An iridoid, lamiide, its extraction method from *Phlomis bruguieri*, and evaluation of its antioxidant capacity

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Abstract

Phlomis bruguieri is a native plant in Iran, which deserves phytochemical study. The aim of this study was evaluation of its iridoid contents along with its antioxidant capacity. Aerial parts of the plant material were extracted with methanol and applied on repeated normal column chromatography using hexane: chloroform (70:30), chloroform: methanol (90:10), and methanol (100%). The fraction eluted with methanol (100%) containing polar glycosides was selected and medium pressure liquid chromatography (MPLC) on a RP-18 cartridge. Fraction elute by MeOH: water 30:70 was loaded on HPLC on RP-18 column for final purification. Structure elucidation was done using 1D and 2D NMR, and mass spectra. Antioxidant activity including total antioxidant capacity, DPPH and FRAP methods were designated to assess the *in vitro* antioxidant capacities. This study indicated the presence of lamiide as iridoid compound in the aerial parts of *Phlomis bruguieri* for the first time in this plant [350 mg/2 kg; 0.0175 % (w/w)]. Lamiide showed moderate antioxidant activity using TAC (EC_{50}: 55 µg/mL; ascorbic acid EC_{50}: 7.5 µg/mL), DPPH (EC_{50}: 116.2 ± 3.51 µg/mL; ascorbic acid:34.7 ± 0.97 µg/mL), and FRAP (EC_{50}:>200 µg/mL; quercetin: 7.5 ± 0.68 µg/mL) methods.

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Introduction

Phlomis species from Lamiaceae family are distributed in North Africa, throughout Europe, and Asia.[1] They have been used *as* herbal to protect the kidney, liver, and cardiovascular system,[2] for treatment of painful swellings, psoriasis, and skin eruptions,[3] lesion and burns,[4] stomach ache and gastrointestinal troubles,[5] antidiabetic.[6] Even, grilled leaves of some species have reported for culinary use.[7] Essential oils, flavonoids, Iridoids, phenylethyl alcohol glycosides are their major secondary metabolites.[1] In the case of Iridoids, 8-O-acetylshanzhiside methyl ester, shanzhiside methyl ester, and lamiide are more frequent which are distributed in most of the *Phlomis* species.[8] In pharmacological properties, they are reported for reducing the oxidative stress in diabetes or by stimulating the production of enzymes implicated in glucose metabolism.[9] Mohajer *et al.* also reported pain reliving and analgesic properties of different *Phlomis* species.[10] Their aqueous extract in some species have been studied for the gastroprotective activity.[11] In another study conducted by Ismailoglu and coworkers, phenylethyl alcohol fraction and iridoid fraction of *Phlomis pungens* prevented the inhibition of acetylcholine response induced by electrolysis in aortic rings.[12] They showed also protective activity in free radical-induced impairment of endothelium-dependent relaxation which probably id because of free radical scavenging activity of these compounds.[12] *Phlomis bruguieri* (*P. bruguieri*) is distributed in flora of Turkey, Iran, and Iraq.[13] Previous biological studies reported its antimicrobial against Streptococcus *sanguis* and Staphylococcus aureus, antioxidant, and α-amylase inhibitory activities.[14] Previous phytochemical studies showed that the main constituents of its essential oil are bicyclogermacrene (4.1%), γ-elemene (16.5%), germacrene D (60.5%), and germacrene B (7.1%).[15] In another study LC mass analysis reported presence of phenylethanoid glycosides: verbascoside, leucosceptosides A, isoverbascoside, and martynoside.[16]

Another phytochemical study by the authors, leaded in isolation of one new 4'-methoxy-luteolin-7-phosphate as well as three flavones: luteolin, tricin, and apigenin[17] in semi polar partition of this plant. In this study we investigated the major component in aqueous partition of *Phlomis bruguieri* in addition to its antioxidant activity.

Materials and Methods

NMR spectra were taken in DMSO-d6 as the solvent on a Bruker AV400 NMR spectrometer (Bruker, Germany). High resolution mass spectra were carried out on an Agilent 1100 SL series mass spectrometer. Column Chromatography (CC) was done on flash silica gel particle size 40-63 µm, and Size Exclusion Chromatography (SEC) on Sephadex LH-20 (Sigma-Aldrich). HPLC was done on a waters 501 pump and YMC RP-18 column (250 x 20 mm) with AcCN:H20 (10:90) at 3.0 mL/min.

Plant material

Aerial parts of *P. bruguieri* were collected from Kermanshah, Iran. It was identified according to the voucher specimen (2182) deposited in the herbarium of biology department, Faculty of Science, University of Isfahan, Isfahan, I.R. Iran.

Extraction and isolation

Dried powder of (2.0 Kg) was extracted with methanol (20 L) for one week. It was filtered and concentrated (361 g) by rotary evaporator at 40 °C. The extract was defatted in a separating funnel by partitioning between methanol: water and hexane. Methanolic phase was concentrated (80 g) and applied on column chromatography (silica gel, 400 g) for preliminary fractionation using hexane: chloroform (70:30), chloroform: methanol (90:10), and methanol (100%). Fr. 2 eluted with chloroform: methanol (90:10) was analyzed and reported previously [17] and Fr.3, eluted with methanol (100%) containing polar glycosides selected and loaded on MPLC on a RP-18 cartridge (50 * 150 mm) using MeOH: water 30:70; 40:60, and 50:50. Based on TLC, fraction elute by MeOH: water 30:70 was loaded on HPLC column on YMC RP-18 column (20 * 250 mm) using acetonitril: water (10:90) as solvent and yielded lamiide as major iridoid component (350 mg).

To check its antioxidant activity, total antioxidant capacity, DPPH and FRAP methods were designated to assess the *in vitro* antioxidant capacities.

Evaluation of total antioxidant capacity

The Total Antioxidant Capacity (TAC) was done using the method of Salmanian *et al.* and by some adaptation method to micro titer plates.[18] 96 well plate resistant to temperature was poured with 10 µL of sample in different concentrations of 1, 10, 25, 50, 100, and 200 µg/mL and in triplicate wells combined with 100 µL of reagent solution containing $(NH_4)_2MoO_4$ (4 mM), Na_3PO4 (28 mM), and H_2SO_4 (0.6M). Well plate was incubated at 95ºC for 1.5 hr. After cooling to room temperature, the absorbance was read at 695 nm. Blank was 10 µL of sample solutions solvent and 100 µL of reagent. Ascorbic acid 200 µg/mL was used as control. The concentration of the sample which had 50% of total antioxidant capacity, (EC_{50}), was determined for sample and standard using Excel 2010 graph software.

TAC (%) =[(OD control - OD sample)/OD control]*100

DPPH free radical scavenging activity

It was done using the method of West *et al.* and by adaptation to 96 well plates.[19] Ninety-six well plate resistant to temperature were poured with 250 µL of sample in different concentrations of 1, 10, 25, 50, 100, and 200 µg/mL and in triplicate wells; 100 µL of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) ethanol solution (0.3 mM) was added. The reaction would be finished after 30 min at room temperature, and the absorbance values could be measured at 518 nm. Radical scavenging activity (RSA) was calculated as follows:

RSA (%) =[(OD blank - OD sample)/OD blank]*100

The concentration of the sample which could scavenge 50% of DPPH radical, (EC_{50}) was determined for sample and standard using Excel 2010 graph software.

Ferric ion reducing antioxidant power by potassium ferric cyanide

The Ferric ion Reducing Antioxidant Power (FRAP) was determined using the method described by West *et al.*[19,20] but by some modification in final calculation. To 1 mL of sample, quercetin as standard in different concentrations of 1, 10, 25, 50, 100, and 200 µg/mL and in triplicate, 2.5 mL of sodium phosphate buffer (0.2M, pH=6.6) and 2.5 mL of 1% potassium ferric cyanide: $K_3[Fe(CN)_6]$ were added. The solution was incubated at 50 ºC for 20 min. Methanol as negative control and quercetin in the same concentrations as positive control were added. Then after incubation, 2.5 mL of trichloroacetic acid (TCA, 10%) was added, and the solution was centrifuged at 10000g for 10 min. Then, 2.5 mL of the supernatant, with 2.5 mL deionized water and 0.5 mL $FeCl_3$ (1%) were added. The mixture was incubated at room temperature for 30 min, and the absorbance OD was measured at 700 nm. Quercetin 200 µg/mL was used as control.

FRAP (%) =[(OD control -OD sample)/OD control]*100

The concentration of the sample which had 50% of ferric ion reducing antioxidant power, (EC_{50}), was determined for sample and standard using Excel 2010 graph software.

Results and Discussion

Compound 1 was isolated in good quantities using MPLC on a RP-18 cartridge followed by prep HPLC column on YMC RP-18 column using acetonitrile: water (10:90) as solvent. It was obtained as a white, amorphous powder and its structure was determined based on 1H-NMR, ^{13}C-NMR, HSQC, and HMBC experiments. In the HR-ESIMS spectra pseudomolecular ion at *m/z* 445.1316 was indicative to the molecular formula $C_{17}H_{26}O_{12}$ $+Na^+$ (Calcd: 445.1323). ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC analysis showed signals related to a beta pyranoglucoside substituent δ_C 97.9 [δ_H 4.38 (1H, d, J=7.9 Hz, H-1')], 72.9 [δ_H 2.94 (1H, ddd, $J_{1',2'}$=8.7, $J_{2',3'}$=8.7, $J_{2',OH}$=3.6 Hz, H-2')], 75.6 [δ_H 3.11-3.17 (overlap, H-3')], 70.0 [δ_H 3.06 (1H, ddd, $J_{3',4'}$=9.1, $J_{4',5'}$=9.1, $J_{4',OH}$=5.1 Hz, (H-4')], 77.3 [δ_H 3.11-3.17 (1H, overlap, H-5'), and 60.9 [δ_H 3.45-3.50 (overlap, H-6'a); 3.70 (1H, ddd, $J_{5',6'b}$=1.73, $J_{6'a,6'b}$=11.70, $J_{6'b,OH}$=5.9 Hz, H-6'b), as well as δ_H 5.14 (1H, d, $J_{2',OH}$=3.8, 2'-OH), 5.02 (1H, d, $J_{4',OH}$=5.4, 4'-OH), 4.70 (1H, d, $J_{3',OH}$=3.9, 3'-OH), 4.67 (1H, t, $J_{6',OH}$=5.9, Hz 6'-OH), one methoxy group δ_C 50.9 [δ_H 3.62 (3H, bs)]. Main core was consisted of 10 ^{13}C-NMR resonances: a singlet methyl group δ_C 21.3 [δ_H 0.93 (3H, s)], a three-substituted double bond [δ_C 114.4 and 150.5 (each s);

δ_H 7.33 (1H, s)], conjugated with an α,β unsaturated esteric group [δ_C 165.8], one methylene δ_C 44.6 [δ_H 1.98 (1H, dd, $J_{6a,6b}$=14.5, $J_{6a,7}$=4.2 Hz, H-6_a); 2.21 (1H, dd, $J_{6a,6b}$=14.5, $J_{6b,7}$=5.3 Hz, H-6_b)], three SP3 methines: one attached to a hydroxyl function δ_C 75.6 [δ_H 3.28 (1H, ddd, $J_{6a,7}$ =4.2, $J_{6b,7}$=5.3, $J_{7,OH}$=4.8 Hz); 4.98 (1H, d, $J_{7,OH}$ =4.8, 7-OH], one hemiacetal δ_C 93.0 [δ_H 5.65 (1H, d, $J_{1,9}$ =1.0 Hz, H-1)], and a simple one δ_C 57.3 [δ_H 2.55 (1H, d, $J_{1,9}$ =1.0 Hz, H-1)], in addition to two oxygenated quaternary carbons δ_C 66.9 (C-5), and 77.0 (C-8), and two singlet hydroxyl groups δ_H 4.36 (1H, s), 4.48 (1H, s) with HMBC correlation to C-5, and C-8, respectively. By the complete assignment of the structure using 2D-NMR spectra and comparison with the published data the structure was confirmed as lamiide (Figure 1) and is reported for the first time in this plant.[21]

Total Antioxidant Capacity Assay

In this assay phosphomolybdenum (VI) is reduced to phosphate/Mo (V) complex at acidic pH. As shown in Figure 2, total antioxidant capacity of lamiide increased with increasing concentration (Dose concentration pattern). In concentrations of 1, 10, 25, 50 µg/mL its activity was less than ascorbic acid (P two-tail<0.001) while at concentrations 100 µg/mL (P two-tail=0.089) and 200 µg/mL (P two-tail=0.868) difference was not significant. Lamiide showed EC_{50} value of TAC equal to 55 µg/mL, while ascorbic acid determined to be equivalent to 7.5 µg/mL determined for 100 µL of reagent solution in test (Figure 2).

DPPH free radical scavenging activity

DPPH method is free radical scavenging activity evaluation by proton donation or radical scavenging ability using pink stable radical DPPH (λmax =517 nm). As shown in Figure 3, free radical scavenging by lamiide was in a dose dependent pattern. In concentrations of 25, 50, 100 µg/ml its activity was less than ascorbic acid (P<0.001) while at concentrations 200 µg/mL difference was not significant (P>0.05). Lamiide EC_{50} value at concentration of 116.2 ± 3.51 µg/ml, while ascorbic acid as positive control was 34.7 ± 0.97 µg/mL (Figure 3).

Determination of ferric ion reducing antioxidant power

Reducing power assay method is based on this principle that compounds with FRAP activity, reduce ferric ion (Fe^{3+}) in the form of $K_3[Fe(CN)_6]$ to Fe^{2+} ion in $K_4[Fe(CN)_6]$, which then reacts with $FeCL_3$ to form $Fe_4[Fe(CN)_6]_3$ with Prussian color that has an maximum absorption (λmax) at 700 nm. As shown in Figure 4, ferric ion reducing activity by lamiide was dose dependent. Lamiide EC_{50} value was more than 200 µg/mL, while quercetin was 7.5 ± 0.68 µg/ml in this test. Lamiide at higher concentration of 200 µg/mL, showed 16.28 ± 0.37 % FRAP activity compared with quercetin with 100 % activity (Figure 4).

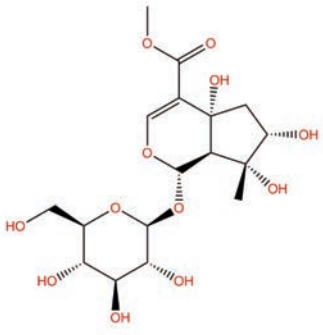

Figure 1. lamiide isolated from *Phlomis bruguieri*.

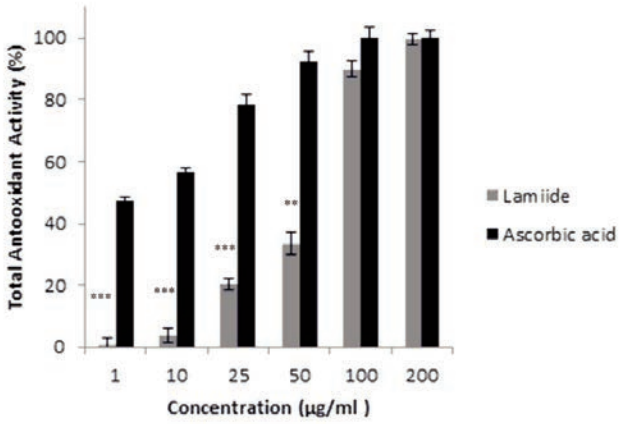

Figure 2. Total antioxidant activity of lamiide isolated from *Phlomis bruguieri*. Ascorbic acid was used as positive control (* p<0.0001, ** p<0.001, * p<0.05 versus control). Values are represented as mean ± standard error (n=3).**

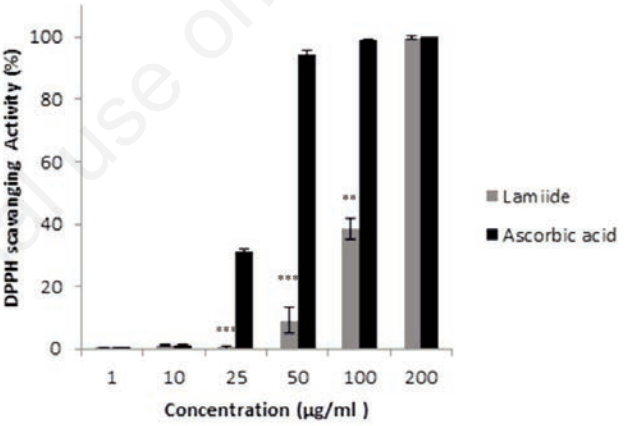

Figure 3. DPPH free radical scavenging activity of lamiide isolated from *Phlomis bruguieri*. Ascorbic acid was used as positive control (* p<0.0001, ** p<0.001, * p<0.05 versus control). Values are represented as mean ± standard error (n=3).**

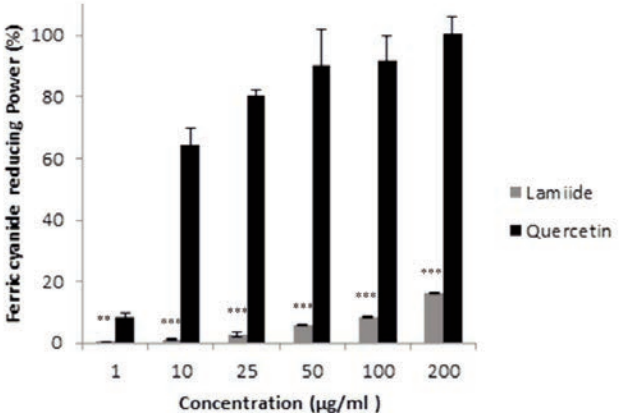

Figure 4. Ferric cyanide reducing power of lamiide isolated from *Phlomis bruguieri*. Quercetin was used as positive control (* p<0.0001, ** p<0.001, * p<0.05 versus control). Values are represented as mean ± standard error (n=3).**

Conclusions

This study indicated using MPLC on reverse phase C-18 followed by HPLC on prep RP C-18 columns using acetonitrile: water as solvent is a good method for purification of Lamiide iridoid. the presence of lamiide as iridoid compound in the aerial parts of *Phlomis bruguieri* for the first time from in this plant [350 mg/^{2} kg; 0.0175 % (w/w)]. Lamiide has been found in other *Phlomis* species by increasing order as following: *P. thapsoides (*9mg/2.7kg; 0.00033%*), P. persian* (4mg/750g; 0.0005%), *P. area* (40mg/5kg; 0.0008%), *P. capitata* (6mg/500g; 0.0012%), *P.integriflora* (17mg/530g; 0.003%)*, P. monocephala* (15mg/500g; 0.003%), *P. syriaca* (14.7mg/450g; 0.0032%), *P.angustissima* (20mg/435g; 0.0045%), *P. fruticosa* (26mg/512g; 0.005%), *P. grandiflora* var. fimbrilligera (26mg/420g; 0.006%), *P. physocalyx* (75mg/600g; 0.0125%), *P. kotschyana* (60mg/280g; 0.021%), *P. nissolii* (300 mg/1.2kg; 0.025%), *P. oppositiflora* (114mg / 200g; 0.057%), *P. pungens* (3g / 2.5kg; 0.12%), *P. viscosa* (1794mg/ 350g; 0.5%).[22-35] In comparison between *Phlomis* species, *P. viscosa*, *and P. pungens* with 0.1 to 0.5 % (w/w) lamiide content could be considered as most rich sources of lamiide. However, because of this fact that *P. bruguieri* contains only one major iridoid, it could be considered also as a source with 0.035 % lamiide content because of less purification steps.

Lamiide showed moderate antioxidant activity using TAC ($EC_{50;}$ 55 µg/mL; ascorbic acid EC50: 7.5 µg/mL), DPPH (EC_{50}: 116.2 ± 3.51 µg/mL; ascorbic acid:34.7 ± 0.97 µg/mL), and FRAP (EC_{50}:>200 µg/mL; quercetin: 7.5 ± 0.68 µg/mL). These results were in agreement with DPPH activity of other iridoids as follows: 6-methoxy scandoside methyl ester (EC_{50}: 277.38 µg/mL),[36] longifolioside A (EC_{50}: l27.0 µg/mL), longifolioside B (EC_{50}:19.0 µg/mL),[37] Ixoside (EC_{50}: 42.4 µg/mL),[38] and 10-O-caffeoyl scandoside methyl ester (EC_{50}: 2.56 µg/mL).[36]

Previously lamiide showed anti-inflammatory activity which support antioxidant activities. Its activity on rat-brain phospholipid peroxidation showed a prosperous activity with IC_{50} value of 0.92+/-0.01 mM and an anti-inflammatory effect in a carrageenin-induced paw edema in rat test with ED_{50} value of 62.3+/-7 mg/kg.[39]

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