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Original Article

DETERMINATION, ISOLATION, AND IDENTIFICATION OF AUCUBIN AND VERBASCOSIDE IN THE LEAVES OF IRAQI *PLANTAGO LANCOLETA L. USING DIFFERENT DETECTING METHODS*

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ABSTRACT

Objective: *Plantago lanceoleta* L. (ribwort plantain) is one of the important medicinal herbs which is widespread fortune available in Iraq, that have a wide range of medicinal properties. The aim of this work was to determine, isolate and identify verbascoside and aucubin in Iraqi *P. lanceoleta* L. by using different chromatographic and spectrometric methods.

Methods: Verbascoside and aucubin were isolated and quantified by preparative TLC, and then they were determined by the high-performance thin-layer chromatography (HPTLC) fingerprinting. Aucubin and catalpol in the plant extract were analyzed by liquid chromatography-mass spectrometry (LC-MS); aucubin and verbascoside that isolated from the plant sample were examined by fourier-transform infrared spectroscopy (FT-IR) and LC-MS, respectively.

Results: The result showed that the Iraqi *P. lanceoleta* L. contains 1.74 percent (verbascoside) and 0.24 percent (aucubin) of dry powdered leaves. Each TLC-isolated compound showed a single spot on the HPTLC plate, which give an idea about the purity of the isolated compound. Aucubin (with catalpol) and verbascoside both are detected by LC-MS in different ionization mode. Many functional groups were identified in the TLC-isolated aucubin by FT-IR.

Conclusion: The Iraqi *P. lanceoleta* L. showed a high content of verbasoside, and it is a very rich source for this compound, which can be easily isolated by TLC and subjected to many pharmacological studies. The extract of the young leaves of this plant gave a little amount of aucubin, and it is easy to obtain a higher content from the older leaves.

Keywords*:* *Plantago lanceoleta* L.*,* Ribwort plantain*,* Verbascoside*,* Aucubin

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INTRODUCTION

Plantago lanceoleta L. is a flowering plant species of the Plantaginaceae family; the most famous names of this species are ribwort plantain and English plantain which is commonly known in Iraq as sagittal lamb's tongue. Ribwort is a communal, perennial wild plant of arable grounds and grasslands suitable for planting [1].

Ribwort plantain is plentiful throughout Eurasia; it is moderately resistant to the drought and can be cultivated in any relatively fertile soil under sunshine, also seen in very deprived lands and characterized by erect, straight leaves that sheltered with soft minute hairs that reach up to about 17 inches long while flowers set as condensed spikes on the top of the stalks as shown in (fig. 1) [2, 3].

Fig. 1: Iraqi *P. lanceoleta* L. grown onto the sidewalk

P. lanceolata L. has a monograph in British and European Pharmacopoeia and European scientific cooperative on phytotherapy (ESCOP) [4]. Ribwort plantain is rich in many active constituents, including: iridoid glycosides (aucubin and catalpol) [5], tannins [6], phenylethanoid-phenylpropanoid glycosides (e.g. verbascoside) [7], mucilages [8], flavonoids (apigenin and luteolin) [9], coumarins (esculetin) [10], saponins and volatile compounds [4, 11].

Iridoid glycosides that found in the ribwort plantain considered as a group of the most important secondary metabolites, and also as chemotaxonomic markers for Plantago spp [12]; aucubin is one of the major molecules found in ribwort plantain; the aucubin concentration in ribwort plantain becomes higher as the leaves get older [13]. It plays many important roles in the medicinal effects of ribwort plantain that include its hepatoprotective [14], pancreas-protective [15], antiarthritic [16], spasmolytic [17], collagen synthesis promoting effects [18], neuroprotective and anti-atherogenic [19]; both aucubin and catalpol (fig. 2) play important role in neuroprotection against many pathological disorders [20].

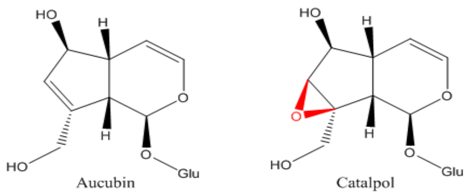

Fig. 2: Aucubin and catalpol structures

On the other hand, verbascoside is also one of the main active constituents of ribwort plantain as presented in (fig. 3), which has many pharmacological effects that include its role in the treatments of neurodegenerative disorders, pains and aches [21, 22], in addition to its antioxidant [23], antitumor [24, 25], anti-allergic and anti-inflammatory effects [26-29].

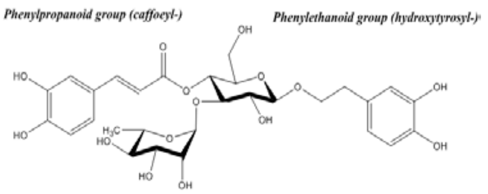

Fig. 3: Verbascoside

Medicinally, a tea of this plant can be used internally for its soothing effect and for treating many complaints involving: cough, bronchitis, sinusitis, asthma, diarrhea, gastritis, peptic ulcers, irritable bowel disease, internal bleeding, hemorrhoids, anal fissure, cervicitis and bladder inflammation [30-33]; externally, it is used in treating skin problems like ulcers, inflammations, stings, cuts, and bleeding [34]. Its astringent effect made this plant effective and safe for treatment of bleeding and inspires the healing of injured tissue [35].

The objective of this work was to determine, isolate and identify verbascoside and aucubin in Iraqi *P. lanceoleta* L. by using different chromatographic and spectrometric methods.

MATERIALS AND METHODS

Plant material

Young *Plantago lanceoleta* (fig. 1) was collected in the month of January/ 2018 from the medicinal plant garden of Mustinsirya University/College of Pharmacy, district of Al-Qadisiyah/Baghdad-Iraq. The sample was authenticated by Assist Prof. Ibrahim S. Abbas, Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, Mustansiriya University, Baghdad-Iraq, voucher specimens were deposited in the National herbarium of Iraq (no. 52749). Collected sample was washed and cleaned directly by water to remove the dust and other debris; after that, the plant was dried at room temperature under a stream of air to avoid the fermentation. After drying, the plant was powdered and homogenized by automatic mortar; the powder kept in the securely closed container and stored at 4 °C for further studies.

Chemicals and reagents

Aucubin, catalpol and verbascoside standards were obtained from Sigma-Aldrich. Ammonia, Ethyl acetate, n-Hexane, and Methanol were provided by Sharlau Chemicals. Copper (ll) sulfate 5 hydrate, Formic acid, n-butanol, Sulfuric acid, and Trifluroacetic acid were obtained from BDH Chemicals. Anisaldehyde and Glacial acetic acid were supplied by Riedel-de Haen. All solvents and reagents that used were of analytical grade.

Extraction of aucubin and verbascoside

The leaves powder (20 gm) of Iraqi *P. lanceoleta* L. was defatted with n-hexane (350 ml) by using soxhlet apparatus [36]. After that, absolute methanol was used as extracting solvent (330 ml); the extraction conducting for about 12 h. After that, the extract was concentrated and dried by using a rotatory vacuum evaporator at 50 °C. The dry extract was saved in tightly sealed amber glass container under 4 °C until analysis time.

General procedure

The high-performance thin-layer chromatography (HPTLC) analysis was done on 60 GF254 (10*10 cm) glass HPTLC plates (Merck, Germany) with 0.2 mm thickness; all samples and standards applied on the HPTLC plates automatically by means of thin-layer chromatography (TLC) sampler 3 (Camag, Switzerland), while densitograms were gained by use of TLC Scanner 3, using WinCat software (Camag, Switzerland). Preparative TLC plates that used for isolation were coated with silica gel GF 254 with dimensions of (20 * 20 cm) and thickness of 0.5 mm. 6410 Series LC system was used (Agilent technologies, Germany); the separation by liquid chromatography-mass spectrometry (LC-MS) was done by Zobrex C18 analytical column with length of 150 mm, diameter of 4.6 mm and particle size of 5 µm. The infrared (IR) spectrum of aucubin was obtained using KBr disk on foureir-transform (FT-IR) JASCO, 6100.

Chromatographic analysis for detection of aucubin and verbascoside

TLC analysis

A small amount of methanolic extract was dissolved in water, filtered and cleaned-up by partitioning the aqueous layer with ethyl acetate (ETAC) (this partitioning did not used for verbascoside due to its moderate solubility in ethyl acetate), the water layer was used for the TLC analysis of aucubin.

Three mobile phases were used for the analytical TLC of aucubin including n-butanol (n-BuOH): distilled water (DW) in a proportion of 9 ml: 1 ml [37], n-BuOH: acetic acid (AcOH): DW (8 ml: 2 ml: 10 ml) and isopropanol (IsoPrOH): DW (6 ml: 4 ml) [38]. Three mobile phases were used for the analytical TLC of verbascoside including Ethyl acetate (ETAC): methanol (MeOH): DW (77 ml: 15 ml: 8 ml) [39], ETAC: DW: formic acid (FA) (10 ml: 3 ml: 2 ml) and ETAC: MeOH: DW (100 ml: 16.5 ml: 13.5 ml) [40, 41].

The solvent system that used for isolation of aucubin by preparative TLC was n-BuOH: DW (9:1); while ETAC: MeOH: DW (77:15:8) was used as a mobile phase for isolation of verbascoside. The dry methanolic extract (0.5 gm) was dissolved in water and partitioned with ETAC, and both layers were subjected to Trim-Hill test; after that, the concentrated solution applied on the TLC plates for isolation of aucubin; the same amount was dissolved in methanol and applied on the TLC plates for isolation of verbascoside without the partitioning step due to the solubility of verbascoside in the ethyl acetate. Therefore, the cleaning-up step was done by applying TLC-isolated verbascoside again on the TLC plates for maximum purity.

Derivatization with other compound is necessary for visualization of aucubin spot. So, two reagents were used for post-derivatization of aucubin that includes 10% alcoholic H_2SO_4 which burns the glucose molecule in the aucubin giving colored spot and 10% anisaldehyde that gives a colored spot for aucubin molecule [41, 42]. These reagents were applied by dipping TLC plates in the reagent solutions or by spraying the solutions on the TLC plates

HPTLC analysis

The solvent systems that used for HPTLC analysis were chloroform ($CHCl_3$)**:** MeOH**:** 0.25M trifluoroacetic acid in ammonia (7:4:1) and ETAC**:** MeOH**:** DW (77:15:8) for separating aucubin and verbascoside, respectively.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Detection of aucubin and catalpol

LC-MS conditions: LC-MS analysis was accomplished in the Shahid Bahashti University/College of Pharmacy in Iran; the mobile phase was consisted of acetonitrile (ACN) and water at 0.5 ml/min flow rate, with 20 min running time; the volume of injection was 25µL at ambient temperature; the gradient elution program is presented in (table 1).

Table 1: Gradient elution program

Time (min)	Mobile phase fraction (% ACN)
0	95
4	95
8	10
12	10
16	95
20	95

After that, 1% of acetic acid in sodium acetate 200μM and water at 0.07 ml/min flow rate was added. The mass spectrometer was triple quadrupole, that worked in the electrospray positive ionization mode (ESI+), which set for isolating the sodium ions of catalpol and aucubin with mass to size ratio (m/z) of 384 and 368 respectively; the full scan was done in m/z range of 200-800 Dalton. The pressure of nebulizer was 60 psi N2 at 12L/min; the voltage of fragmentor was set at 65V and the capillary voltage was 4000V.

Detection of verbascoside

LC condition

The same instrument was used with different parameters; column: ZORBOX ODS (4.6 X150 mm, 5 μm); column temperature 25 °C; Mobile phase: A= 0.1% acetic acid (aqueous sol), B= 0.1% acetic acid (acetonitrile solution), (A: B) = 20:80; flow rate: 0.3 ml/min; injection volumes were 10 μl.

MS condition

The mode was negative electrospray ionization (ESI-) using the Agilent G6410 Triple Quadrupole Mass spectrometer; nebulizer = 15 psi; drying gas flow = 6 ml/min; V capillary = 4000v; drying gas temperature: 300 °C; Dwell time: 500msec; fragment or voltage = 135 v.

FT-IR analysis of TLC-isolated aucubin

The analysis was done in Shahid Beheshti University/College of Pharmacy in Iran. TLC-isolated aucubin was subjected to FT-IR analysis for observing some of the important functional groups in the compound; the sample of aucubin was in the solid state (2 mg) that mixed with perfectly dried KBr (150 mg), which then mulled together by mortar and pestle for producing small particle size for avoiding the Christiansen effect [43].

RESULTS

Aucubin and verbascoside were analyzed by TLC with their analytical standards in three different mobile phases for each one, and compared with that reported in the literatures. For aucubin, only the aqueous layer gave a positive result in Trim-Hill test; all TLC tests revealed the presence of both compounds in the plant samples; 10% alcoholic H_2SO_4 solution was gave the best result; the best method for applying the visualizing reagent was dipping the plate in the solution, as shown in (table 2).

Table 2: R_f values for aucubin and verbascoside in different mobile phases

Mobile phases	R_f values of Aucubin		Mobile phases	R_f values of verbascoside	
	Sample	Standard		Sample	Standard
n-BuOH: DW 9:1	0.55	0.57	ETAC: MeOH: DW 77:15:8	0.62	0.60
n-BuOH: conc. AcOH: DW 4:1:5	0.36	0.35	ETAC: DW: FA 10:3:2	0.65	0.64
IsoPrOH: DW 6:4	0.78	0.79	ETAC: MeOH: DW 100:16.5:13.5	0.55	0.56

It was found that the dry powder of Iraqi *P. lanceoleta* L. leaves (young plant) contain 1.74 percent of verbascoside, making this plant a rich source for verbascoside; while the isolated aucubin was found to be 0.24 percent of the powdered plant material; the preparative TLC fig. is presented in (fig. 3).

The development of HPTLC plates for aucubin accomplished after 45 min; then the plates dried by an electrical dryer and dipped in alcoholic H_2SO_4 (10%) and then dried for visualizing the spots, as shown in (fig. 4B). The development of HPTLC plate for verbascoside accomplished after 35 min; then the plate dried by an electrical dryer and visualized under UV light (365 nm), as presented in (fig. 4A).

Both aucubin and catalpol (M. W 346.3 and 362.3 respectively) were separated by LC and detected in Iraqi *P. lanceoleta* L. by electrospray ionization mass spectroscopy (ESI-MS) in the positive mode (ESI+) as sodium adducts [M+Na^{+2}] as presented in the (fig. 5).

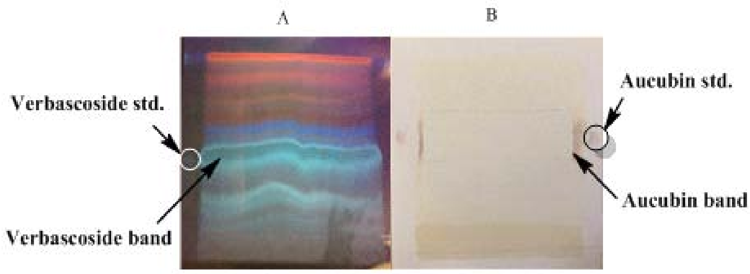

Fig. 3: Preparative TLC (A isolation of verbascoside, B isolation of aucubin)

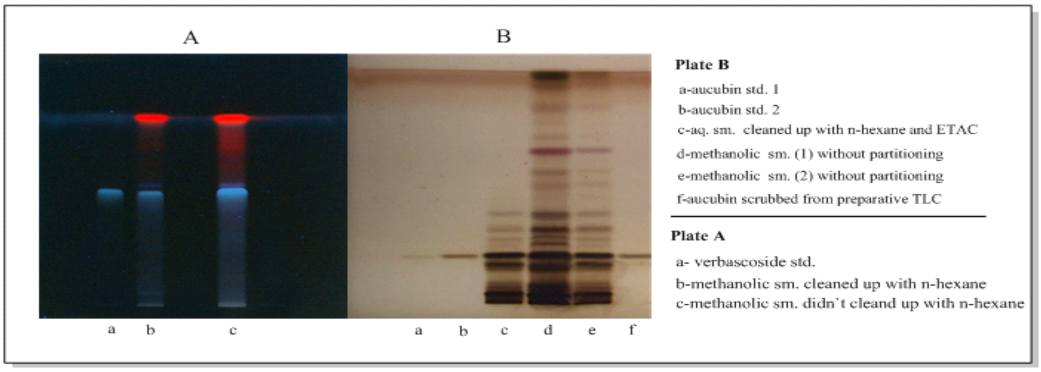

Fig. 4: HPTLC plates for qualitative studies of aucubin and verbascoside in Iraqi *P. lanceoleta* L.

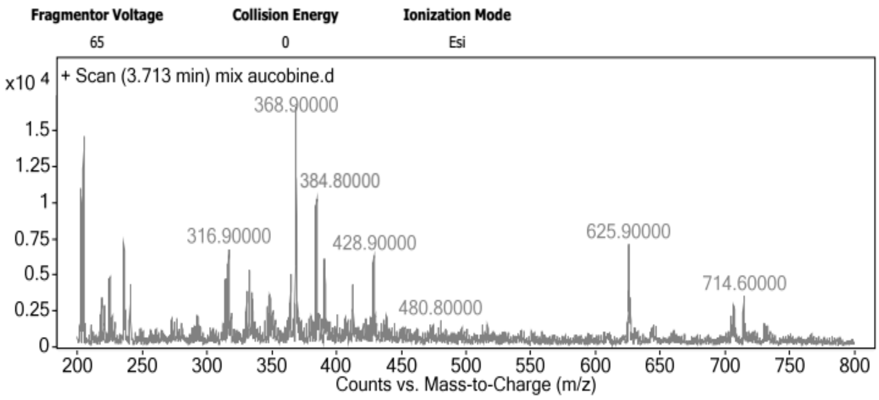

Fig. 5: Determination of aucubin and catalpol in the Iraqi *P. lanceoleta* L. by LC-MS analysis

Verbascoside (M. W 624.2) was determined in the Iraqi *P. lanceoleta* L. by means of LC-MS analysis using ESI-MS in negative ionization mode (ESI-) as quasi-molecular ion [M-H+]; which gave the mother ion [M-H+] with two product ions, as presented in (fig. 6).

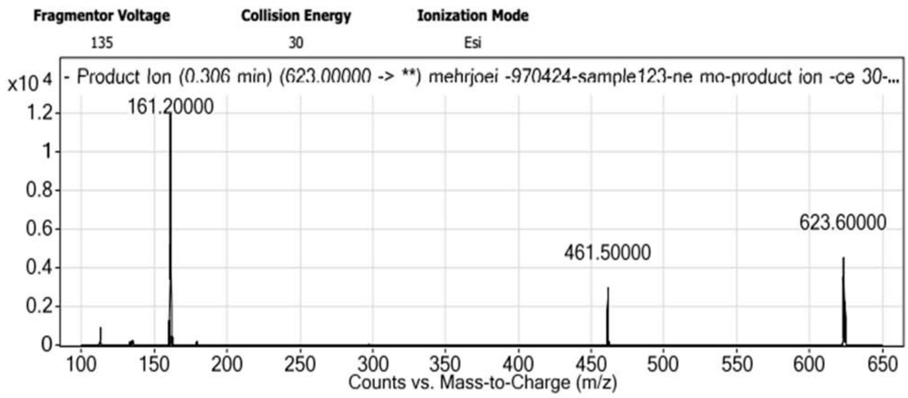

Fig. 6: Determination of verbascoside in the Iraqi *P. lanceoleta* L.

Verbascoside that isolated from the preparative TLC plates was also analyzed and compared to that detected in the plant sample; the result shown the high purity of verbascoside that cleared-up by preparative TLC, as clarified in (fig. 7).

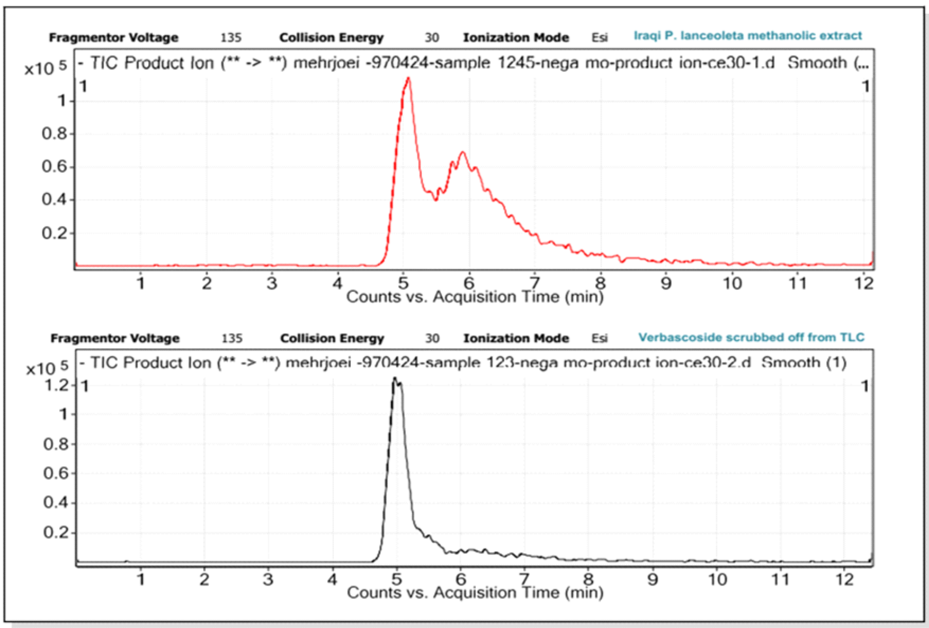

Fig. 7: Plant sample vs. TLC-isolated verbascosideunder LC-MS

After confirming its purity by HPTLC chromatography [44-46], TLC-isolatedaucubin was subjected to FT-IR analysis, in which the absorption of many important functional groups of aucubin was observed, as shown in (fig. 8) and (table 3).

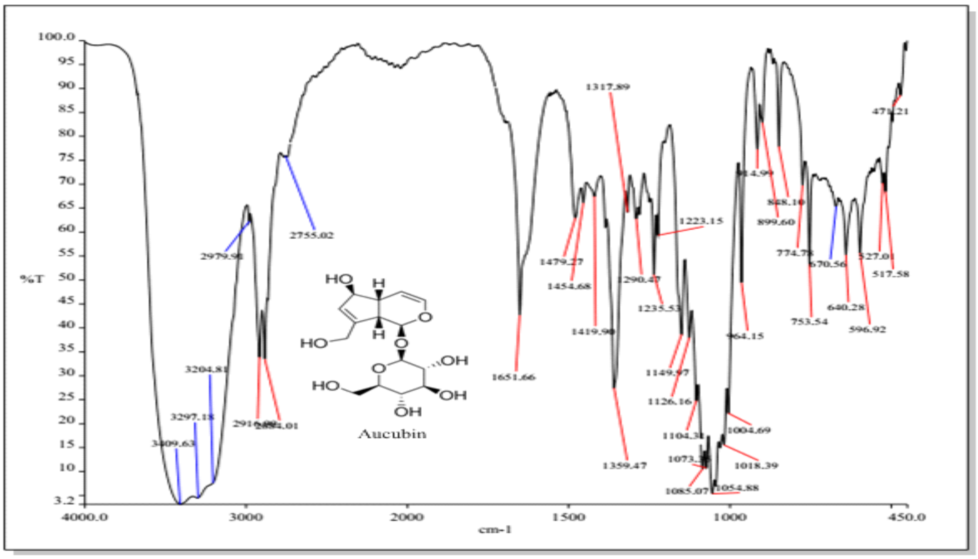

Fig. 8: FT-IR analysis of the aucubin isolated from Iraqi *P. lanceoleta* L. by TLC

Table 3: IR absorption of some important functional groups observed in the IR chart of the aucubin isolated from Iraqi *P. lanceoleta* L.

Functional group	Absorption (cm^{-1})	Intensity	Vibration
O—H of 1 ° and 2 ° alcohol	3409-3204	Broad, strong	Stretching
C=C of alkene	1651	Sharp, medium	Stretching
Alcoholic O—H	1359	Sharp, medium	Bending
C—O of ether	1126	Sharp, medium	Stretching
Alcoholic C—O	1054	Sharp, strong	Stretching

DISCUSSION

From the above findings, 1.74 percent of verbascoside obtained from Iraqi *P. lanceoleta* L. in first growing season considered as high percent and compatible to that highlighted in British pharmacopeia "*P. lanceoleta* L. contains 1.5 percent of verbascoside as minimum", while another study showed that the *P. lanceolate* L. cultivated in New Zealand showed an increment in the concentration of verbascoside from 2.36 to 3.54 percent in the first growing season, which is higher than that obtained from Iraqi plant; the same study showed an increment in aucubin concentration from 0.178 to 0.380 percent which is compatible to that obtained from Iraqi plant (0.240 percent) [13].

Post-derivatization with the specific reagent is essential for visualization of aucubin spot because there is no any chromophore in aucubin molecule. Verbascoside on another hand, its molecule contains two chromophores with many auxochromes attached to both chromophores; these properties of verbascoside molecule allow it to be seen under UV lamp (254 and 365 nm).

The result of LC-MS showed that the difference in the molecular weight of aucubin and catalpol sodium adducts was 16 Dalton, due to the difference in one oxygen atom, as presented in (fig. 5). The LC-MS result for verbascoside showed two daughter ions with high abundance at m/z 461.5 and 161.2, that resulted from the loss of a hexose sugar moiety [$M-H^{+}-162$] and loss of water molecule from the caffoeyl moiety (weak fragment at 179 m/z), respectively. The LC-MS result also showed the high purity of TLC-isolated verbascoside compared to that detected in the *P. lanceoleta* L. extract.

The FT-IR result for aucubin showed that C=C stretch result in the generation of the most characteristic peak in the aucubin structure, which occurs at 1651 cm^{-1}, unlike the conjugated or aromatic C=C, which their absorptions occur at lower frequencies in the range of 1630-1600 cm^{-1}[47].

CONCLUSION

All the results (e. g. TLC, HPTLC, LC-MS, FT-IR) proved the presence of aucubin and verbasoside in the Iraqi *P. lanceoleta* L.; the plant showed a high content (1.74%) of verbasoside. Therefore, it is considered as a very rich source for this compound, which can easily be isolated from the Iraqi *P. lanceoleta* L. and subjected to numerous pharmacological studies. The extract of the young leaves of this plant gave 0.24% of aucubin, and it is easy to obtain a higher percent from the older leaves to be a source for an important compound that needs more attention and pharmacological studies.

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AUTHORS CONTRIBUTIONS

Hasan A. Khalaf was responsible for HPTLC and advanced chemical analysis, including liquid chromatography-mass spectrometry (LC-MS) and fourier-transform infrared spectroscopy (FT-IR). Assist Prof. Dr. Ibrahim S. Abbas was responsible for authentication, collection, and drying of the plant. Amani A. Tawfeeq was responsible for plant extraction and isolation of active constituents. Prof. Dr. Monther F. Mahdi was responsible for an explanation of the results and revision of the manuscript.

CONFLICT OF INTERESTS

Declared none

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