a-b): (a) CAT inhibition exerted by the flower extracts and (b) CAT inhibition profile by the leaf extracts

flavonoid used as a standard[41,42]. However, the recent studies speculated that some plant species with higher antioxidant activities, measured by means of chemical methods, may also exert oxidative effects at the cellular level[8-10]. These controversial results have shown that the chemical methods employed to measure the antioxidant potential of plant polyphenols may not reflect the actual effect of plant extracts on biological targets[43]. This is attributed to the complex nature of polyphenols which possibly have complex interactions with other biomolecules inside the cell, such as proteins, coenzymes and enzymes, as reviewed before[13,14,22,44]. Moreover, the enzymes engaged in detoxification of reactive oxygen species, such as GPX, CAT and SOD, should be considered as biological targets of the polyphenols in extracts to evaluate their role in cytosolic cellular defense in relation to their antioxidant capacity. In addition to those, as a secondary way of defense, GR and G6PD are found functional to indirectly reduce cellular oxidation status. Obviously, neither

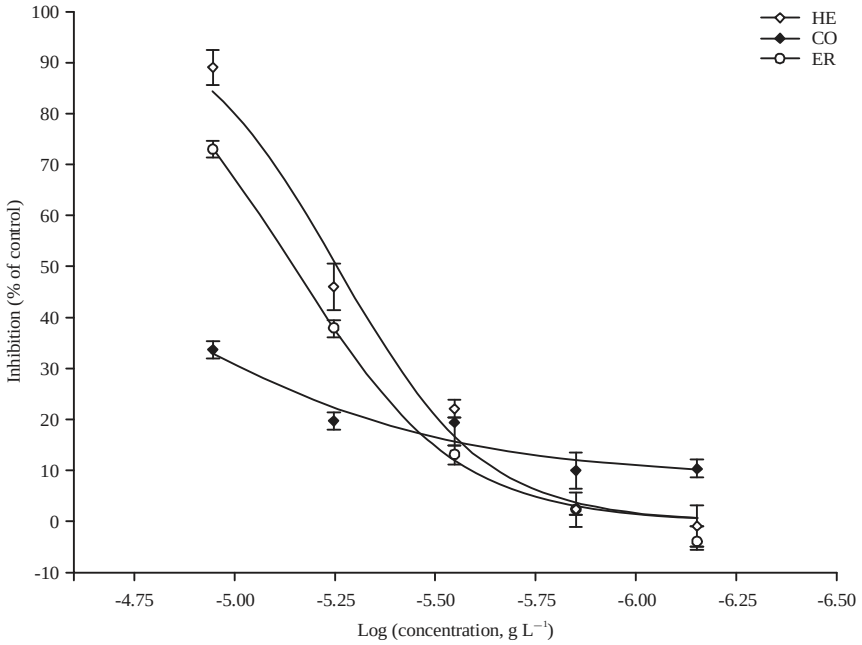


Fig. 6: SOD inhibition exerted by the flower extracts

GR nor G6PD have capacity to utilize peroxides or free radical, instead, the former is employed in GSH homeostasis and the latter is in regulating NADPH levels. In this context, GST as a phase II enzyme (Scheme 1) may be considered as a better target for cellular secondary defense, due to its peroxidase and GSH utilization functions[18,19]. Besides, GST can utilize secondary plant metabolites with highly oxidative chemical structure[45]. Although some clinical contradictions due to the limitations in experimental plan, still GST and G6PD are directly and positively correlated with each other in most of the cases[46]. Therefore, it is most beneficial to consider GST rather than G6PD for plant secondary product utilization.

Although, the inhibition of antioxidant defense enzymes by polyphenol rich extracts may appear as controversial, *in vitro* assay conditions depict the behavior of the molecule or extract under study. In this approach, to standardize the assay, the enzyme activity screens were designed to mimic oxidative stress conditions, under which the enzymes display maximum activity and it is almost 70-80% of overall enzyme activity for screening purposes[37]. If the plant extracts contribute to oxidative status of the assay medium, then it is expected to observe no change, very low inhibition or slight increase in enzyme activity.

In contrast, if extract content contribute to reduce the oxidative status of the assay medium, then the decrease in enzyme activity was expected. Moreover, there is no clear statement in current literature regarding the direct effect of phenolic constituents of these plant extracts on antioxidant and cytosolic defense enzymes. Keeping these in mind, the leaves of *E. ritro* appear to be a strong ROS scavenger according to the DPPH scavenging capacity, but exerting a mild antioxidant effect with respect to GPX assay. Beside the DPPH scavenging activity of flower extracts (0.0982 mg L^{-1}) was higher than leaf extracts (0.224 mg L^{-1}), the flower extract did not exhibit GPX, GST and CAT, but SOD inhibition. Moreover, *H. europaeum* with the lowest DPPH scavenging capacity, showed the best inhibitory profile of antioxidant enzymes. For SOD, no inhibition by leaves was observed, but inhibition by flowers was. For *C. oligocephala* flower and leaf extracts, although a good DPPH scavenging capacity was observed, both fractions only exerted mild GST and CAT inhibitions, but no SOD inhibition. For GPX, only leaf extract showed inhibition. Therefore, the chemically determined antioxidant capacity of extracts does not reflect their effect on antioxidant thiol metabolizing enzymes.

Recently employed research methods are, therefore, more focused on bio-assay guided analysis of plant sources, as extracts or partially purified fractions. It should be performed to decide whether the isolation of most active components are feasible or not and if the product should be provided as extracts or standardized finished products, for human use. Therefore, the discovery of traditional plants with less known medicinal properties, verifying their effects on various biological targets and hence, the conservation of plant species

and sources, are of particular interest to many scientists[47]. In this concept, among all plant fractions analyzed, *H. europaeum*, which has been used topically to relieve the symptoms of warts, insect bites and swollen joints due to inflammation, appears to have strong antioxidant effects. Due to cultured region or the natural habitat conditions, oral consumption may have limitations. However, affordable extracts should be tested further on disease models, to provide benefit for inflammatory conditions, as well as for the use of therapeutics with reduced effectiveness caused by overactive enzymes, such as GST. Compared to *H. europaeum*, *C. oligocephala* may not beneficial for GST overactive situations. However, its strong GPX inhibition makes it a good candidate for anti-inflammatory use by both orally and topically. Considering most of the joint inflammations require highly toxic cancer therapeutics for treatments, *C. oligocephala* extracts may be a good supplement, after optimizing the best product for use in clinics. *E. ritro* oral consumption may be limited due to well known dose dependent hepatotoxicity issues. In addition to roots[28,29] which are generally consumed for soothing anti-inflammatory conditions, here, the leaf extracts also appear to induce a good anti-inflammatory effect. However, similar to *C. oligocephala*, it does not appear to be beneficial for use with therapeutics interfered with overactive GST cases.

CONCLUSION AND FUTURE RECOMMENDATIONS

The overall results revealed that the best enzyme inhibition profile was observed for *H. europaeum*. In contrast, the best DPPH scavenging was observed by *E. ritro*. These results are found to be consistent with both the literature and the hypothesis proposed with the current study that chemical methods are inadequate to evaluate the medicinal value and the biological antioxidant capacity of natural products[13,14]. Moreover, this study showed that traditional plant use may have scientific reasons to induce a therapeutic effect due to biological targets of extract components available in metabolism. Furthermore, biological target screening may help to improve the preparation of therapeutic or dietary supplements. Knowing the possible metabolic targets shared by therapeutics and dietary supplements may help to improve the benefits of therapy. If the supplement activates an enzyme that interferes with drug action, this may reduce the therapeutic benefit, but the opposite may enhance it. Similarly, if the expected therapeutic outcome is the induction of cellular oxidation, therefore, use of the supplement with antioxidant potential should be avoided. Besides health benefits of an antioxidant rich diet, it should be noted that supplements are more concentrated in components of plants and should be used with caution. Such limitation is possibly overcomed by determining the overlapping or conflicting cellular targets of therapeutics and supplements .

These plants and several others with traditional use are generally wild plants. Based on the results of the current study, it is suggested that these plants are cultivated under more controlled conditions and further studies should be performed to provide the best supplementary formulation of extracts for inflammatory cases. Without overlooking the previous studies, which have mainly evaluated their toxic effects, these plants should be further analyzed for all their possible cellular targets.

SIGNIFICANCE STATEMENT

Considering the current reports, 20% of the world population rely on herbs, herbal preparations, or finished products with standardized herbal materials as plant-based TCM practices. From this view point, the plants presented in this current article are commonly consumed as herbal remedies in Eastern Europe and Turkey to reduce inflammatory conditions, soothing the inflammatory symptoms and facilitating the treatment via topical or oral use. However, none of those plants or their extracts have been evaluated for possible biological targets that could be related with inflammatory conditions. In this context, the oxidative status of cells regulated by antioxidant and xenobiotic defense enzymes were found as the best approach for such evaluation. This study discovers the effect of plants on xenobiotic metabolism and cellular defense enzymes for the first time. The study also reveals that the antioxidant efficiency of plants by chemical methods is not a way to define their real potential on cellular oxidative status, where enzymes are critical for cellular defense. Therefore, this study will help the researcher to uncover the role of enzyme profiling to define the medicinal value of plants of traditional or TCM use. This study will not only help to develop new preparations as dietary or therapeutic supplements, but also to evaluate their synergistic or adverse effects upon use with certain and commonly used medications.

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