

Original Article

MICROMORPHOLOGICAL CHARACTERIZATIONS AND PHYTOCHEMICAL CONTENTS OF SOME *PHLOMIS* SPECIES FROM IRAN

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ABSTRACT

Objective: Microscopic characterization of a plant is a valuable method for accurate identification of the plant powder. The plants of *Phlomis* genus (Lamiaceae) are mostly distributed in the north and west of Iran with about 10 endemic species. In the present investigation, microscopic characterization of some *Phlomis* species including *Phlomis bruguieri*, *Phlomis rigida*, *Phlomis kurdica*, and *Phlomis olivieri* were assessed along with their phytochemical contents.

Methods: The powders of the mentioned plants were analyzed using Zeiss microscope attached to a digital camera. Phytochemical contents of the plants extracts including total phenol, tannin, and polysaccharide were measured as well as a radical scavenging activity using the 2,2-diphenyl-1-picryl-hydrazyl method.

Results: The results of this study indicated that diacytic stomata, glandular trichome, stellate trichome, and crystals were the characteristic features of the examined species. Total phenol, tannins, and polysaccharides of the plants were assessed ranging 66.0-101.8 µg gallic acid equivalent in mg dry extract (µg GAE/mg EXT), 6.9-9.5 µg tannic acid equivalent in mg dry extract (TAE/mg EXT), and 512-559 µg glucose equivalent in mg dry extract (GE/mg EXT), respectively. Moreover, half maximal inhibitory concentration (IC₅₀) values of radical scavenging activity of the extracts were calculated according to the plot of inhibition percentage against different concentrations of each extract as 218.6, 112.0, 113.3, and 58.7 µg/ml, respectively.

Conclusion: The observed differences between *Phlomis* species can be applied in the accurate identification of these medicinal plants particularly in dried powdered materials regarding their microscopic characterizations and phytochemical contents.

Keywords: *Phlomis*, Microscopic characterization, Phytochemical contents, Free radical scavenging.

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INTRODUCTION

Accurate identification of a plant is a prerequisite step for scientists before using them for other applications. Although molecular assessment is attracted more attention, it is not sufficient for identification of plants as well as quality control. Botanical microscopy is a valuable method especially in the identification of dried plant materials, in which the characteristics were changed compared with the fresh materials while drying and fragmentation of the plant do not alter the most microscopic features [1,2]. In addition, chemical profile of a plant dramatically changed through drying and fragmentation processes, which can limit the usefulness of chemistry for the plant identification [2]. This method is also extremely valuable in the detection of adulterated plant materials with other plants and/or different parts of the same plant, as well as mixture of inorganic materials such as dirt, insect parts and rodent hairs that are not detectable using chemical assessment [2, 3]. For instance, specialized glandular trichomes (scales or hairs on the surface of the leaf) are typical characteristics of the family Lamiaceae. The presence of such glandular trichomes in a sample that belongs to another family indicates the adulteration of that sample with some types of mint. On the other hand, the absence of a particular trichome in an examined sample shows that the plant does not belong to the Lamiaceae family [2]. Therefore, knowledge of the diagnostic properties associated with the genus or family provides a direction toward identification of unknown samples. However, the microscopic characterization is appropriate only for identification not for quality assessment or the extracts evaluation [2].

In the microscopic characterization of a plant material, there are two major diagnostic parameters including cell types and crystals of calcium oxalate [4]. Soluble oxalic acid is detoxified by many plants to insoluble calcium oxalate, which crystallizes in characteristic forms like prismatic crystals, raphides, acicular crystals and cluster crystals that are critical for diagnostic purposes. Prismatic crystals are

rhomboidal in shape. Cluster crystals or druses of calcium oxalate are spheroidal aggregates with numerous faces and sharp points [2].

The genus *Phlomis* belongs to Lamiaceae family with 17 species, 10 of which are endemic of Iran, distributed in the Azerbaijan, Fars, Gilan, Hamedan, Isfahan, Kurdistan, and Mazandaran provinces [5, 6]. The plants of this genus demonstrate several medicinal properties including wound healing, anti-tussive, sedative, tonic, carminative, astringent, anti-diabetic, diuretic along with ulcer treatment, hemorrhoids, and respiratory tract disorders [7-11].

Furthermore, recent studies indicated various activities for some species of *Phlomis* like anti-inflammatory, immunosuppressive, anti-nociceptive, anti-mutagenic, anti-allergic, antimalarial, antibacterial, and antioxidant properties [7, 12-16]. Phytochemical analysis of these plants resulted in the identification of several secondary metabolites comprising lignans, neolignans, alkaloids, terpenoids, iridoids, flavonoids, and phenolic compounds like phenylpropanoids, phenylethanoids, as well as their glycoside derivatives [10, 17-19]. In the present article, anatomical structures of the aerial parts of some *Phlomis* species including *P. bruguieri*, *P. rigida*, *P. kurdica* and *P. olivieri* along with their phytochemical contents and radical scavenging activities were clarified.

MATERIALS AND METHODS

Chemicals and reagents

All the substances including solvents and reagents were purchased with the analytical grade. Solvents, glucose, gallic acid and Folin-Ciocalteu were purchased from Merck (Darmstadt, Germany).

Plant materials and extraction

All the *Phlomis* species in this study including *P. bruguieri*, *P. rigida*, *P. kurdica*, and *P. olivieri* were collected from Kurdistan province in the west parts of Iran. The plants were identified by Mr. Yousef Ajani

from Institute of Medicinal Plants, Karaj, Iran. The voucher specimens were deposited at the same institute, Iranian Academic Centre for Education, Culture and Research (ACECR) as 1581, 1582, 1612, and 1557 for the above-mentioned species, respectively. Plant materials were dried at shade and subjected to extraction twice by aqueous methanol (80 %) using percolation method for one week.

Total phenol

Total phenol contents of the plants extracts were determined using Folin-Ciocalteu method [20]. Five ml of Folin-Ciocalteu reagent (diluted 1:10) was added to 1 ml of each extract with a concentration of 1 mg/ml and incubated at room temperature for 10 min. Four ml of sodium bicarbonate (75 mg/ml) was transferred to every glass tube and they were made up to 10 ml with distilled water incubating for 30 min at room temperature. The absorbance of all samples was measured at 765 nm and compared to gallic acid absorbance. All the tests were performed in triplicate and total phenol contents of the plants were expressed as gallic acid equivalent in dry extract ($\mu\text{g GAE/mg EXT}$) using gallic acid calibration curve ($Y=0.0074X-0.075$, $r^2=0.98$).

Total tannin

Total tannins of the extracts were evaluated using Folin-Ciocalteu method coupling with the application of the insoluble matrix, polyvinyl poly pyrrolidone (PVPP), which causes precipitation with tannins in the samples [21]. All the tests were carried out in triplicate and total tannin contents of the plants were expressed as tannic acid equivalent in the dry extract ($\mu\text{g TAE/mg EXT}$) using tannic acid calibration curve ($Y = 0.042X+0.077$, $r^2= 0.97$).

Total polysaccharide

Polysaccharide contents of the plants were examined regarding the preliminary study [21, 22]. Sulfuric acid breaks all the glycosidic linkages and colored complex obtained from phenol and carbohydrate. The absorbance of the complex was assessed at 490 nm. Calibration curve was depicted using different concentrations of glucose and the total polysaccharide contents of all samples provided as glucose equivalent in the extract ($\mu\text{g GE/mg EXT}$) using calibration curve ($Y = 0.014X+0.024$, $r^2=0.99$).

Free radical scavenging assay

The free radical scavenging activity of the extracts with different concentrations was evaluated using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a free stable radical [20, 21]. Free radical 50% inhibition (IC_{50}) of the extracts was obtained using the plot of inhibition percentage against different concentrations of each extract. Furthermore, vitamin E and butyl hydroxyl anisole (BHA) were applied as conventional natural and synthetic antioxidants, respectively.

Microscopic observations

Powder of each tissue (1 g) including flower, leaf, and stem of all the plants were individually boiled in 10 ml of potassium hydroxide (10%) for 30 s for flower and leaf parts and 1 min for stem fragments on the heater. The tissues were subsequently washed with distilled water three times. Sodium hypochlorite was used afterwards to eliminate colors and then all samples were washed with distilled water [21]. All the treated samples were conserved in aqueous glycerin in the closed glass until anatomical investigation. Zeiss microscope attached with a digital camera was applied taking photomicrographs with different magnification regarding the tissue details.

RESULTS

Phytochemicals contents of the examined plants were briefly presented in the table 1. Total phenol, total tannins, and total polysaccharides of the plants were assessed ranging 66.0-101.8 $\mu\text{g GAE/mg EXT}$, 6.9-9.5 $\mu\text{g TAE/mg EXT}$, and 512-559 $\mu\text{g GE/mg EXT}$, respectively. The IC_{50} values of radical scavenging activity of *P. bruguieri*, *P. rigida*, *P. kurdica*, and *P. olivieri* extracts were calculated according to the plot of inhibition percentage against different concentrations of each extract as 218.6, 58.7, 112.0, and 113.3

$\mu\text{g/ml}$, respectively. The IC_{50} values of the positive controls vitamin E and BHA were calculated as 14.2 and 7.8 $\mu\text{g/ml}$, respectively. Although all the plant extracts inhibited free radical moderately, *P. rigida* displayed the highest antioxidant activity (IC_{50} : 58.7 $\mu\text{g/ml}$) and total phenol content ($101.8\pm 3.3 \mu\text{g GAE/mg EXT}$) among other extracts.

Microscopic differentiation of the examined species was briefly depicted in table 2. Trichomes are cells or group of cells that present as hairs on the epidermal surface if they break off from the plant surface leaving cicatrices, which are characteristic scars. The family of Lamiaceae has special glandular trichomes [2]. In the examined species, all plants represent stellate trichomes in their fragments but the size of these stellate trichomes, and the number of them are different in the plant parts. For instance, stellate trichomes are rare in the fragments of *P. bruguieri* calix, while this kind of trichome is abundantly found in *P. kurdica* calix in a smaller size than *P. olivieri* and *P. rigida* (fig. 1). In addition, glandular trichomes in fragments of *P. kurdica* (fig. 1:3) and *P. bruguieri* (fig. 2:1) are smaller than glandular trichomes of *P. olivieri* (fig. 1:4 and 2:4) and *P. rigida* (fig. 3:2). Glandular trichomes in two latter species have longer stalk than that same kind of trichomes in *P. kurdica* and *P. bruguieri*. Fragments of calix from *P. olivieri* have lots of elongated unicellular covering trichomes compared to other species. Guard cell in stomata for all species are diacytic (fig. 1:1, 1:3, 2:1, 2:2, and 2:3), in which stomata is surrounded by two subsidiary cells with the common wall at a right angle to the longitudinal axis of guard cells. This type of stomata is common in Lamiaceae family [2]. Crystals show highly diagnostic features in the plant anatomical structure.

The results of this study revealed that calcium oxalate crystallize is a characteristic feature in the examined plants. For example, *P. kurdica* represented long prismatic crystals in flower and stem fragments, as well as sand crystals in the flower parts (fig. 1:3). Moreover, in the flower parts of *P. rigida* acicular crystals were observed, which are elongated needle-like crystals tapering at the both sides (fig. 1:2). Vessel elements in all the powdered samples were fairly small mostly in groups with spiral thickening (fig. 1:2, 2:2, 2:3, 2:4, 3:2, 3:3, and 3:4), while fragments of pitted vessels were found in leaf parts of *P. rigida* (fig. 2:2). Tissues in essential oil-containing plants become orange-red while treated with Sudan red solution. In all the plants, glandular trichomes became orange-red when colored with Sudan red indicating they reserve essential oil in the plants (fig. 1:4, 2:3, 2:4, and 3:3). Additionally, in the parenchyma of the plants, droplets of essential oil were clearly observed in the presence of Sudan red (fig. 1:2, 1:4, 2:1, 2:3, and 3:4).

DISCUSSION

Literature revealed that the antioxidant activity of a methanolic extract obtained from *P. armeniaca* was mainly related to its phenol content [23]. Both *P. kurdica* and *P. olivieri* showed similar antioxidant activity (IC_{50} : 112 and 113.3, respectively) with similar tannin contents (7.1 ± 1.5 and $7.1\pm 0.2 \mu\text{g TAE/mg EXT}$, respectively), and total phenol (87 ± 3.7 and $100.6\pm 6.0 \mu\text{g GAE/mg EXT}$, respectively). The extract of *P. bruguieri* demonstrated weak radical scavenging activity (IC_{50} : 218 $\mu\text{g/ml}$) and the lowest total phenol content ($66\pm 0.8 \mu\text{g GAE/mg EXT}$) as well. The previous study demonstrated that polysaccharides possess antioxidant activity in a dose-dependent manner [24]. Free radical scavenging activities of several polymers of carbohydrate were described mostly due to their monosaccharide constituents [25]. Some studies showed that phenolic compounds like protocatechic, rosmarinic acid, phenyl propanoids, and verbascoside are major constituents of the observed antioxidant activity [26, 27]. A recent study reported a quantitative analysis of verbascoside, a well-known antioxidant, in the methanolic extracts of same *Phlomis* plants using HPTLC method. The results indicated that the amount of verbascoside in the extracts of *P. bruguieri*, *P. kurdica* and *P. rigida* were measured as 9.6 ± 0.5 , 7.0 ± 0.1 , $9.8\pm 0.1 \text{ mg/ml}$, while different amounts of verbascoside were reported from *P. olivieri* depends on the plant origin (3.9 ± 0.2 , from Azerbaijan, 8.6 ± 0.2 , from Tabriz, and 9.1 ± 0.4 , from Mazandaran) [28]. However, there is no direct relation between antioxidant activity of the plants and their verbascoside contents.

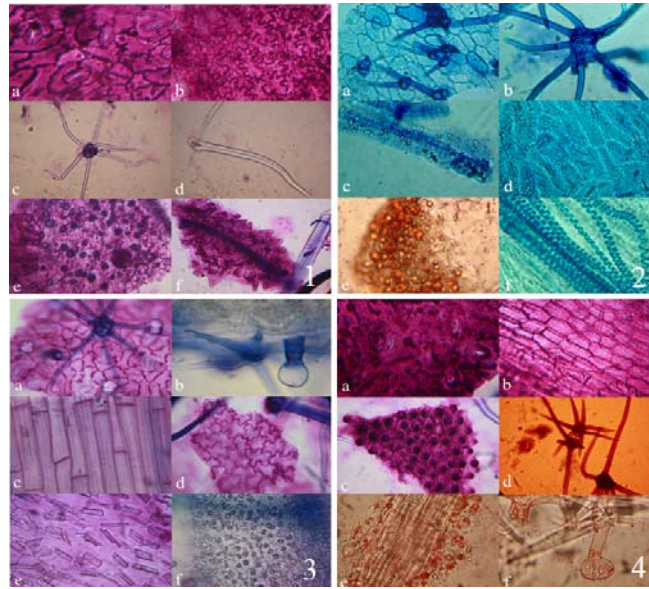


Fig. 1: (1) Microscopic characterization of *P. bruguieri* flower; (a) Epidermis with sinuous thick-wall cells and diacytic stomata, (b) outer epidermis of the corolla in surface view with sinuous anticlinal cell wall, (c) a branched stellate covering trichome, (d) a covering trichome, (e) cluster of calcium oxalate, (f) inner epidermis of the corolla in surface view showing papillae. (2) Microscopic characterization of *P. rigida* flower. (a) Epidermis of petiole with elongated cells, covering trichomes and cicatrices, (b) a stellate covering trichome, (c) crystal sands of calcium oxalate, (d) acicular crystal of calcium oxalate, (e) parenchyma cells containing droplets of essential oil, (f) vessels with spirally thickening. (3) Microscopic characterization of *P. kurdica* flower; (a) Epidermis cells with pitted cell wall, diacytic stomata and stellate covering trichome, (b) glandular trichomes with uniseriate stalk and unicellular spherical head, (c) large undifferentiated parenchyma arranged in rows with thin walls, (d) outer epidermis of the corolla in surface view with sinuous anticlinal cell walls, (e) prismatic crystals of calcium oxalate, (f) crystal sands. (4) Microscopic characterization of *P. olivieri* flower; (a) Epidermis of flower with diacytic stomata, (b) parenchyma with pitted walls arranged in rows, (c) inner epidermis of the corolla in surface view showing papillae, (d) stellate non-glandular trichomes with unicellular thick-wall cells, (e) parenchyma cells containing droplets of essential oil, (f) a glandular trichome with a uniseriate stalk and unicellular head containing oil

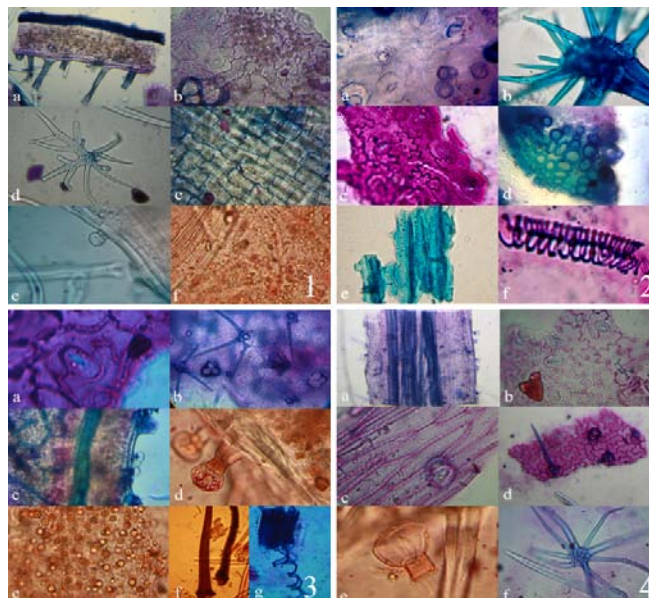


Fig. 2: (1) Microscopic characterization of *P. bruguieri* leaf; (a) Epidermis of leaf with unicellular covering trichomes and vessels, (b) epidermis with sinuous cell walls and diacytic stomata, (d) a branched multicellular covering trichome, (c) parenchyma of the leaf with thin-wall cells arranged in rows, (e) glandular trichome with unicellular stalk and short unicellular head (f) parenchyma cells containing oil and spirally vessels. (2) Microscopic characterization of *P. rigida* leaf. (a) Epidermis of the leaf in surface view with glandular trichomes and stomata, (b) covering trichome with tick-wall cells, (c) epidermis with thick sinuous wall cells and diacytic stomata, (d) transverse section of xylem of petiole, (e) fragment of pitted vessel and fibres, (f) part of spiral vessel. (3) Microscopic characterization of *P. kurdica* leaf; (a) epidermal cells with sinuous walls and diacytic stomata, (b) stellate covering trichomes and cicatrices, (c) sectional view of leaf showing glandular and non-glandular covering trichomes and groups of vessels, (d) glandular covering trichomes with unicellular spherical head and uniseriate stalk containing oil, (e) parenchyma cells containing droplets of essential oil, (f) unicellular covering trichomes with tick-wall cell, (g) vessels with spiral thickening. (4) Microscopic characterization of *P. olivieri* leaf; (a) Sectional view of lamina with glandular trichomes, non-glandular covering trichomes attached to the both epidermis and groups of vessels with spiral thickening, (b) epidermis in surface view with sinuous wall cells and diacytic stomata, (c) elongated cells of epidermis of petiole with a cicatrix, (d) epidermis in surface view with attached covering trichome and cicatrix, (e) glandular trichome-containing oil, (f) stellate covering trichome with thick-wall cells

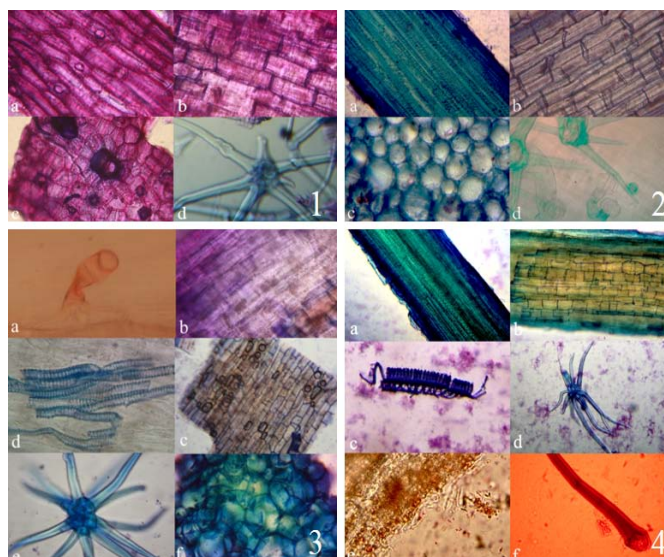


Fig. 3: (1) Microscopic characterization of *P. bruguieri* stem; (a) Parenchyma of the stem with pitted walls arranged in rows with cicatrices, (b) parenchyma with thin-walled cells, (c) epidermis cells with striated cuticle and rounded cicatrices, (d) stellate covering trichome. (2) Microscopic characterization of *P. rigida* stem. (a) Vascular tissue of the stem with spiral thickening and fibres in longitudinal view, (b) parenchyma with elongated thin-wall cells arranged in rows, (c) cork with thin-walled cells in surface view, (d) epidermis with covering trichomes and glandular trichomes. (3) Microscopic characterization of *P. kurdica* stem; (a) Covering trichome-containing oil, (b) large undifferentiated parenchyma cells arranged in rows containing calcium oxalate crystals, (d) a group of spirally thickened vessels, (c) storage parenchyma cells containing starch, (e) stellate covering trichome with thickened wall, (f) cork with thin-walled cells in surface view. (4) Microscopic characterization of *P. olivieri* stem; (a) Vascular tissue of the stem with spiral thickening and fibres in longitudinal view, (b) parenchyma with thin-wall cells arranged in rows, (c) vessel with spiral thickening, (d) stellate covering trichome with thick-wall cells, (e) parenchyma cells containing droplets of essential oil, (f) a covering trichome with thick-wall cell

Table 1: Total phenol, total tannin, and total polysaccharide contents of tested *Phlomis* spp. along with their radical scavenging activity

Sample	Total phenol ¹	Total tannin ²	Total polysaccharide ³	Radical scavenging activity ⁴
<i>P. bruguieri</i>	66.0±0.8	9.5±0.0	559±4.8	218.6
<i>P. rigida</i>	101.8±3.3	6.9±0.7	513±6.2	58.7
<i>P. kurdica</i>	87±3.7	7.1±1.5	546±7.8	112
<i>P. olivieri</i>	100.6±6.0	7.1±0.2	512±1.3	113.3

Data is given as mean±standard deviation (SD), n=3, ¹µg GAE/mg EXT, ²µg TAE/mg EXT, ³µg GE/mg EXT, ⁴IC₅₀ (µg/ml).

Table 2: Microscopic differentiation of four *Phlomis* spp. with each other

Plant name	<i>P. bruguieri</i>	<i>P. kurdica</i>	<i>P. olivieri</i>	<i>P. rigida</i>
Organ	Flower			
Stomata	diacytic	Diacytic	diacytic	diacytic
Crystals	cluster	prismatic and sand	prismatic	acicular, cluster and sand
Trichome	rare stellate and elongated unicellular covering trichome	lots of stellate trichomes, elongated unicellular covering trichomes, glandular trichomes	lots of elongated unicellular covering trichome and glandular trichome, stellate trichomes are rare, all are larger than other species	lots of stellate trichomes, rare glandular trichomes and branched multicellular trichomes
Epidermis	elongated cells with sinuous cell wall	sinuous cell wall	sinuous cell wall	sinuous cell wall
Organ	Leaf			
Stomata	diacytic	Diacytic	diacytic	diacytic
Crystal	cluster and acicular	cluster and acicular	cluster	needle-like
Trichome	rare multicellular, stellate and elongated unicellular covering trichome	stellate trichomes, elongated unicellular covering trichomes, glandular trichomes	unicellular covering trichomes, lots of glandular trichomes and stellate trichomes	glandular and stellate
Epidermis	wavy cell wall	sinuous cell wall	larger cell than others, sinuous cell wall	sinuous cell wall
Organ	Stem			
Crystal	sand crystals	prismatic crystals	sand crystals	needle-like crystals
Trichome	stellate trichome	stellate trichome	stellate trichome	stellate and glandular trichomes
Epidermis and parenchyma	epidermis with pitted wall and striated cuticle	parenchyma containing starch	parenchyma containing starch	epidermis have pitted wall
Vessels	spiral thickening	spiral thickening	spiral thickening	spiral thickening

Therefore, free radical scavenging activity of the extracts was probable outcome of all phytochemicals presented including phenols, tannins and polysaccharides along with other compounds like flavonoids.

All the extracts of the examined plants demonstrated moderate antioxidant activity, which can be related at least partly to the phenol contents thereof. Microscopic structures of the mentioned plants' powders exhibited characterizing glandular trichomes (unicellular head, uniseriate stalk and stellate covering) in their various organs, as well as particular epidermis and parenchyma features, different shapes of oxalate crystals, and also diacytic stomata, which all could be important in determination of the plant in the mixed herbal powders.

CONCLUSION

The observed differences between *Phlomis* species can be applied in the accurate identification of these medicinal plants particularly in dried powdered materials regarding their microscopic characterizations like unicellular head, uniseriate stalk and stellate covering trichomes along with phytochemical contents.

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CONFLICT OF INTERESTS

Declared None

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