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Dracocephalum kotschyi: Inhibition of critical enzyme relevant to type-2 diabetes, essential oil composition, bactericidal and anti-oxidant activity

Mehri Kazempour, S. Shirin Shahangian*, Reyhaneh Sariri

Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

** Corresponding author's E-mail: shahangian@guilan.ac.ir*

ABSTRACT

Dracocephalum kotschyi, an endemic medicinal plant in Iran, has long been used in folk medicine to treat various disorders. This study aimed to determine the phytochemical composition and biological activities of various extracts of leaves and flowers of *D. kotschyi*. Different solvent extracts (aqueous solution of acetone, methanol, ethanol, and a mixture of acetone and methanol) of *D. kotschyi* were screened for anti-amylase activity, antioxidant potential, and antibacterial efficiency. Antioxidant effects were elucidated by the methods such as DPPH and FRAP. The inhibitory potential of the extracts against amylase, a key enzyme involved in diabetes, was investigated by the Bernfeld method. The total phenolic, flavonoid, and anthocyanin contents of these extracts were also calculated. Disc diffusion, MIC, and MBC methods were applied to analyze the antibacterial efficiency of the extracts. GC-MS analysis of the essential oil was performed. Additionally, the HPLC method was used for the identification and quantification of caffeic acid. Based on antioxidant assays, the acetonic extract showed the highest antioxidant ability, due to its highest total antioxidant content. Also, the acetonic extract strongly inhibited α-amylase activity. Various extracts of *D. kotschyi* displayed inhibitory effects against both Gram-positive and Gram-negative bacteria. Analyzing the essential oils of the leaves and flowers of *D. kotschyi* by GC-MS led to identifying 6 and 19 compounds, respectively. These results suggest that *D. kotschyi* can be considered a promising source of natural antioxidant, antimicrobial, and anti-amylase agents for managing oxidative damage, as well as pharmaceutical and food purposes.

Keywords: *Dracocephalum kotschyi*, Chemical composition, Anti-amylase, Antioxidant, Antibacterial.
Article type: Research Article.

INTRODUCTION

Oxidative stress occurs from the imbalance between pro-oxidant and antioxidant defence systems, leading to the formation of toxic forms of oxygen and other free radicals. Different types of oxidative stress can cause the development of various chronic disorders, including atherosclerosis, cancer, aging, neurodegenerative disorders (Alzheimer's and Parkinson's disease), and type 2 diabetes by damaging macromolecules such as proteins, DNA, membrane lipids as well as inactivation of enzymes (Bjelakovic *et al.* 2014; Jiang 2014). Free radicals can also cause damage in different industries. For instance, they may oxidize the fatty acids during the production process, transport, and storage of food, which leads to rancidity in foods. Oxidized fatty acid consumption leads to cell damage and therefore causes the progression of different chronic diseases (Sarmadi & Ismail, 2010). Synthetic antioxidants such as tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxyl toluene (BHT) are widely used in food to prevent the oxidation of lipids (Girgih *et al.* 2015). Since using synthetic antioxidants for a long time may cause serious health problems, many investigations have become more interested in natural antioxidants (Chalamaiah *et al.* 2012; Farvin *et al.* 2014). Natural ingredients in

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medicinal plants are potential candidates to prevent oxidative stress and reduce its harmful effects (Girgih *et al.* 2015). As a well-known type of secondary metabolite, phenolic compounds are widely distributed in plants and possess different biological activities (anti-oxidant, anti-bacterial, anti-mutagenic effects, etc.; Shin *et al.* 2018). The antioxidant activity of phenolic compounds occurs through various mechanisms, including scavenging free radicals by donation of hydrogen atoms or electrons, converting dangerous oxidative products to non-oxidant molecules, reducing oxygen levels, and acting as chelators of metals (Rice-Evans *et al.* 1997). In addition, some polyphenol compounds exhibit an antimicrobial activity that can act effectively against a broad range of microorganisms (Rice-Evans *et al.* 1997). Beside the antioxidant activity, the secondary metabolites could also play other different roles, including the inhibition of certain disease-associated enzymes. For instance, voglibose, galantamine, and kojic acid are currently used as pharmaceuticals to reduce the actions of α-amylase, cholinesterase, and tyrosinase, respectively. However, due to their side effects such as gastrointestinal disturbance and cytotoxicity, many attempts have been made to find more efficient and safer alternatives than natural sources (Lee *et al.* 2011; Saha & Verma 2012; Zahrae Redouan *et al.* 2020; Khademian Amiri *et al.* 2022; Obaid *et al.* 2022). *Dracocephalum* genus, belonging to the Lamiaceae family, has about 186 species, eight of which are in the flora of Iran. *Dracocephalum kotschyi* Boiss (locally known as Zarrin-giah or Badrandjboie-Dennaie) is among the most important endemic species (Heydari *et al.* 2019). *D. kotschyi* is used in Iranian traditional medicine as a natural remedy for the treatment of stomach disorders. Traditional medicine documents show that it is also useful for headaches, inflammatory pain, congestion, and liver disorders (Jahaniani *et al.* 2005). Anti-hyperlipidemic, anti-nociceptive and immunomodulatory activities have already been reported for *D. kotschyi* (Golshani *et al.* 2004). Many effective constituents have recently been identified from the essential oils of *D. kotschyi* collected from different regions of Iran. These results demonstrate significant regional variation in the chemical compositions of *D. kotschyi* (Ashrafi *et al.* 2017). The extraction method of the antioxidants affects the amount of phenolic and antioxidant activities of the extracts. It is ideal if the process could completely extract compounds of interest without their chemical modification (Zuo *et al.* 2002). The antioxidant activity of extract is not only affected by the method of extraction, but also by the solvent for its preparation. Different solvent systems are used to extract the plant's phenolic compounds (Pokorný & Korczak 2001). However, the extraction yields and the antioxidant capacities of plant materials significantly depend on the nature of the solvent used for extraction, owing to the existence of various bioactive compounds of different chemical properties and polarities, which makes them soluble or not in a particular solvent (Turkmen *et al.* 2006). Polar solvents are the most widely-used solvents for recovering phenolic compounds from plant materials, so that, aqueous mixtures of ethanol, methanol, and acetone are the best solvents (Do *et al.* 2014). To our knowledge, there is no study focusing on the optimization of the extraction process of phenolic compounds from *D. kotschyi*. Accordingly, the present study objective was to evaluate the effect of different extraction solvents on the bioactive components (total phenolic, flavonoid, and anthocyanin), antioxidant, anti-bacterial, and anti-α-amylase activities of its leaves and flowers. In addition to the study of biological activity, phytochemical compositions of its essential oil inside its leaves and flowers were determined by GC-MS. Furthermore, the amount of caffeic acid in various extracts of *D. kotschyi* was determined by the HPLC method.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu (FC) reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), quercetin, gallic acid, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), DNS (3,5-Dinitrosalicylic acid), and pancreatic α-amylase (3.2.1.1) were obtained from Sigma Co. (St. Louis, MO, USA). Potassium ferricyanide [$K_3Fe(CN)_6$], ammonium iron (II) sulfate hexahydrate [$(NH_4)_2Fe(SO_4)_2.6H_2O$], aluminum chloride hexahydrate ($AlCl_3.6H_2O$), and ferric chloride ($FeCl_3$) were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents (such as methanol, acetone and ethanol) were of analytical-grade purity. The specific buffers were freshly prepared in our biochemistry laboratory.

Plant collection

Dracocephalum kotschyi was collected from the Rostamabad region in Gilan Province, northern part of Iran, in June 2020. It was identified by a senior taxonomist and confirmed based on its microscopical, and macromorphological characteristics.

Preparation of plant extracts

The aerial parts, flowers, and leaves, of the *D. kotschyi* were separated, washed, air-dried in the shade, and then ground to a fine powder. Extraction of samples was performed using the aqueous solution (70%) of acetone, methanol, ethanol, and a mixture of acetone, and methanol (80:20 v/v) for one day. The mixture was then filtered through Whatman No. 1 filter papers and, the second extraction was carried out. Both supernatants were mixed and concentrated under the vacuum in a rotary evaporator. The extraction for each solvent was performed three times. The residues obtained were preserved at 4 °C until further examinations.

Plant extraction yield (EY)

The yield of the extraction was calculated by $\{(W_1/W_2) \times 100\}$, where W_1 is the weight of extract after evaporation of the solvent and W_2 is the dry weight of the plant sample (Metrouh-Amir *et al.* 2015).

Preparation of essential oil

The extraction of essential oils of *D. kotschyi* was carried out by hydro-distillation method for 3.5 h using a Clevenger-type apparatus. The oils were dried under anhydrous sodium sulfate and kept in the refrigerator until analysed.

GC-MS analysis

The Gas chromatography-Mass spectroscopy on HP-6890/HP5 equipment (Agilent-7890A) was used for chemical constituent analysis. The GC was equipped with a TRB-5MS column (32 m × 0.25 mm × 0.25 mm). The carrier gas was Helium, split injecting was 1 mL with 1 mL min^{-1} flow rate and the injecting temperature was 60 °C to 220 °C with an increased rate of 6 °C min^{-1}.

Total phenols content (TPC) determination

The modified Folin-Ciocalteu assay (Meda *et al.* 2005) with gallic acid as standard was conducted to determine the amount of total phenols of extracts. Briefly, 100 µL of appropriately diluted extract or standard solutions, 2.8 mL dH_2O, 2 mL Na_2CO_3 (2%), and 100 µL Folin-Ciocalteu reagent were mixed, vortexed, and left for 25 min at ambient temperature. The absorbance of the resulting mixture was read at 720 nm. The TPC was expressed as mg GAE/g DW (mg gallic acid equivalent / g dried weight).

Total flavonoids content (TFC) determination

The aluminium chloride colorimetric assay was used for the quantification of the content of total flavonoid (Chang *et al.* 2002). Quercetin was employed as standard. In brief, the appropriately diluted extracts (500 µL) were mixed with $AlCl_3(6H_2O)$ [100 µL, 10% (w/v)], potassium acetate (100 µL, 1.0 M), and dH_2O (2.8 mL). The absorbance of the mixture was measured at 415 nm after 40 min of incubation at ambient temperature. The TFC was reported as milligram of quercetin equivalent/g DW.

Total anthocyanin content (TAC) determination

The amount of total anthocyanin was assessed by a previously described method (Mita *et al.* 1997). Briefly, 0.02 g of flowers and leaves of *D. kotschyi* was homogenized in acidified solvents [ethanol, methanol, and acetone containing 1% HCl (v/v)] and kept for one day in the refrigerator. The extracts were then centrifuged and the absorbance was measured at 530 and 657 nm for each supernatant. TAC was calculated using the following equation.

Anthocyanin content (mg/g DW) = absorbance at 530 nm - (0.25 × absorbance at 657 nm)

DPPH radical scavenging activity

The ability of different extracts to scavenging of DPPH radical was evaluated as described before (Miliauskas *et al.* 2004). The DPPH solution (1.0 mL, 0.1 mM) was mixed with 1.0 mL of different concentrations (0.05-3 mg mL^{-1}) of each extract. The mixture was placed in the dark at room temperature for 35 min, afterward its absorbance was recorded at 517 nm. DPPH solution (0.1 mM) was used separately as control. The percentage of scavenging activity was determined from $[(A_{control}-A_{extract})/A_{control} \times 100]$, where $A_{control}$ and $A_{extract}$ are the absorb of the control

and extract, respectively. IC_{50} was reported as the amount of extracts causing a 50% inhibition of DPPH radical. Butylated hydroxyanisole (BHA) served as the positive control.

FRAP assay

A formerly described protocol was used to evaluate the ferric reducing power of extracts (Wijekoon *et al.* 2011). Briefly, FRAP reagent included acetate buffer (10 mL, 0.3 M, pH 3.6), TPTZ solution (1 mL, 10 mM) in 40 mM HCl and $FeCl_3$ (1 mL, 20 mM). 1.5 mL of prepared reagent was mixed with 0.5 mL of extract and thoroughly vortexed. After the mixture was incubated for 25 min in the dark at 25 °C, the absorbance was read at 593 nm. Ammonium iron (II) sulfate hexahydrate was used as the standard. The FRAP value was expressed as mM $FeSO_4$ equivalents per gram of dry weight.

Determination of caffeic acid content by HPLC

HPLC analysis of caffeic acid was carried out using Agilent 1200 series HPLC system. The chromatographic separation was performed on a C18 reversed-phase analytical column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of water: methanol (95:5) as solvent A and methanol: acetonitrile (50:50) as solvent B. The gradient elution was operated at a flow rate of 1.0 mL min^{-1} as follows: from 0 to 15 min A:B (98:2), from 15 to 35 min A:B (70:30), from 35 to 45 min A:B (55:45) and from 45 to 60 min A:B (5:95).

α-amylase inhibitory activity

The inhibition effect of various extracts on α-amylase activity was assessed according to a previously described method (Bernfeld 1955). At first, the enzyme (50 µL, 0.012 mg mL), and different extracts concentrations were mixed and incubated at 37 °C for 15 min. The enzyme reaction was then started by adding 100 µL starch (1% w/v). After 3 min, 200 µL DNS was added to terminate the reaction. The mixture was incubated for 5 min at 100 °C, cooled, and diluted with 3.6 mL distilled water. The absorbance was recorded at 540 nm against the blank (The blank sample was the same as the other samples except that the enzyme was removed). The control sample was prepared with the same procedure, except that distilled water was added instead of the extracts. The inhibition rate (%) was calculated by $I\ (\%) = (\Delta A_{control} - \Delta A_{sample}) / \Delta A_{control} \times 100$. Based on the obtained results, the value of IC_{50} was calculated by plotting the Iα-amylase (%) versus the extract concentrations. Acarbose (α-amylase inhibitory drug) at a concentration range of 2-50 µg mL^{-1} was used as the positive control.

Antibacterial activity

The antibacterial activity of various extracts of *D. kotschyi* was examined on both Gram-negative (*Escherichia coli* PTCC-1338 and, *Pseudomonas aeruginosa* PTCC-1430) and Gram-positive (*Staphylococcus aureus* PTCC-1112 and *Micrococcus luteus* PTCC-1110) bacteria.

Disc diffusion method

The Inoculum density of the bacteria was standardized according to the 0.5 McFarland standard and uniformly spread on the Luria-Bertani (LB) agar plates. The sterilized blank disks (6 mm in diameter) were placed on the plates and 20 µL of the test sample was poured on the discs. Thereafter, the plates were kept in a refrigerator (4 °C) for 1 h, followed by further incubation at 37 °C for 24 h. Evaluation of antibacterial activity was performed by measuring the inhibitory zone surrounding the disks.

Microdilution assays

The Minimum inhibitory concentration (MIC) was evaluated using the micro-dilution broth method in a 96-well micro-plate. The plate was prepared by dispensing LB medium (50 µL) and bacterial suspensions (50 µL) into each well. Different concentrations of extracts (50 µL) were then added into the wells, and the absorbance was read immediately at 600 nm. The absorbance was measured again after 18 h of incubation at 37 °C. By definition, the lowest concentration, which decreased the absorbance, was taken as MIC. To obtain the value of minimum bactericidal concentration (MBC), 50 µL of material in MIC well was cultured in Luria-Bertani (LB) agar. The lowest concentration of extract that did not show any bacterial growth was taken as the MBC.

Statistical analysis

All data were reported as mean ± SD (standard deviation) and carried out with triplicates. Statistical analysis was carried out with ANOVA and Tukey post hoc test with $p < 0.05$. SPSS software (version 26, SPSS Inc., Chicago, IL) was used for all analyses.

RESULTS AND DISCUSSION

Chemical composition of EOs

Essential oils (EOs) from medicinal plants are a complex mixture of volatile secondary metabolites that are obtained from different parts of plants through various methods. GC/MS chromatogram of compounds identified in the leaves and flowers essential oils of *D. kotschyi* was shown in Fig. 1. The results showed that EOs from the leaves and flowers of *D. kotschyi* possess 6 and 19 different chemical compounds, respectively. Results presented in Table 1 depict that the major compounds of flower EOs were perillaldehyde (26.041%), Z-citral (14.875%), E-citral (14.076%), and dl-limonene (14.039%). The leaf EOs contained E-citral (23.951%), Limonene (21.574%), 2-cyclohexen-1-one (21.404%), and Z-citral (17.305%) as the major compounds (Table 1). Perilla aldehyde, as one of the major compounds of *D. kotschyi*, exhibits a broad spectrum of biological effects, including anticancer, antimicrobial, and vasodilatory activities. Also, perylla aldehyde shows activation effects on Transient Receptor Potential A1 (TRPA1) channel, which may be related to its anti-inflammatory effects (Khodaei *et al.* 2018). Several studies have been carried out regarding the chemical composition of *D. kotschyi* from different regions of Iran. Despite some similarities in reported ingredients in previous analyses with the present study, the samples obtained from various areas of Iran exhibit substantial qualitative and quantitative differences.

Table 1. Chemical composition of essential oils of flower and leaf of *D. kotschyi*.

Flower			Leaf		
Compounds	**Retention time (min)**	**Area (%)**	**Compounds**	**Retention time (min)**	**Area (%)**
α-Pinene	3.405	2.606	Limonene	4.860	21.574
Sabinene	3.975	0.627	2-Cyclohexen-1-one	8.043	21.404
Beta-Myrcene	4.229	1.532	Z-Citral	8.659	17.305
dl-Limonene	5.091	14.039	E-Citral	9.171	23.951
α-Campholene aldehyde	6.180	0.529	Neric acid	10.772	8.854
Cis-Sabinene hydrate	6.265	0.684	Laurobtusol	12.789	6.912
α-campholenal	6.677	2.294	-	-	-
Trans-Limonene oxide	6.873	2.880	-	-	-
Phellandral	7.327	1.602	-	-	-
α -Thujone	7.651	1.121	-	-	-
Pulegone	7.974	2.272	-	-	-
Perilla aldehyde	8.455	26.041	-	-	-
Z-Citral	9.163	14.875	-	-	-
E-Citral	9.752	14.076	-	-	-
Perilla alcohol	9.906	1.511	-	-	-
Geranic acid	10.380	5.670	-	-	-
Geranyl acetate	11.230	4.690	-	-	-
Germacrene D	12.747	0.527	-	-	-
Farnesol	15.079	0.923	-	-	-
Total	-	98.449	-	-	100

According to an investigation conducted by Javidnia *et al.* (2005), α-pinene (10.5%), caryophyllene oxide (9.2%), terpinen- 4-ol (5.7%), and germacrene D (5.6%) were major components in *D. kotschyi* obtained from Muteh protected region in Isfahan Province. In addition, in EO of *D. kotschyi*, collected from Garin mountains (3200 m) beside Alshtar in Lorestan Province, Iran, Geranial (12.1%), α-Pinene (10.34%), Geraniol acetate (10.27%), Geraniol (9.55%), Neral (8.9%) and Limonene (6.95%) were determined as main compounds (Ashrafi *et al.* 2017). These differences may be linked to climatic and seasonal conditions, the geographical location of collection sites, harvest periods, and experimental conditions (Heydari *et al.* 2019).

Extract yields

The results showed that the extraction rates obtained were affected by the type of solvent used (Table 1). The difference in the extraction rates of extracts is related to the polarities of various compounds present in *D. kotschyi*. The highest yield in extracts was achieved by the aqueous acetone solvent. These results indicate that the plant contains more polar substances than the others. Extraction rate, total polyphenol content, antioxidant, and antibacterial activities depend considerably on the extraction method and the solvent nature (Metrouh-Amir *et al.* 2015).

Determination of antioxidant activity

Medicinal plants have a special place in traditional medicine and the management of different diseases, especially in developing countries. They possess antioxidant, antibacterial, antidiabetic, and many other biological activities. Most of their antioxidant activity may be associated with phenolic and flavonoid groups that are known as plant secondary metabolites (Heydari *et al.* 2019). In this study, the total phenolic contents of various extracts of *D. kotschyi* were determined using the Folin-Ciocalteu method. It was found that the total phenolic content of various extracts ranged between 0.13 ± 0.03 and 0.71 ± 0.11 mg GAE/g DW (Table 2).

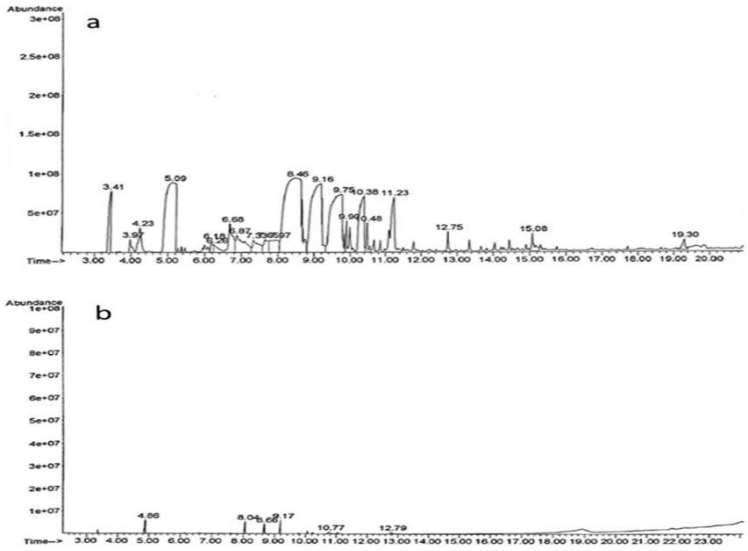

Fig. 1. GC/MS chromatogram of compounds identified in the flower (a) and leaf (b) essential oils of *D. kotschyi*.

Leaf and flower extracts of *D. kotschyi* obtained by acetone exhibited a meaningful difference ($p < 0.05$) compared to other extracts (0.71 ± 0.11 and 0.63 ± 0.07 mg GAE/g DW for leaves and flower extracts, respectively). The effects of various solvents on the content of the plants total phenolic and antioxidant activity have been investigated in several studies. In support of our results, it has been reported that the highest content of total phenolic compounds in cumin seeds, *Cuminum cyminum* L. (Rebey *et al.* 2012), the by-product of eggplant and lychee, *Litchi chinenesis* Sonn. and flowers (Liu *et al.* 2009) were obtained using an aqueous-acetone solvent.

Flavonoids, as the main group of natural compounds, are related to a wide range of health-promoting properties such as reducing the incidence of many diseases, cardioprotective, and neuroprotective effects. Due to the existence of the OH group in their structure, flavonoids are mostly responsible for the antioxidant activity of many plants (Panche *et al.* 2016; Ruiz-Cruz *et al.* 2017). The total flavonoid contents of the *D. kotschyi* extracts are shown in Table 2 exhibiting that the flavonoid content was statistically different in various extracts ($p < 0.05$) and

was between 0.16 ± 0.07 and 0.99 ± 0.16 mg QE/g DW. The effects of the solvent types on the extraction of the total content of flavonoids followed a similar trend to total phenol, so that, the acetonic extract displayed the highest level of flavonoid content, compared to other extracts. An aqueous mixture of acetone is the most appropriate solvent for extracting the higher molecular weight flavanols (Dai & Mumper 2010). Our findings were consistent with the results of some previous studies (Zieliński & Kozłowska 2000; Sulaiman *et al.* 2011). Anthocyanins are one of the most important groups of plant phytochemicals that have numerous health benefits (antioxidant and anti-inflammatory) or nutraceutical effects on the cognitive abilities of the brain, obesity, cardiovascular disease, and prevention of cancer (Lila 2004). In this study, total anthocyanins extraction was performed using three solvent systems (acidified methanol, ethanol, and acetone). As shown in Table 2, the best solvent for extracting anthocyanins is acidified methanol. The total anthocyanin contents were 0.26 ± 0.08 and 0.16 ± 0.04 mg g^{-1} DW for the methanolic extracts of flowers and leaves, respectively. Acidified organic solvents, especially methanol, are widely employed for anthocyanins extraction from plant materials (Boeing *et al.* 2014). The acidic nature of methanol can dissolve and stabilize the anthocyanins after disrupting the cell membrane (Naczk & Shahidi 2006). This finding is consistent with previous studies (Wijekoon *et al.* 2011; Boulekbache-Makhlouf *et al.* 2013). The DPPH method is the most common assay for assessing the antioxidant capacity of plant extracts based on the reduction of the DPPH radical. The DPPH radical exhibits maximum absorption at 517 nm, which loses this absorption by accepting an electron or hydrogen atom from an antioxidant (Noipa *et al.* 2011). DPPH radical-scavenging ability of plant extracts expressed by the IC_{50} value. The lowest value of IC_{50} indicates the highest antioxidant activity of extracts. Various extracts of *D. kotschyi* showed significant potential in DPPH free radicals scavenging. According to Table 2, the acetonic extracts from leaves and flowers exhibited the most significant antioxidant activity with the IC_{50} value of 1.02 ± 0.18 and 3.17 ± 0.31 mg mL^{-1}, respectively. The highest total phenolic and flavonoid content were found in the acetonic extract, reflecting in the lower IC_{50} of this extract compared to others. The synthetic antioxidant, BHA exhibited a higher DPPH radical scavenging effect than all extracts of *D. kotschyi*. Noteworthy, the type of extracted antioxidants depends on the polarity, viscosity, and vapor pressure of the solvent. It has been demonstrated that fewer viscose solvents can diffuse more easily into the plant pores leading to the extraction of more diverse bioactive secondary metabolites (Naczk & Shahidi 2006). Following several studies, aqueous acetone is the most suitable solvent for high efficient extraction of antioxidants and subsequent antioxidant activity. According to a study carried out by Zhao *et al*. (2006), the acetone/water (8:2) solvent system is most preferred to achieve the greatest antioxidant activity of *Hordeum vulgare* L. In addition, Kchaou *et al*. (Kchaou *et al.* 2013) reported that the extract obtained using aqueous acetone had the maximum polyphenolic compounds and displayed higher DPPH radical-scavenging activity than other solvents, which is in accordance with the present study. The antioxidant capacity of extracts was also evaluated using FRAP assay, which is based on the reduction of the Fe^{3+}–TPTZ complex to the intensely blue-coloured Fe^{2+}–TPTZ exhibiting maximum absorption at 593 nm (Moon & Shibamoto 2009). Table 2 depicts the FRAP values of different extracts, and the values were consistent with the antioxidant potential obtained by the DPPH assay. The result showed that acetonic extracts from leaves and flowers possessed the strongest ferric-reducing power compared to other extracts [0.64 ± 0.07 and 0.47 ± 0.01 mM Fe (II)/ g DW for leaves and flowers acetonic extract, respectively]. The antioxidant activity exhibited by plants is of considerable interest in both scientific research and industrial applications. Natural food additives are rapidly replacing synthetic ones. Our present results showed that the antioxidant activity of *D. kotschyi* extract is associated with the chemical composition obtained from GC-MS analysis. It is suggested that these extracts are appropriate candidates to be used as a potential preservative in food products, thereby acquiring protection against microbial spoilage and acting against their oxidative deterioration.

Analysis of extracts by HPLC

The determination of phenolic compounds by HPLC is one of the most dominant analytical procedures. This rapid instrumental analysis possesses many advantages including short analysis time, comfortable sample treatment, changing the mobile phase's polarity during the examination, and being highly reproducible (Kumar 2017). In the present study, HPLC analysis was used for the identification and quantification of caffeic acid, present in various extracts of *D. kotschyi* (Fig. 2). The results showed that the highest amount of caffeic acid in leaf and flower of *D. kotschyi* was found in acetonic (1.711 µg g^{-1}DW) and methanolic (1.152 µg g^{-1}DW) extracts, respectively (Table 3). Caffeic acid consists of both phenolic and acrylic functional groups and possesses many health benefits,

including anti-inflammatory, anticancer, antidiabetic, and antiviral abilities (Espíndola *et al.* 2019). Also, caffeic acid has anti-prostaglandin effects, a feature of conventional medicines used to treat dysmenorrhea (Zhu *et al.* 2009). Previous studies reported that caffeic acid and other phenolic compounds such as chlorogenic acid, phenylpropanoids, and flavonoids are probably responsible for the antioxidant properties of *D. kotschyi* (Heydari *et al.* 2019).

Table 2. Extraction yields, Total bioactive components and antioxidant activities of various extracts of *D. kotschyi*.

Sample	Solvent	EY (%)	TPC (mgGAE/gDW)	TFC (mgQE/gDW)	TAC (mg/gDW)	DPPH IC_{50} (mg/ml)	FRAP (mMFe(II)/gDW)
Leaf	Acetone	25.58 ± 1.04	0.71 ± 0.11^{a}	0.99 ± 0.16^{a}	0.09 ± 0.03^{b}	1.02 ± 0.18^{a}	0.64 ± 0.07^{a}
	Methanol	21.87 ± 1.23	0.58 ± 0.09^{b}	0.83 ± 0.13^{b}	0.16 ± 0.04^{a}	1.83 ± 0.23^{b}	0.41 ± 0.08^{b}
	Ethanol	20.23 ± 1.01	0.43 ± 0.07^{c}	0.70 ± 0.12^{c}	0.08 ± 0.02^{b}	2.11 ± 0.2^{c}	0.26 ± 0.03^{c}
	Acetone:Methanol	18.92 ± 0.94	0.23 ± 0.05^{d}	0.28 ± 0.09^{d}	0.03 ± 0.01^{c}	2.84 ± 0.24^{d}	0.19 ± 0.04^{d}
Flower	Acetone	17.03 ± 1.12	0.63 ± 0.07^{a}	0.52 ± 0.11^{a}	0.13 ± 0.04^{b}	3.17 ± 0.31^{a}	0.47 ± 0.01^{a}
	Methanol	15.75 ± 1.45	0.49 ± 0.05^{b}	0.35 ± 0.1^{b}	0.26 ± 0.08^{a}	3.86 ± 0.26^{b}	0.34 ± 0.03^{b}
	Ethanol	13.85 ± 1.61	0.34 ± 0.05^{c}	0.24 ± 0.07^{c}	0.15 ± 0.03^{b}	4.34 ± 0.33^{c}	0.2 ± 0.06^{c}
	Acetone:Methanol	10.39 ± 1.14	0.13 ± 0.03^{d}	0.16 ± 0.07^{d}	0.06 ± 0.02^{c}	5.4 ± 0.4^{d}	0.12 ± 0.04^{d}
BHT	-		-	-	-	0.03 ± 0.001	-

Note: Each value is expressed as mean ± SD (n = 3). Means, in the same column, with different letters are significantly different ($p < 0.05$).
TPC: Total phenolic content; TFC: Total flavonoid content; TAC: Total anthocyanin content; EY: Extraction yield.

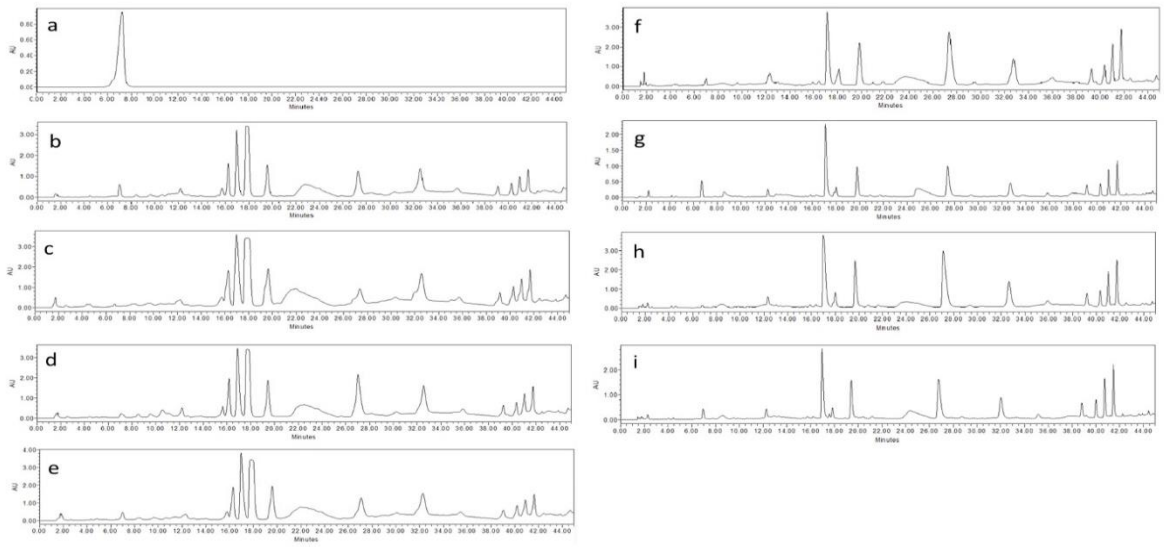

Fig. 2. HPLC chromatogram of standard (a) and caffeic acid content in various extracts of *D. kotschyi*; b, c, d and e related to acetone, methanol, ethanol, and acetone:methanol extracts of leaf and f, g, h and i related to acetone, methanol, ethanol, and acetone: methanol extracts of the flower.

Table 3. Caffeic acid content and enzyme inhibitory activity of various extracts of *D. kotschyi.*

Sample	Solvent	Caffeic acid (µg/gDW)	IC_{50} (mg mL^{-1})
Leaf	Acetone	1.711	0.34 ± 0.036
	Methanol	0.190	0.45 ± 0.05
	Ethanol	0.245	0.51 ± 0.03
	Acetone:Methanol	0.548	0.59 ± 0.05
Flower	Acetone	0.439	1.72 ± 0.1
	Methanol	1.152	1.93 ± 0.07
	Ethanol	0.096	2.31 ± 0.17
	Acetone:Methanol	0.947	2.67 ± 0.09
Acarbose			0.023 ± 0.003

Effect of extracts on the activity of amylase

α-Amylase is one of the important digestive enzymes that hydrolyse starch to simple sugars. Inhibition of this enzyme can delay the digestion of carbohydrates, and cause a reduction in the absorption of glucose, thereby blunting the postprandial plasma glucose rise (Ali *et al.* 2006). On the other hand, natural phenolic compounds from medicinal plants have been reported that possess an inhibitory effect on carbohydrate-hydrolysing enzymes (Aryaeian *et al.* 2017). These together led us to explore the α-amylase inhibitory activity of extracts obtained from *D. kotschyi*. The inhibition of α-amylase by extracts is presented by the IC_{50} value (Fig. 3). The data in Table 3 indicated that acetone extracts from leaves and flowers exhibited the highest inhibitory activity with the IC_{50} value of 0.34 ± 0.036 and 1.72 ± 0.1 mg mL^{-1}, respectively. Interestingly, the acetone extract possessed the highest level of phenolic content (Table 1). Based on our literature survey, the anti-amylase activity of our plant parts has not been reported so far. The effect of plant extracts on α-amylase and a-glucosidase has been investigated, aiming to find a cure for diabetes. Recently, Vadivelan *et al.* (2019) have reported an inhibitory effect of *Asparagus racemosus* Willd extract on α-amylase and α-glucosidase. They used various solvents for the extraction process. Similar to our results, they found the *in-vitro* inhibitory effect on α-amylase activity. In their discussion, they suggested that the extract of *Asparagus racemosus* Willd can be used as a good candidate for the management of type 2 diabetes mellitus. The natural α-amylase inhibitors from the food-grade plant sources offer a potential therapeutic strategy to treat postprandial hyperglycaemia, which could help manage diabetes mellitus. The results of the α-amylase test in our study confirmed the *in-vitro* potential of *D. kotschyi* to inhibit this essential carbohydrate-digesting enzyme.

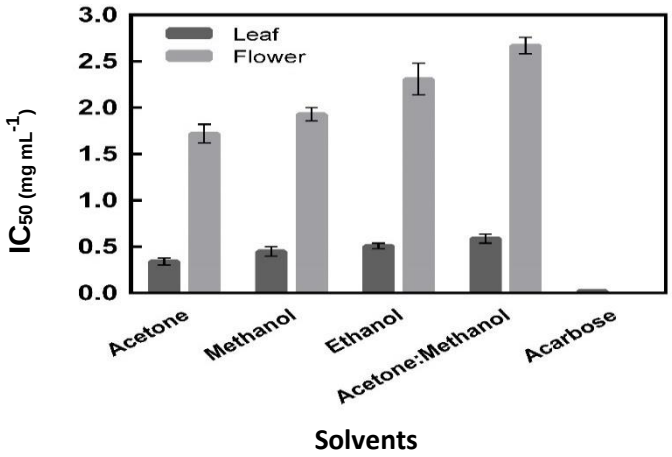

Fig. 3. Enzyme inhibitory activity of the solvent extracts from various extracts of *D. kotschyi*.

3.6. Antibacterial activity

The antibacterial potential of various extracts of *D. kotschyi* was investigated against some Gram-positive and Gram-negative bacteria, which are well-known foodborne pathogens by agar disc diffusion and broth microdilution methods. As depicted in Table 4, almost all extracts of our plant exhibited varying levels of antibacterial activity. It is suggested that the antibacterial effect of plant extracts is mainly dependent on the extract concentration, extraction solvent used for extract preparation, and the specific part of the plant (Anyanwu & Okoye 2017). We found that the flower extracts exhibited higher antibacterial activity than various extracts from the leaf. Also, the flower extract obtained using acetone as the solvent showed a more powerful antibacterial effect than other extracts. The highest activity observed for this extract was 21.0 mm against, *S. aureus*, followed by *E. coli*, *M. luteus* and *P. aeruginosa* (inhibition zones of 18.0, 15.0 and 10.0 mm respectively). Higher contents of phenolic compounds and flavonoids in acetone extract may lead to its better anti-bacterial effect. The data obtained from the MIC and MBC are presented in Table 5. The extracts were active against all tested strains, with average MIC ranging from 3.75 to 30.0 mg mL^{-1} and 3.75 to 240.0 mg mL^{-1} for MBC. The correlation between antibacterial properties and phytochemical compositions has been investigated by some studies. For instance, It was reported that the high antibacterial activity of the methanolic extract of *Rumex dentatus* L. could be due to the presence of the highest polyphenol content in the extract (Humeera *et al.* 2013). As critical bioactive constituents of plants, phenolic compounds, flavonoids, tannins, and alkaloids play critical roles in the antibacterial properties of plants. They could bind proteins, act as chelating agents and induce inflammation in bacterial cells, thereby interfering with biochemical processes, and threatening their life (Ahumada-Santos *et al.* 2013). Phenolic compounds could present their antibacterial ability via interacting with enzymes, abrogating synthesis of nucleic acids, deprivation of metal ions, adsorption to the cell membrane, and disrupting the permeability barrier of bacterial cell envelopes (Scalbert 1991). In agreement with our study, chemical screening of another species of *Dracocephalum* has been reported. The authors have stated that the presence of some secondary metabolites, including monoterpenoids, phenolic, alcoholic compounds, terpenoids, and alkaloids in the plant, could be responsible for its considerable antibacterial effect (Fallah *et al.* 2018). We suggest that an altered percentage of chemical compounds present in different parts of the plant could be among the important contributing factors for the substantial antimicrobial activity of flower and leaf extracts of *D. kotschyi*.

Table 4. Anti-bacterial activity of various extracts of *D. kotschyi* at different concentrations against Gram-positive and Gram-negative bacterial strains.

Sample	Solvent	Conc. (mg mL^{-1})	Zone of inhibition (mm)			
			M. luteus	*S. aureus*	*E. coli*	*P. aeruginosa*
Leaf	Acetone	120	17	18.5	16	15
		60	14.5	15	13	10
		30	10	12	10	7.5
		15	7	9	7	-
	Methanol	120	13	17	12	10
		60	9	14	8	-
		30	7	9.5	-	-
		15	-	-	-	-
	Ethanol	120	12	15	13	10
		60	7	11	8	-
		30	-	8	-	-
		15	-	-	-	-

	Acetone:Methanol	120	11	13	10	8.5
		60	7.5	8.5	7	-
		30	-	-	-	-
		15	-	-	-	-
Flower	Acetone	120	15	21	18	10
		60	10	17	14	7
		30	7	12	9.5	-
		15	-	8.5	7	-
	Methanol	120	13	17	15	10.5
		60	9.5	14	11	7.5
		30	7	10.5	9	-
		15	-	8	6	-
	Ethanol	120	12	14	11	10
		60	9.5	11	8	7
		30	7.5	8	6.5	-
		15	-	-	-	-
	Acetone:Methanol	120	11	13	11	8
		60	9	9	7.5	-
		30	-	7	-	-
		15	-	-	-	-
Gentamicin (10 µg/disc)			-	-	27	24
Tetracycline (30 µg/disc)			26.5	28.5	-	-

Table 5. Minimal Inhibitory Concentration (MIC, mg/ml) and Minimal Bactericidal Concentration (MBC, mg/ml) of various extracts of *D. kotschyi*.

Sample	**Solvent**	***M. luteus***		***S. aureus***		***E. coli***		***P. aeruginosa***	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Leaf	Acetone	3.75	15	3.75	15	3.75	15	3.75	30
	Methanol	7.5	15	30	30	7.5	-	15	30
	Ethanol	-	-	30	30	15	-	15	-
	Acetone:Methanol	7.5	-	15	-	7.5	-	7.5	-
Flower	Acetone	3.75	15	3.75	3.75	3.75	15	3.75	30

Methanol	3.75	15	7.5	15	3.75	15	3.75	15
Ethanol	3.75	30	3.75	30	3.75	30	3.75	-
Acetone:Methanol	3.75	-	3.75	30	3.75	-	3.75	-

Note: Evaluated extract concentrations were 30, 15, 7.5 and 3.75 mg mL^{-1}.

CONCLUSION

Medicinal plants are potential sources of phenolic compounds with impressive antioxidant, antimicrobial, and disease-associated enzyme inhibitory activity. To our knowledge, this study was the first report on the evaluation of bioactivities of *D. kotschyi* extracts prepared using different extraction solvents. The results of the present study support the traditional uses of *D. kotschyi*. Our finding showed that the various extracts from leaves and flowers of *D. kotschyi* exhibit strong antibacterial and antioxidant activities. The novel finding in the present study was a considerable inhibitory effect of *D. kotschyi* extracts on α-amylase enzymatic activity. Thus, *D. kotschyi* may be suggested to employ in the management of type 2 diabetes mellitus. However, further, both *in-vitro* and *in-vivo* studies are required to evaluate the safety and efficacy of *D. kotschyi*. Also, these results corroborate the idea that bioactive compounds present in the essential oils of *D. kotschyi*, can be used for the development of new drugs as well as a source of pharmaceutical raw materials.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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