Accepted Manuscript

Title: Health attributes of an endemic orchid form Eastern Anatolia, Dactylorhiza chuhensis RenzandTaub in vitro investigations

Author: Abdullah Dalar Yu Guo Nevzat Esim Aydin Sukru Bengu Izaela Konczak

PII: S2210-8033(15)00008-1
DOI: http://dx.doi.org/doi:10.1016/j.hermed.2015.02.001
Reference: HERMED 98

To appear in:

Received date: 28-8-2014
Revised date: 23-12-2014
Accepted date: 2-2-2015

Please cite this article as: Dalar A, Guo Y, Esim N, Bengu AS, Konczak I, Health attributes of an endemic orchid form Eastern Anatolia, Dactylorhiza chuhensis RenzandTaub in vitro investigations, Journal of Herbal Medicine (2015), http://dx.doi.org/10.1016/j.hermed.2015.02.001

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Health attributes of an endemic orchid form Eastern Anatolia, Dactylorhiza chuhensis Renz & Taub. – in vitro investigations

Abdullah Dalar\textsuperscript{a,b}, Yu Guo\textsuperscript{a,c}, Nevzat Esim\textsuperscript{d}, Aydin Sukru Bengü\textsuperscript{e}, Izabela Konczak\textsuperscript{a,f,g}*

\textsuperscript{a} CSIRO Animal, Food and Health Sciences, North Ryde, NSW, Australia
\textsuperscript{b} Yüzyüncü Yıl University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Van, Turkey
\textsuperscript{c} Shanxi Agricultural University, Department of Food Science and Engineering, Shanxi, China
\textsuperscript{d} Bingol University, Vocational Training School, Bingöl, Turkey
\textsuperscript{e} Bingol University, Central Research Laboratory, Bingöl, Turkey
\textsuperscript{f} Food Science and Technology, School of Chemical Sciences and Engineering, The University of New South Wales, Sydney, NSW 2052, Australia
\textsuperscript{g} Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, St Lucia, Queensland 4072, Australia

*Corresponding author. E-mail address: i.konczak@unsw.edu.au
Abstract

Phytochemical composition and potential health attributes of *Dactylorhiza chuhensis* Renz&Taub., an endemic orchid from Eastern Anatolia, were investigated. Lyophilised methanol-based extracts obtained from leaf, flower, stem and tuber were investigated for the presence of phenolic compounds [(Folin-Ciocalteu assay and high performance liquid chromatography analysis (HPLC)], antioxidant capacities [ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay] and enzyme-inhibitory activities [lipase, $\alpha$-amylase, $\alpha$-glucosidase and angiotensin converting enzyme (ACE)]. The tuber, used as a traditional remedy and utilized by the pharmaceutical industry, had the lowest content of total phenolics, inferior antioxidant and enzyme-inhibitory activities. The highest phenolic content (44.9±0.8 mg GAE/g DW) exhibited leaf extract, which also shown superior reducing (736.8±16.2 µmol Fe$^{2+}$/g DW) and oxygen radical scavenging capacities (2715.8±83.5 µmol Trolox E/g DW). The inhibitory activities of leaf extract toward $\alpha$-amylase, $\alpha$-glucosidase and ACE were moderate. Applied at non-toxic concentrations, the leaf extract effectively reduced accumulation of nitric oxide (NO) in lipopolysaccharide (LPS)-activated hepatocellular carcinoma (HepG2) cells. Further studies towards potential utilization of *D. chuhensis* leaf as a source of physiologically active phytochemicals are justified.

*Keywords: Dactylorhiza chuhensis* Renz&Taub.; antioxidant; enzyme-inhibitory activity; total phenolics
1. Introduction

The Orchidaceae family is one of the largest and widespread families in the plant kingdom with 26567 species recorded worldwide (World Checklist of Selected Plant Families, Kew WCSP, 2011). The economic importance of orchids arises from their ornamental and therapeutic properties. Although predominantly known for their ornamental qualities, numerous orchid species are also used in food and pharmaceutical industries. Vanilla flavour derived from fruit of orchid belonging to the genus Vanilla is an example of a widespread commercial application of orchid products.

High consumption of orchid tubers has been recorded for southern African countries. A meatless sausage "chikanda" made of Disa, Habenaria and Satyrium orchid tuber, traditionally consumed as a midday snack food, is a popular Zambian dish. The high popularity of chikanda sausage lead to a severe decline of orchid species in Zambia. Subsequently, a large export of edible orchid tubers from Tanzania to Zambia followed (Challe and Price, 2009).

In Mediterranean countries flour made of Orchis mascula and Orchis molitaris tuber is traditionally used for the preparation of a nutritional drink called ‘salep’. The main component of orchid flour is glucomannan, a polysaccharide, comprising of mannose and glucose (Kaya and Tekin, 2001). Glucomannans are neutral water soluble fibers, reported to possess nutritive and demulcent properties (Kayacier and Dogan, 2006). Traditional preparation of orchid flour involves immersing washed tubers in boiling water to deactivate enzymes, removing the outer layer of skin and drying the tubers in the sun or in an oven. Dried tubers are ground into a fine yellowish powder, later used
to prepare beverages (salep) and desserts (salep pudding and salep ice cream) (Kaya and Tekin, 2001). In Eastern Anatolia salep drink is still used by the locals to cure common colds, bronchitis and diarrhoea. Extensive use of *O. mascula* and *O. molitaris* tubers by the local population lead to the plants near extinction, and other orchid species are being explored for salep preparation. At present in Turkey salep is produced from approximately 120 taxa of orchids, representing the genera *Ophrys*, *Orchis*, *Himantoglossum*, *Serapis*, *Anacamptis*, *Compreria*, *Barlia*, *Dactylorhiza*, *Aceras*, and *Neotinea* (Sezik, 2002).

Therapeutic preparations made of orchids are also used in traditional Chinese medicine (Bulpitt et al., 2007) and in traditional Indian medicine, Ayurveda (Giri et al., 2012a). In India an orchid *Habenaria edgeworthi* is used for preparation of a popular tonic, chyawanprash. The tonic is used to cure coughs, colds, anemia and to strengthen vitality (Giri et al., 2012a).

Eastern Anatolia is home to a large number of endemic orchids. *Dactylorhiza chuhensis* Renz&Taub. is an endemic plant growing in a restricted area of Van city in the Eastern Anatolia Region. The plant is locally known as ‘kartolik’, ‘șepirze’ and ‘kulîlka qamiş’. The herb is robust (15-45 cm), the leaves are oblong, lanceolate (7–13 × 1–5 cm) and the flowers are rose-purple. The plant produces one edible tuber (roughly the size of one small potato) and the entire plant is removed in the harvesting process. The tuber is collected in spring, air dried in the dark and ground to obtain a fine powder for later use; the aerial parts are not used. Utilization of the aerial part, especially the leaf, may bring additional economical benefits.

Orchids accumulate a large number of diverse phytochemicals, responsible for their pharmacological activities, however research on phytochemical composition of
edible and medicinal orchids and their physiological activities has been limited to only a few (Hossain, 2011). Therefore, this study aimed at evaluation of *D. chuhensis* leaf as a potential source of physiologically active phytochemicals for therapeutic applications. The antioxidant capacities and total phenolic content of lyophilized extracts prepared from tuber, stem, leaf and flower of *D. chuhensis* were evaluated. Subsequently, inhibitory activities towards selected digestive enzymes and potential anti-inflammatory properties of leaf extract were investigated.

2. Materials and Methods

2.1. Plant materials

Plants were harvested in the Eastern Anatolia Region of Turkey (Van city, Chuh path, from Hoşap town to Başkale County; 38° 09' 04.17" N 043° 59' 19.54"E; 2307 m) during May - August, 2010. Plant samples with no apparent physical damage were collected from Chuh path, Başkale, Van City. The plant material, sealed in pre-cleaned polythene bags, was brought to a laboratory within a maximum of 3 hours after harvest. The identity of the plant material was confirmed by Dr. Sinan İşler at the Biological Sciences Department, Science Faculty, Yüzüncü Yıl University, Turkey, and a voucher specimen stored in the university’s herbarium (Herbarium code: VANF-4418). Tubers, stems, leaves and flowers of fresh plants were divided, washed with distilled water to remove surface dust and left at room temperature in the dark until dry. Subsequently, samples were ground into a fine powder using a laboratory mill and stored at -20 °C until analysis.

2.2. Reagents
Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, Inc. (Sydney, Australia) and were of analytical or HPLC grade. Soluble starch, iodine reagent and colouring reagent (Glucose C2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium carbonate was purchased from Ajax Chemicals (Sydney, Australia). Acetic acid and sodium hydroxide were purchased from Ajax Finechem Pty. Ltd., Sydney, Australia. Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). α-Amylase from porcine, intestinal acetone powders from rat, angiotensin converting enzyme (ACE) from rabbit lung, porcine pancreatic lipase (type II), orlistat and captopril were purchased from Sigma–Aldrich, Inc. (USA). Acarbose was purchased as ‘glucobay’ from Bayer (Bayer Australia Ltd., Pymble, NSW).

2.3. Cell lines

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% carbon dioxide (CO₂) atmosphere in media containing 10% foetal bovine serum (FBS; Invitrogen Corporation, Carlsbad, CA, USA), 100 µg/ml streptomycin and 100 units/ml penicillin (Invitrogen Corporation, Carlsbad, CA, USA). HepG2 (hepatocellular carcinoma) cells were cultured in Eagle’s minimum essential medium (EMEM; Sigma–Aldrich, USA). RAW 264.7 (murine macrophage) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corporation, Carlsbad, CA, USA). Experiments were conducted using cell lines with less than 40 passages.

2.4. Preparation of lyophilized extracts
Ground plant material was mixed with a 20-fold volume of acidified ethanol (80% ethanol, 19.9% H₂O and 0.1% trifluoroacetic acid, v/v/v), shaken for 2 h at room temperature (22°C) and centrifuged for 20 min at 15320g (10000 rpm) at 4°C (Sorvall RC-5B; DuPont, Wilmington, DE, USA; rotor Beckman JA14 (137 mm) serial No. 02U8152, USA) with the supernatant collected. The extraction was repeated once more. The supernatants from the consecutive extractions were combined and the solvent evaporated under reduced pressure at 37°C using a rotary evaporator (Rotavapor R-205; Buchi, Switzerland). The derived fraction was dissolved in purified water (Synergy UV, Millipore, Sydney, Australia) and freeze-dried under a vacuum at -51°C to obtain a fine lyophilized powder.

2.5. Total phenolic content
Total phenolics were quantified by Folin-Ciocalteu method, as described previously (Dalar et al., 2012).

2.6. Sugar analysis
The analysis of sugars in lyophilized extracts was carried out according to Cardozo and collaborators (Cardozo et al., 2011). Neutral sugars were determined using the phenol–sulphuric acid method. Diluted extracts (0.8 ml) were mixed with 0.04 ml of 80% phenol and 2 ml of H₂SO₄, incubated at 100°C for 20 minutes and the absorbance was measured at 490 nm (Shimadzu 1601 spectrophotometer, Tokyo, Japan). Reducing sugars were measured using the Somogyi–Nelson method. Copper tartrate reagent (0.5 ml) was added to 0.1 ml aliquots of different extract dilutions. The solution was heated at 100 °C for 15 min and 0.5 mL of arsénomolybolic acid reagent was added. The
absorbance was measured at 520 nm. The results were expressed as milligrams of glucose/gram dry weight (mg Glucose Eq./g DW) of lyophilized extracts based on glucose calibration curve.

2.7. Antioxidant capacity
Antioxidant capacity was investigated using the ferric reducing antioxidant power (FRAP) assay to estimate the total reducing capacities and the oxygen radical absorbance capacity (ORAC) assay to estimate the oxygen radicals scavenging capacities, as previously described (Dalar et al., 2012).

2.8. Inhibitory activities towards selected enzymes

2.8.1. Alpha-amylase inhibitory activity
Inhibitory activity of plant extracts toward α-amylase [from porcine pancreas, Type 1-A; diisopropylfluorophosphate (DFP)-treated] was determined as described previously (Dalar and Konczak, 2013), using Wako Amylase kit. The decrease in starch concentration [40 mg starch/100 ml (in PBS buffer, pH = 6.8)] due to the activity of α-amylase was measured at 660 nm using a Shimadzu 1601 spectrophotometer (Tokyo, Japan). The enzyme inhibitory activities of plant extracts were calculated as a percentage of the control using the following equation: \[\text{Inhibition (\%)} = \frac{(\text{ACB–AC}) – (\text{ASB–AS})}{(\text{ACB–AC})} \times 100\], where ACB is the absorbance of the control blank, AC is the absorbance of the control, ASB is the absorbance of the sample blank and AS is the absorbance of the sample.

2.8.2. Alpha-glucosidase inhibitory activity
Inhibition of α-glucosidase (from rat’s intestinal acetone powders) by plant extracts was determined according to Sakulnarmrat and Konczak (2012), using sucrose (2g of sucrose in 100 ml of maleic acid buffer) as a substrate. The relative α-glucosidase inhibition was calculated using the following formula: % Inhibition = (ACB–AC) – (ASB–AS) / (ACB –AC) x 100, where AS and AC were the absorbance of sample and negative control, and where ASB and ACB were the absorbance of sample blank and control blank, respectively. The absorbance was measured at 505 nm using a Shimadzu 1601 spectrophotometer (Tokyo, Japan).

2.8.3. Pancreatic lipase inhibitory activity

Lipase inhibitory activity of D. chuhensis plant extracts was assayed according to Sakulnarmrat and Konczak (2012), using 4-methylumbelliferyl oleate (0.1 mM) as substrate, with the exception of porcine pancreatic lipase (Sigma type II), which was prepared using a concentration of 0.085 g/ml. The relative lipase inhibition activity was calculated using the following formula: % Inhibition = (1- (FS-FSB) / (FC-FCB) x100, where FS and FC were the values of samples and negative control measured fluorometrically at an emission wavelength of 460 nm and excitation of 320 nm with slit widths of 5 nm (POLARstar Omega, BMG Labtech, Germany), and where FSB and FCB were the fluorescence readings of sample blank and control blank, respectively.

2.8.4. Angiotensin converting enzyme (ACE) inhibitory activity

Inhibitory activity toward ACE was conducted using furanacroloyl-Phe-Glu-Glu (FAPGG) as a substrate and the results were expressed as a percentage of ACE inhibition as described by Sakulnarmrat and Konczak (2012). The color intensity was measured at 340 nm (POLARstar Omega, BMG Labtech, Germany).
2.9. Assessment of cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to evaluate the potential of plant extract to suppress the proliferation of hepatocellular carcinoma (HepG2) and murine macrophages (RAW 264.7) cells as described by Sakulnarmrat et al. (2013).

2.10. Effect of plant extract on nitric oxide accumulation.

RAW 264.7 and HepG2 cells (3 x 10^5 per well) were pretreated with D. chuhensis leaf extract, applied at concentrations from 0.2 to 2.0 mg/ml for 1 h, and subsequently exposed to 40 ng/mL lipopolysaccharides (LPS) over 12 hours. The concentration of nitric oxide (NO) in culture supernatant was measured by the Griess reaction, according to Tan et al. (2011).

2.11. Analysis of phenolic compounds by high performance liquid chromatography-diode array detector (HPLC-DAD) in lyophilized leaf extract.

The levels of phenolic compounds in leaf extract were evaluated by HPLC-DAD at 280, 326, 370 and 520 nm wavelengths, as described previously (Dalar and Konczak, 2013). The amounts of phenolic compounds detected at 280 nm were quantified as mg gallic acid equivalent per gram dry weight of lyophilized extract (mg GA Eq./g DW). Phenolic compounds detected at 326 nm were calculated as mg of chlorogenic acid equivalent per gram dry weight of lyophilized extract (mg CHA E/g DW) and those detected at 370 nm were quantified as mg rutin equivalent per gram dry weight of lyophilized extract (mg R Eq./g DW).
2.12. GC-MS Analysis

Fatty acids/fatty acids methyl esters (FAMEs) were analysed on Varian 3800 gas chromatograph directly interfaced with Varian 2000 ion trap mass spectrometer (Varian Spa, Milan, Italy) equipped with a flame ionisation detector (FID), used with injector temperature of 260°C in injection mode, splitless. For volatile compounds analysis an aliquot (1 μl) of ethanolic extract was chromatographed on a 60 x 0.25 mm i.d., 0.2 μm HP 88 column. Oven temperature was programmed as follows: starting at 120°C and increased to 200°C at a rate of 5°C/min, then held at 200°C for 2 min. subsequently increased to 280°C at a rate of 5°C/min, with following 8 minute hold time. The carrier gas was helium, used at a constant pressure of 10 psi, electron impact (EI) ionisation mode and acquisition range 40 to 200 m/z. The transfer line temperature was 250°C. The compounds were identified using the National Institute of Standards and Technology (NIST) library (NIST/WILEY/EPA/NIH), mass spectral library and verified by the retention indices, calculated as described by Van den Dool and Kratz (1963). Results were expressed as Flame Ionization Detector (FID) response area in relative percentages.

2.13. Statistical Analysis

The mean values were calculated based on at least three independent evaluations (n = 3). One way ANOVA followed by the Bonferroni post-hoc test were performed to assess differences between the samples at the level of p<0.05. Statistical correlation analyses were performed using Graphpad Prism 5 (Graphpad Software, CA, USA). The results of correlation analysis were considered statistically significant when p<0.05.
3. Results and Discussion

3.1. Extraction yields, total phenolics and sugar contents

High yields of alcohol-based crude lyophilized extracts were obtained from all *D. chuhensis* plant parts, with leaf extract representing 28.2% of leaf dry weight (DW), followed by stem extract (24.5% DW), tuber (19.6% DW) and flower extract (19.4% DW; Table 1). The highest levels of total phenolics (TP), as evaluated using the Folin-Ciocalteu method, were found in leaf and flower extracts, followed by stem and tuber extracts (Table 1). The TP of leaf and flower extracts were slightly lower (12%) than those of *Vanilla planifolia* (vanilla orchid) (51.64±0.35 mg Gallic acid Eq./g DW) (Dudonnné *et al.* 2009). The TP content of *D. chuhensis* tuber was approximately 2.5 times that of a rare endemic orchid from Himalayans, *Habenaria edgeworthii*, used as a therapeutic agent (Giri *et al.*, 2012b).

The levels of phenolic compounds in lyophilized extracts were also evaluated by the HPLC in order to minimize the interferences from non-phenolic compounds (Table 1). The HPLC profile revealed that the majority of compounds eluted at 370 nm wavelength and at 326 nm. These results indicate that hydroxycinnamic acids, flavonoids and possibly chalcones dominated the composition of the phenolic mixtures. No detectable compounds were found at 520 nm, indicating the lack of anthocyanins.

The levels of phenolic compounds in leaf and flower extracts, evaluated by the HPLC and quantified as gallic acid equivalent (at 280 nm) were similar to those obtained by the Folin-Ciocalteu method and also quantified as gallic acid equivalent. However, the level of phenolic compounds in tuber and stem extracts quantified at 280 nm were lower by 38 and 23%, respectively. This indicates that beside phenolic compounds, tuber and stem contain also other redox active phytochemicals, potentially sugars (Table 1), which
interfered in the Folin-Ciocalteu assays. Moreover, the HPLC chromatogram showed that in this investigation, the majority of phenolic compounds eluted at 370 nm and quantification of these compounds showed that their levels were significantly higher than the levels obtained by the Folin-Ciocalteu assay. Although the Folin-Ciocalteu assay is commonly used to quantify the levels of phenolic compounds in plant extracts, the nature of phenolic compounds significantly affects the results.

3.2. Volatile compounds

Volatile compounds are important natural compounds used as therapeutic and industrial agents, such as taxol – an anticancer drug (Kintzios and Barberaki, 2004), phloroglucinol – an analgesic (Chassany et al., 2007) or benzaldehyde – a flavouring agent (Brühne and Wright, 2011). Volatile compounds present in *D. chuhensis* extracts were analyzed by gas chromatography mass spectrometry (GC/MS) and identified by the relative retention indices of their peaks with those of the MS library standards (Table 2). Maximum number of volatile compounds were detected in leaf extract (9), followed by tuber/flower (7) and stem (5) extracts. The volatile composition of leaf comprised mainly 2-furancarboxaldehyde (4.5%), 2-methoxy-4-vinylphenol (3.9%) and *p*-cresol (3.8%). Other volatile compounds, oxetane, phloroglucinol, 4h-pyran-4-one, benzaldehyde, oxirane and ethyl palmitate were present at traces amounts (% area ≤ 2). Multiple volatile compounds detected in the *D. chuhensis* leaf extract are of therapeutic and industrial value (Table 2).

Tuber extract contained 2-furancarboxaldehyde (9.7%), nonadecane (9.7%), cyclopentane (14.8%) and octadecane (17.9%). Additionally 4h-pyran-4-one, 4-vinylphenol, *p*-cresol, salicyaldehyde and octadecane were also found (% area ≤ 2)
Flower extract contained \( p \)-cresol (4.4%), benzenemethanol (5.6%), cyclopentane (12.8) and linoleic acid (4.2%). Furthermore, traces amounts (\% area \( \leq 2 \)) of 2-furancarboxaldehyde, 4-vinyl-2-methoxy-phenol, benzaldehyde and ethyl palmitate were also found (Table 2). 2-Furancarboxaldehyde (18.6%) was the major compound of stem extract, accompanied by 4h-pyran-4-one, 2-methoxy-4-vinylphenol, \( p \)-cresol and benzaldehyde present at low levels (\% area \( \leq 2 \)) (Table 2). Among the identified volatile compounds oxetane, phloroglucinol and oxirane were found only in leaf extract; 4-vinylphenol, salicyaldehyde and octadecane were present only in tuber extract; while 4-vinyl-2-methoxy-phenol, cyclopentane and linoleic acid were detected only in flower extract (Table 2). The common volatile compounds present in all extracts were \( p \)-cresol and 2-furancarboxaldehyde (Table 2). Phloroglucinol, 4h-pyran-4-one, 4-vinylphenol, 2-methoxy-4-vinylphenol, and 4-vinyl-2-methoxy-phenol are of phenolic nature.

3.2. Antioxidant capacity of lyophilized extracts

The antioxidant profile of crude extracts obtained from tubers, stems, leaves and flowers of *D. chuhensis*, as characterized using the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays, presents Table 3. The ORAC values ranged from 633.9±42.4 (tuber) to 2715.0±83.5 (leaf) \( \mu \text{mol Trolox E/g DW} \). The distribution of ORAC activities in various plant parts followed the order tuber \( < \) stem \( < \) flower \( < \) leaf. The oxygen radical absorbance capacities of leaf and flower extracts were higher than those of *Vanilla planifolia* (vanilla orchid) (1593±12 \( \mu \text{mol Trolox E/g DW} \)), one of the most important commercial plants selected and evaluated by Dudonné et al. (2009).
The reducing capacities (FRAP values) of *D. chuhensis* ranged from 85.8±8.6 (tuber) to 756.8±31.8 (leaf) µmol Fe²⁺E/g DW (Table 3). The weakest total reducing capacity exhibited the extract of tuber, followed by stem, flower and leaf, similarly to the pattern displayed by the ORAC values (Table 3). On the contrary to the ORAC values, the reducing power of leaf extract was lower than that of vanilla orchid leaf (970 µmol Fe²⁺ E/g DW) (Dudonnné et al., 2009).

3.3. Lipase-inhibitory activities of *D. chuhensis*

The inhibitory capacity of crude *D. chuhensis* plant extracts against pancreatic lipase presents Table 3. Tuber and stem extracts exhibited similar lipase-inhibitory potential, inferior to that of flower and leaf. The IC₅₀ values ranged from 19.5 mg/ml (tuber and/or stem) to 6.3 mg/ml (leaf), and were lower than the activity of orlistat – a commercially available drug used in the treatment of obesity. *Dactylorhiza chuhensis* leaf extract exhibited the most pronounced activity of 0.44 µmol orlistat equivalent/g crude extract (Table 3). Similar lipase-inhibitory activities were previously reported for a crude extract of Thai herb *Antigonon leptosus* (7.87±0.69; Kaisoon et al., 2012) and for a purified polyphenolic-rich bay leaf extract (6.3±0.27; Sakulnarmrat and Konczak, 2012). The lipase-inhibitory activities of plant extracts were dose-depended (Figure 1). At the concentration of 5 mg/ml, the enzyme inhibitory activity of leaf extract reached 43% and was comparable to that of *Rheum ribes*, which displayed the 8th highest lipase-inhibitory potential among 92 plant extracts evaluated by Gholamhoseinian and collaborators (Gholamhoseinian et al., 2012).

3.4. Potential health attributes of *D. chuhensis* leaf
Utilization of orchid plant for food requires the whole plant to be removed from soil. After separation of tuber for consumption or processing, leaf represents the major waste material. The extract of leaf obtained in this study exhibited the highest antioxidant capacity and – together with the flower extract – had the highest level of phenolic compounds. Therefore, an extended study towards potential utilization of leaf extract as a source of physiologically active phytochemicals was carried out.

3.4.1. Inhibition of α-amylase, α-glucosidase and angiotensin converting enzyme of leaf extract

Leaf extract had the most pronounced inhibitory activity towards α-amylase, a moderate activity towards α-glucosidase and low towards ACE (Table 3). The inhibitory activities of D. chuhensis leaf extract towards these enzymes were inferior to commercially available drugs, utilized as reference samples in this study.

3.4.2. Suppression of NO accumulation

Potential anti-inflammatory activity of lyophilized crude leaf extract was evaluated in LPS-activated murine macrophages (RAW 264.7) and hepatocellular carcinoma (HepG2) cells. The cells were treated for 1h with a range of concentrations from 6.25 to 400 μg/ml of D. chuhensis leaf extract, which did not affect the proliferation of cells (Figure 2A and 2B, respectively), before exposure to 40 ng/ml LPS over 12 h. The exposure of cells to LPS induces inflammatory responses, associated with upregulation of the key pro-inflammatory enzyme – inducible nitric oxide synthase (iNOS), and leads towards enhanced accumulation of the iNOS product, nitric oxide (NO) (Figure 3A and 3B). A strong effect of cell pre-treatment with leaf extract significantly reduced NO accumulation in LPS-activated HepG2 cells, while the effect on murine macrophage
RAW 456.7 cells was weak. In both cases a dose-dependent reduction of NO accumulation occurred. Following pre-treatment with 6.25, 12.5 and 25 µg/ml of *D.chuhensis* leaf extract the inhibition of NO accumulation in HepG2 cells was, respectively, 94.3, 95.5 and 95.8% (Fig. 3B), which suggests reduction of inflammatory responses by the leaf extract.

3.5. Correlation analysis

Table 4 presents the correlation analysis between total phenolics levels and antioxidant capacities. The TP values obtained by Folin-Ciocalteu method positively correlated with FRAP values ($r^2=0.8757$) and ORAC values ($r^2=0.9456$). The correlation analysis of TP values obtained by HPLC method revealed similar patterns with high correlation coefficients detected for FRAP ($r^2 \geq 0.847$) and ORAC values ($r^2 \geq 0.922$) (Table 4).

These results suggest that phenolic compounds present in the extracts are the primary contributors of antioxidant capacities of the lyophilized extracts. The positive and high linear correlation ($r^2 = 0.9683$) (Table 4) between ORAC and FRAP values suggests that plant extracts of *D. chuhensis* comprise of a mixture of antioxidant compounds which are able to donate a hydrogen atom and a single electron. Correlation analysis between ORAC and lipase-inhibitory activities ($r^2 =0.9691$) as well as FRAP and lipase-inhibitory activities ($r^2 =0.9542$; Table 4) suggests that phytochemicals present in the extracts are able to deactivate oxidant compounds as well as suppress the activity of pancreatic lipase. Moreover, the positive and high correlations ($r^2 \geq 0.8050$) detected between lipase inhibitory activities and total phenolic contents, suggest that phenolic compounds are the major contributors of lipase-inhibitory activities of the extracts.

4. Conclusion
Among ethanol-based crude extracts obtained from flower, leaf, stem and tuber of an endemic orchid *D. chuhensis* from Eastern Anatolia the leaf and flower extracts contained the highest levels of total phenolics and volatile compounds and exhibited the highest antioxidant capacities. Leaf extract was the most efficient inhibitor of pancreatic lipase. The same extract mildly suppressed α-amylase, α-glucosidase and angiotensin converting enzyme (ACE). Moreover, leaf extract inhibited the accumulation of NO in LPS-activated HepG2 cells, but the effect in RAW 246.7 cells was negligible. This study suggests that leaf of orchid plant, not utilized to date, represents a new source of phytochemicals for therapeutic applications.

**Acknowledgements**

The authors thank Dr. Sinan İşler of the Biology Department in Yüzüncü Yıl University for help in plant collection and identification.

**References**


4. Cardozo ML, Ordonez RM, Alberto MR, Zampini IC, Isla MI. Antioxidant and antiinflammatory activity characterization and genotoxicity evaluation of


18. Jeong JB, Jeong HJ. 2-Methoxy-4-vinylphenol can induce cell cycle arrest by blocking the hyper-phosphorylation of retinoblastoma protein in benzo[a]pyrene-treated NIH3T3 cells. Biochem Biophys Res 2010; 400: 752-757.


Table 1. Extraction yields, total phenolics and sugar content of hydrophilic extracts obtained from *Dactylorhiza chuhensis* plant.

<table>
<thead>
<tr>
<th></th>
<th>Tuber</th>
<th>Stem</th>
<th>Leaf</th>
<th>Flower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)</td>
<td>19.6</td>
<td>24.5</td>
<td>28.22</td>
<td>19.4</td>
</tr>
<tr>
<td>Total phenolic compounds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolics 1 (mg Gallic acid E/g DW)</td>
<td>13.9±0.6c</td>
<td>17.3±0.3b</td>
<td>44.9±0.8a</td>
<td>44.2±2.0a</td>
</tr>
<tr>
<td>Phenolics 2 (mg Gallic acid E/g DW)</td>
<td>8.6±0.0d</td>
<td>12.9±0.2c</td>
<td>46.8±0.1a</td>
<td>44.6±0.1b</td>
</tr>
<tr>
<td>Phenolics 3 (mg CHA E/g DW)</td>
<td>12.7±0.6c</td>
<td>22.0±0.4b</td>
<td>71.5±2.4a</td>
<td>73.0±2.9a</td>
</tr>
<tr>
<td>Phenolics 4 (mg Rutin E/g DW)</td>
<td>22.9±1.4b</td>
<td>33.0±1.2b</td>
<td>118.3±5.8a</td>
<td>122.3±111a</td>
</tr>
<tr>
<td>Total sugars (mg Glucose E/g DW)</td>
<td>720.2±14.5b</td>
<td>768.2±18.4a</td>
<td>439.7±9.4d</td>
<td>598.4±11.6c</td>
</tr>
<tr>
<td>Reducing sugars (mg Glucose E/g DW)</td>
<td>167.3±1.1b</td>
<td>189.3±8.1a</td>
<td>105.0±1.3c</td>
<td>174.1±2.8b</td>
</tr>
</tbody>
</table>

Means with different letters in the same row were significantly different at the level (p<0.05); n=3.

1 Folin-Ciocalteu method, 2 at 280 nm by HPLC, 3 at 326 nm by HPLC, 4 at 370 nm by HPLC.
### Table 2. Volatile compounds identified in hydrophilic extracts of *Dactylorhiza chuhensis* plant.

<table>
<thead>
<tr>
<th>No</th>
<th>Retention time</th>
<th>Name of compound</th>
<th>Compound nature</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Therapeutic/Industrial use</th>
<th>Relative concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.76</td>
<td>Oxetane</td>
<td>Heterocyclic organic compound</td>
<td>C₃H₆O</td>
<td>58</td>
<td>Component of taxol (^1)</td>
<td>ND 0.6 ND ND</td>
</tr>
<tr>
<td>2</td>
<td>10.25</td>
<td>Phloroglucinol</td>
<td>Phenol derivative</td>
<td>C₆H₆O₃</td>
<td>126</td>
<td>Gastrointestinal disorders (^2)</td>
<td>ND 1.8 ND ND</td>
</tr>
<tr>
<td>3</td>
<td>12.43</td>
<td>4h-Pyran-4-One</td>
<td>Polyphenol</td>
<td>C₅H₄O₂</td>
<td>96</td>
<td>Component of antioxidant and antiulcer DDMP (^3)</td>
<td>0.5 2.2 2.1 ND</td>
</tr>
<tr>
<td>4</td>
<td>13.93</td>
<td>4-Vinylphenol</td>
<td>Polyphenol</td>
<td>C₈H₈O</td>
<td>120</td>
<td>Flavoring agent, component of polymers and resins (^4)</td>
<td>0.4 ND ND ND</td>
</tr>
<tr>
<td>5</td>
<td>15.46</td>
<td>2-Furancarboxaldehyde</td>
<td>Heterocyclic aldehyde</td>
<td>C₅H₄O₂</td>
<td>96</td>
<td>Liquid biofuel, flavoring agent, resin manufacture (^5)</td>
<td>9.7 18.6 4.5 1.4</td>
</tr>
<tr>
<td>6</td>
<td>17.07</td>
<td>2-Methoxy-4-Vinylphenol</td>
<td>Polyphenol</td>
<td>C₉H₁₀O₂</td>
<td>150</td>
<td>Flavoring agent, anticancer (^6)</td>
<td>ND 1.1 3.9 1.1</td>
</tr>
<tr>
<td>7</td>
<td>18.29</td>
<td>(p)-Cresol</td>
<td>Polyphenol</td>
<td>CH₃</td>
<td>108</td>
<td>Used in BHT production (^7)</td>
<td>0.8 1.2 3.8 4.4</td>
</tr>
<tr>
<td>8</td>
<td>19.31</td>
<td>Benzaldehyde</td>
<td>Aromatic aldehyde</td>
<td>C₇H₆O</td>
<td>106</td>
<td>Flavouring agent - foods and and pharmaceuticals (^8)</td>
<td>ND 0.3 0.9 0.7</td>
</tr>
<tr>
<td>9</td>
<td>19.34</td>
<td>Salicylaldehyde</td>
<td>Hydroxybenzaldehydes</td>
<td>C₇H₆O₂</td>
<td>122</td>
<td>Manufacture of perfumes, dyes, pharmaceuticals (^8)</td>
<td>0.5 ND ND ND</td>
</tr>
<tr>
<td>10</td>
<td>22.18</td>
<td>Nonadecane</td>
<td>Alkanes</td>
<td>C₁₉H₄₀</td>
<td>268</td>
<td>-</td>
<td>9.7 ND ND ND</td>
</tr>
<tr>
<td>11</td>
<td>25.47</td>
<td>Ethylene oxide</td>
<td>Cyclic ether</td>
<td>C₂H₄O</td>
<td>44</td>
<td>Disinfectant, sterilizing and fumigant agent (^9)</td>
<td>ND ND 0.6 ND</td>
</tr>
<tr>
<td>12</td>
<td>29.00</td>
<td>Octadecane</td>
<td>Alkanes</td>
<td>C₁₈H₃₈</td>
<td>254</td>
<td>-</td>
<td>17.9 ND ND ND</td>
</tr>
<tr>
<td>13</td>
<td>30.64</td>
<td>Cyclopentane</td>
<td>Cycloalkanes</td>
<td>C₅H₁₀</td>
<td>70</td>
<td>Production of polypentenamer (^10)</td>
<td>ND ND ND 12.8</td>
</tr>
<tr>
<td>14</td>
<td>33.97</td>
<td>Ethyl palmitate</td>
<td>Fatty acid ester</td>
<td>C₁₈H₃₆O₂</td>
<td>284</td>
<td>-</td>
<td>ND ND 1.6 0.9</td>
</tr>
<tr>
<td>15</td>
<td>39.60</td>
<td>Linoleic acid</td>
<td>Fatty acid</td>
<td>C₁₈H₃₂O₂</td>
<td>280</td>
<td>Nematicide, cancer preventative (^3)</td>
<td>ND ND ND 4.3</td>
</tr>
</tbody>
</table>

ND: not detected. \(^1\) Willenbring and Tantillo (2008); \(^2\) Chassany et al., 2007; \(^3\) Suman et al. (2013); \(^4\) Burdock (1995); \(^5\) Cary et al. (2000); \(^6\) Jeong and Jeong (2010); \(^7\) Fiege (2000); \(^8\) Brühne and Wright (2011); \(^9\) Rebsdat and Mayer (2001); \(^10\) Hönicke et al. (2000).
Table 3. Antioxidant capacities and inhibitory activities towards selected enzymes of hydrophilic extracts obtained from *Dactylorhiza chuhensis* plant.

<table>
<thead>
<tr>
<th></th>
<th>Tuber</th>
<th>Stem</th>
<th>Leaf</th>
<th>Flower</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidant capacity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORAC (^1) (µmol TE/g DW)</td>
<td>633.9±42.4d</td>
<td>792.5±22.7c</td>
<td>2715.8±83.5a</td>
<td>2097.0±47.3b</td>
</tr>
<tr>
<td>FRAP (^2) (µmol Fe(^{2+})/g DW)</td>
<td>85.3±8.6d</td>
<td>239.7±20.1c</td>
<td>736.8±16.2a</td>
<td>511.6±252b</td>
</tr>
<tr>
<td><strong>Lipase inhibitory activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50 (^3) (mg/ml)</td>
<td>19.5±0.8c</td>
<td>19.1±0.8c</td>
<td>6.3±0.0a</td>
<td>9.3±0.2b</td>
</tr>
<tr>
<td>Orlistat Eq. (µmol/g DW)</td>
<td>0.14±0.01c</td>
<td>0.14±0.01c</td>
<td>0.44±0.00a</td>
<td>0.30±0.01b</td>
</tr>
<tr>
<td><strong>α-Amylase inhibitory activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (^a) (%)</td>
<td>-</td>
<td>-</td>
<td>75.8±0.4</td>
<td>-</td>
</tr>
<tr>
<td>Acarbose Eq. (µmol/g DW)</td>
<td>-</td>
<td>-</td>
<td>49.4±1.3</td>
<td>-</td>
</tr>
<tr>
<td><strong>α-Glucosidase inhibitory activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (^b) (%)</td>
<td>-</td>
<td>-</td>
<td>51.8±0.3</td>
<td>-</td>
</tr>
<tr>
<td>Acarbose Eq. (µmol/g DW)</td>
<td>-</td>
<td>-</td>
<td>7.2±0.1</td>
<td>-</td>
</tr>
<tr>
<td><strong>ACE inhibitory activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (^c) (%)</td>
<td>-</td>
<td>-</td>
<td>23.0±2.0</td>
<td>-</td>
</tr>
<tr>
<td>Captoprill Eq. (µmol/g DW)</td>
<td>-</td>
<td>-</td>
<td>1.7±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

All data represent the mean ± standard deviation of at least three independent experiments. Means with different letters in the same row were significantly different at the level (p< 0.05).

\(^1\) Oxygen radical absorbance capacity; \(^2\) Ferric reducing antioxidant power; \(^3\) IC50-Half minimal inhibitory concentration. The concentration of extract used in enzymatic reaction was: \(^a\) 4 mg/ml, \(^b\) 6.7 mg/ml, \(^c\) 0.6 mg/ml.
Table 4. Correlation analysis

<table>
<thead>
<tr>
<th></th>
<th>FRAP</th>
<th>ORAC</th>
<th>Pancreatic lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics (Folin-Ciocalteu method)</td>
<td>0.8757</td>
<td>0.9456</td>
<td>0.8424</td>
</tr>
<tr>
<td>Phenolics (at 280 nm; HPLC method)</td>
<td>0.8953</td>
<td>0.9570</td>
<td>0.8599</td>
</tr>
<tr>
<td>Phenolics (at 326 nm; HPLC method)</td>
<td>0.8603</td>
<td>0.9250</td>
<td>0.8080</td>
</tr>
<tr>
<td>Phenolics (at 370 nm; HPLC method)</td>
<td>0.8470</td>
<td>0.9220</td>
<td>0.8050</td>
</tr>
<tr>
<td>Ferric reducing antioxidant power (FRAP)</td>
<td>1</td>
<td>0.9683</td>
<td>0.9542</td>
</tr>
<tr>
<td>Oxygen radical absorbance capacity</td>
<td>0.9683</td>
<td>1</td>
<td>0.9691</td>
</tr>
</tbody>
</table>
Fig. 1. Dose-dependent inhibition of pancreatic lipase activity by ethanol-based lyophilized crude hydrophilic extracts prepared from Dactylorhiza chuhensis flower, leaf, stem and tuber.
Fig. 2. The effect of *Dactylorhiza chuhensis* crude leaf extract on the proliferation of murine macrophage RAW 264.7 (A) and hepatocellular carcinoma (HepG2) (B) cells.

**A**

![Graph showing the effect of Dactylorhiza chuhensis crude leaf extract on the cell survival rate of RAW 264.7 cells.](image)

**B**

![Graph showing the effect of Dactylorhiza chuhensis crude leaf extract on the cell survival rate of HepG2 cells.](image)
Fig. 3. The effect of *Dactylorhiza chuhensis* crude leaf extract on nitric oxide (NO) production in lipopolysaccharide (LPS) - activated murine macrophage RAW 264.7 (A) and hepatocellular carcinoma (HepG2) (B) cells.
Highlights:

1. *Dactylorhiza chuhensis* leaf extract has moderate enzyme inhibitory activities.
2. *Dactylorhiza chuhensis* leaf extract exhibits anti-inflammatory properties.
3. The leaf of *D. chuhensis* can serve as a source of physiologically active phytochemicals.