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Qualitative and quantitative analysis of alkaloid component in seeds from *Peganum harmala* L. extracts

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Abstract:

Peganum harmala L. represents the major rich plant of alkaloids harmine and harmanline. In this study, one of these alkaloids was detected qualitatively and quantitatively using different extraction methods: aqueous, methanol, and alkaloid. In comparison between standard harmine and the three extracted using TLC technique, different spots were appeared from the three extraction methods and the color looked like the standard. While when run the extracts on HPLC in comparison to standard harmine, three peaks were observed and retention time of each extract was recorded to calculate the concentration of alkaloid. The best extraction method was the alkaloid extraction that give two peaks quite similar to that in the standard and almost the same retention time. This study shows that there are many important compounds in the alkaloid extraction of P. *harmala* of Iraqi species.

Keywords: *Alkaloid extraction, harmine, HPLC, Peganum harmala L., TLC.*

Introduction:

Still high percentage of population depends on plant extracts because it's a source of primary health care (1). These widespread of use plants derived in disease management has led to an interest in the characterization and identification of many active compounds which give the extracts their therapeutic potential. Thus, these active compounds play an important function in the development of many effective synthetic molecules (2).

World health organization (WHO) mentioned that about 11 % of medicines were exclusively produced from plants, and many of these medicines are obtained from natural precursors and about 60% of inflammation, neurological diseases, alzheimer's diseases, pulmonary diseases, diabetes, cardiovascular, arthritis, autoimmune diseases, even anti-infectious drugs, anti-tumor, and cancer in use or under clinical trials were of natural origin (3).

A data base suggest that about 15% of all plant species were studied to some extent for their photochemistry, and only 5% for its biological activities (4). A few plants were studied for their pharmacological properties although the extensive research on medicinal plants were published every year. Also, the medicinal plants and traditional medicines were represent a very interest source of novel medicines and then leads for drug development (5).

Pegan genus Peganum harmala L. belongs to the Zygophyllaceae (the Caltrop plant family) consists of 30 genera and 230 species. Are grows in the tropic, subtropics and warm regions (6,7). This family contains rich Lycorine, Haemanthamine, Galanthamine and Elaeagine alkaloids especially harman and harmine which is normally found in the (Peganum harmala L.) (8).

Alkaloids are low molecular weight nitrogen containing compounds. They have remarkable physiological effects (9), which help to use them as pharmaceuticals, stimulants, and narcotics. These nitrogen in a negative oxidation state which is of limited distribution among living organisms (10). Alkaloids represent group of molecules with a relatively large occurrence in nature. They were diverse chemicals and biomolecules, though secondary compounds and are derived from the transamination process or from amino acids. Also represent a large group of compounds with physiological and chemical, biological, and pharmacological activities.

These pharmacological properties include: analgesics, cardiovascular drugs, dilation of pupil of eye, central nervous system stimulants and depressants, mydriatics, anticholiner-

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gic, sympathomimetic, purgatives, antimalarial, etc. for example the morphine use as narcotic analgesic and quinine use as antimalarial (11).

Alkaloids could be detected by using group of reactions typical of a whole group of alkaloids and specific reactions for an individual alkaloid for their structure, chemical properties, and functional groups presence (11). The reactions are based on the alkaloids ability to yield complex or simple salts with various acids, complex iodides, heavy metal salts, and other substances. The detection reactions are either precipitation or color reactions.

Iraq is an area of high biodiversity with tremendous richness of as yet uninvestigated plant species. In this contemporary world, indigenous people in Iraq still rely mainly on their herbal traditional medicine. Actually there were increasing interest to identify natural products from plant sources that are pharmacologically potent and with low or no side effects for use it in protective medicine and the food industry. So, the main objectives of current study were to:

1) Spotlight on Iraqi Peganum harmala L. seed extracts and screen for phytochemical constituents

2) To characterize and identify the alkaloid extract using chromatographic methods (TLC and HPLC).

Materials and methods:

This study was carried out at laboratories of the Iraqi Center of Cancer and Medical Genetices Research (ICCMGR), Baghdad during 2018- 2019.

Collection and Authentication of Plant Sample:

The P. harmala seeds was bought from the traditional market in Baghdad. Seeds were weighted, cleaned from any stones and washed with tab water. Then they packaged into black paper bags and store at the laboratory until use.

Preparation of aqueous and methanol extracts:

According to Saleem et al. (12), to prepare the aqueous and methanol extracts 10 g of dried P. harmala seeds were soaked in distilled water (100 ml) and methanol (80%), respectively for 24 hours, then they crashed by mortar at room temperature. The extracts mixture was starrier for 1 hour. then filtered (using Watmann filter paper No.1). The extracts were then evaporated at 30 °C until dryness.

Preparation of Alkaloids extract:

According to Manske (13) the alkaloid extract prepared as fallow:

1. 10 g. of P. harmala seeds were crushed then covered with 3:1 (three times their weight) of water dissolved in 30 g of acetic acid per liter of water.
2. The crashed seeds leave 2-3 days to swell as they absorbed the liquid and then formed a thick dough.
3. The seeds were once more treated as above with 2:1 (twice their weight) of dilute acetic acid, then again pressed out.
4. Then, added sodium chloride (100g/ L of liquid) to transform the acetates of both harmine and harmaline (for combined liquors) into the hydrochlorides that insoluble in cold sodium chloride solutions, after that its precipitated during cooling.
5. Then, the supernatant liquid was siphoned off and the crystalline residue was filtered with suction and re-dissolved in hot water.
6. The addition of sodium chloride to the filtered solution was caused the precipitation of hydrochlorides (as a crystalline mush).
7. Then, this preparation was repeated many time until the hydrochlorides have acquired a yellow color.

The final extract was recovered by filtration (using Watmann filter paper No. 1), then extract was then heated to 37°C to get drying.

Chemical Tests Reagents:

Chemical testes reagents were used to detect active compound (secondary metabolites) of alkaloids from plant parts. Mayers and Dregendroffs reagents were used to test the aqueous, methanol and alkaloid extracts. (14)

Sample preparation for TLC and HPLC:

About 2 mg\ml of the three extracts were dissolved in methanol and filtered using polypropylene filter (as 0.45 µm). A 20 µL of aliquot was used for TLC and HPLC analyses.

Calibration solution preparation:

Starting from stock solution of harmine (Catalog NO: H1820- 76; US.Biological, USA) and methanol, which was used as a diluents (0.02g in 10ml) and standard was prepared.

Harmine (banisterine).

Harmine ($C_{13}H_{12}ON_2$) (as showed in figure 1) presents in P. harmala and in some species of Banisteia, viz., B. metallicolor, Spruce. B. lutea, and B. caapi. Alkaloid is optically inactive and forms colorless rhombic prisms from methanol. Solutions of its salts show a deep blue fluorescence. It's found to be slightly soluble in ether, alcohol or water. Pharmacologically, the harmaline looks like harmine in its actions but is more toxic. Hydrochloride has been found to be highly active against Mycobacterium tuberculosis (15).

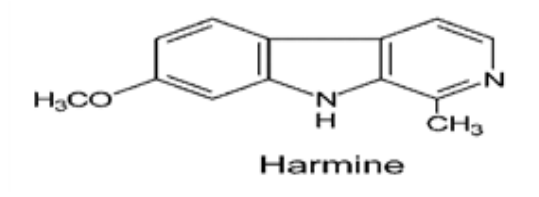

Figure (1): chemical structure of harmine.

Quantitative evaluation of spot by Thin Layer Chromatography (TLC):

In order to separating and analysis the active compounds of plant extractions by thin layer chromatography (TLC). 0.01 g\ml of the three extracts were chromatographed on 20x20 centimeter. The silica gel column (60, F 254, Merch, Germany) plate. The solvent system for the TLC was CHCl3: MeOH (50: 50) as a mobile phase and Harmine as the internal

standard (US. biological, USA). The TLC solvent was made and placed in the chamber. To equilibrate the atmosphere in TLC chamber, the chamber was left for 4-5 minutes. Waiting for few minutes after placing in chamber to let the solvent front run up the plate. Then the TLC plate was dried and visualized on UV visible instrument (16).

Thin layer chromatography TLC preparation

The spots on thin layer chromatography plates was corresponding to the separated compounds that detected under UV light of 365 nm and 254 nm. Dragendorff's and Ehrlich's reagents were followed by heating for 5-10 minutes at 105 °C. After development (the spots appeared after 10 min.), the petri dishes were dried at room temperature and the distance of each spot from the baseline was measured and recorded compared to standard spot. The Rf values of samples were measured as the equation follows (equation number 1) according to (17, 18):

$$Rf = \frac{\text{area moved by the solute}}{\text{area moved by the solvent}}$$

Chromatographic Conditions (HPLC):

The alkaloids qualitative and quantitative estimation was measured by using high performance liquid chromatography. It's done by identifications by detection of retention time obtained at identical chromatographic conditions of all samples use in this study and the standard. The equation below was used to calculate the percentage of the compound in each extraction method (equation number 2) according to (19):

$$\text{percentage of the compound in the plant} = \frac{\frac{AUC\ of\ the\ plant\ sample}{AUC\ of\ standard}}{wt\ of\ plant\ used\ in\ extraction} * C * D * 100$$

Where AUC means the area under curve, wt. means the weight of the plant used in extraction, C is the concentration of standard used in HPLC, and D is the dilution factor.

The separation was performed on a model 600 solvent delivery pump, a 746 data module Integrator, a 486 tunable absorbance detector, and a 600 E system controller. The samples injected in 20 µL loop and were eluted with the mobile phase that containing: potassium phosphate buffer (10 mM, pH 7.0): acetonitrile (100:30, v/v). Chromatography was performed under room temperature 25°C at a flow rate of 1.5 mL/min. and the eluents were monitored at 330 nm as showed in table (1).

Table (1): The experimental condition of HPLC

Experimental condition	Properties
Type of device used	Shimadzu LC- 2010 (Japan) was used to detect the presence and concentration of harmaline in the three types of extraction methods.
Column temperature	25°C
Column details	Hypersil ODS, C-18 250mm x 4.6mm, 5 µm
Flow rate	1.5 ml/min
Mobile Phase	Phosphate Buffer (10 mM pH 6.5): Acetonitrile: Methanol (55:20:25)
Injection volume	20µl
UV Detector	330 nm
Run Time	30 min
Sample compartment temperature	10°C

Results:

The results of isolation and identification of Harmine Alkaloid from Peganum harmala L. extracts were tested by phytochemical analysis (TLC and HPLC) as shown below:

Preliminary screening of alkaloids in P. harmala

Screening of alkaloids from different extraction methods confirms its presence in the extract and the results were presented in Table 2.

Table (2): phytochemical analysis of *Peganum. harmala* seed extractions

Extraction type	Test	Result	Appearance
Aqueous	Mayer's regent	*negative	No precipitate
	Dragendroff's regent	**Positive	precipitate
Methanol	Mayer's regent	Positive	precipitate
	Dragendroff's regent	Positive	precipitate
Alkaloids	Mayer's regent	Positive	precipitate
	Dragendroff's regent	Positive	precipitate

**Positive: The extract contains alkaloid
*Negative: The extract not contains alkaloid

These results showed the general contents tests of aqueous, methanol and alkaloid extracts. These tests were carried out by adding 2-3 drops of Mayer's and Dragendorff's reagents to the test solutions on glass plate. The result shows that all extracts containe Alkaloid (according to Mayers and Dragendroffs regents) except Mayer's regent of aqueous extract, which gave negative results as presented in Table (2).

Quantitative estimation of alkaloids:

Identification and characterization of alkaloids by TLC

The TLC experiments for the three extracts show that only alkaloid extract reveals clear spots on TLC papers. Alkaloid extraction smear exhibits under UV light at 365 nm two spots. This spots were observed at 365 nm under UV light were fluorescent green and purple stains with Rf values for the standard were 0.29 and 0.76 respectively, while Rf values of the extraction were 0.54 and 0.79 respectively. These Rf values were calculated (according to equation number 1) in comparable to Rf values of the standard as showed in Fig. 2 and table 3.

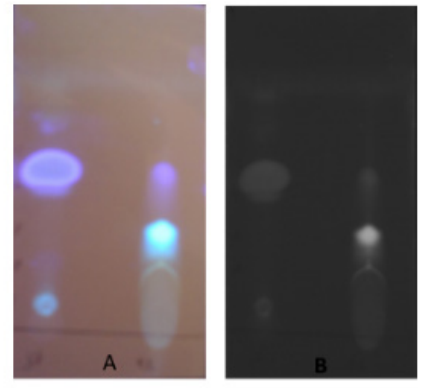

Figure (2): Chromatogram of preparative TLC for standard harmine and alkaloids extracted respectively from left to right in both A and B for *P. harmala* seeds. A: under UV light. B: under fluorescent light.

Table (3): TLC of *P. harmala* alkaloid extraction compared to standard (harmine)

Spot number	Spot type	color	RF value
1	Standard	green	0.29
		purple	0.76
2	Alkaloid extraction	green	0.54
		purple	0.79

Qualitative Identification of harmine using HPLC

The HPLC chromatogram of standard alkaloid Harmine has three peaks with retention time of 1.9, 2.5 and 2.67 Rt, min. Tow peaks where showed in HPLC chromatogram of aqueous extraction with retention time of 2.5 and 8.1. While the methanol extraction has two peaks with retention times 2.5 and 8.0. The alkaloid extraction has three peaks with retention time of 1.9, 2.5 and 7.9 min. The most abundant peak of standard (with 112564 area) was observed at the retention time 2.6 (Rt, min), while the aqueous extraction abundant peak (with 1669604 area) was observed at the retention time 2.5 (Rt,min), this results are matching to HPLC of the known standard alkaloids of harmine. The methanol extraction abundant peak (with 162360 area) was observed at 2.5 retention time which seems to be very close to the known standard of harmine. The alkaloid extraction peak with 2418624 area observed at 2.5 retention time also matching to that of standard harmine.

Hence it seems that P. harmala may contain harmine alkaloid. The concentrations of alkaloid in the three extractions have also been analyzed using equation mentioned in the materials and methods sections, and it is found to be 40.8, 39.7 and 59.1 mg/ml for aqueous, methanol and alkaloid extractions, respectively, as explained below in Table 4.

Table (4): HPLC data of three extractions from *P. harmala* compared to standard harmine

Type of extraction	Retention time RT Peak1	Retention time RT Peak2	Retention time RT Peak3
Harmine standard	1.9	2.56	2.677
Aqueous extraction	2.589	8.189	-
Alkaloidal extraction	1.934	2.599	7.94
Methanolic extraction	2.588	8.073	-

The HPLC spectra of standard alkaloids and P. harmala extractions aqueous, methanol and alkaloid are presented in Figure 3.

The concentrations of the water, methanol and alkaloid extracts are 40.8, 39.7 and 59.1 mg\ml respectively compared to standard which is just 2.0 mg\ml.

In general, the HPLC technique is very useful technique to analysis harmine alkaloid during alkaloid extraction method of P. harmala seeds.

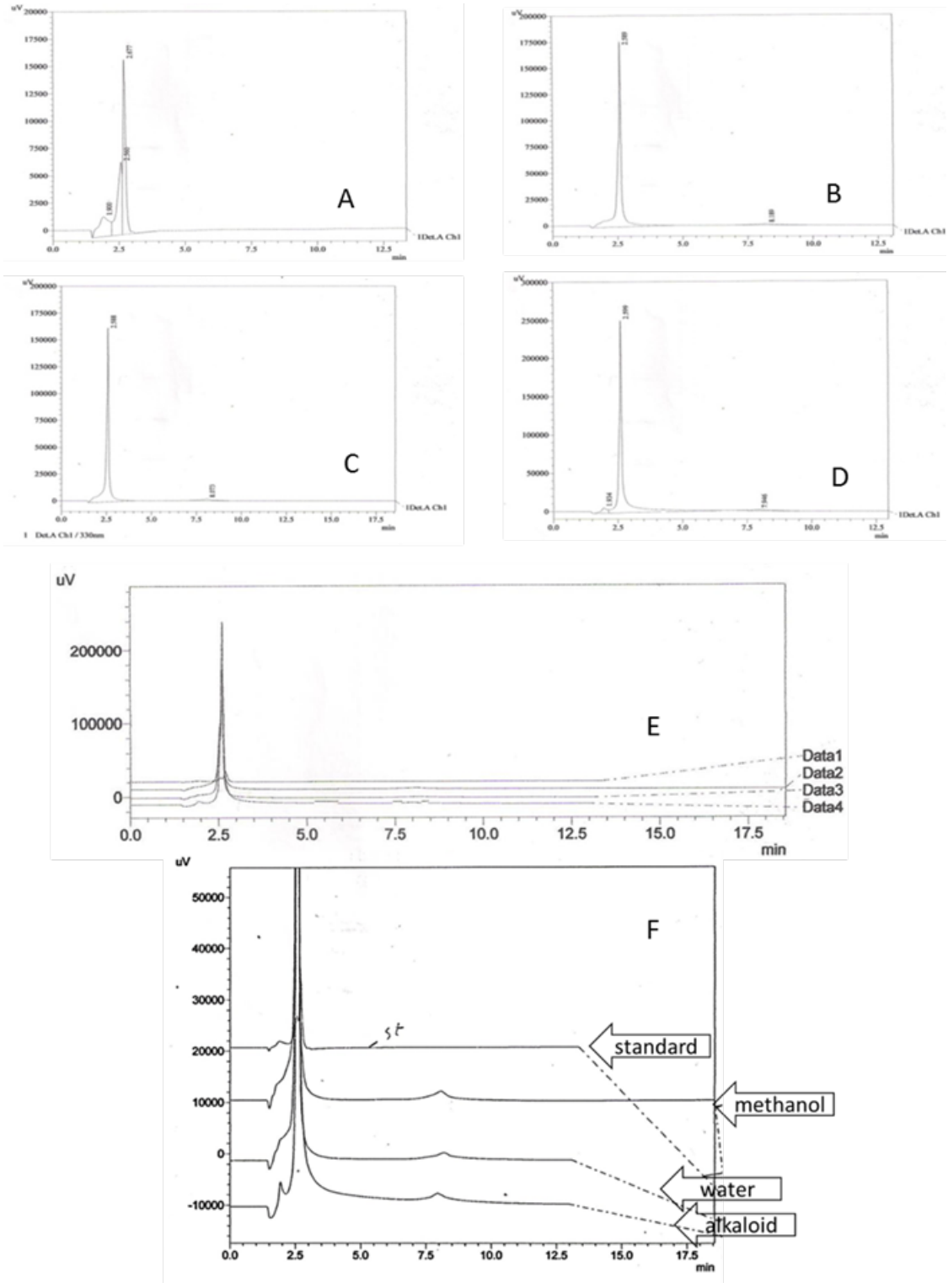

Figure (3): HPLC analysis of combination three different extraction types with standard harmine, harmine standard (A), water extraction (B), methanol extraction (C), alkaloid extraction (D), and matching (E).

Discussion:

The qualitative phytochemical analysis of P. harmala seeds through different extraction types used in this study was given a primarily spot lights about the presence of biological active compound: alkaloid. The medicinal value of the plant depends on the type of active compound and these produce as secondary metabolites to either protect the plant from the harsh environment or play as antibacterial and antifungal action and also play as antioxidant or free radical scavengers. These important alkaloids may employ in industrial and medicine fields. The best extraction type that give indication of the presence of alkaloid in the seeds is alkaloid extraction (17).

A solution of mercury iodide in potassium iodide (Mayer's reagent) with most acidified or neutral alkaloid solutions, it yields white or slightly yellowish precipitates. This reagent precipitates almost all the alkaloids except caffeine and colchicine. A solution of bismuth iodide in potassium iodide (Dragendorff's reagent) the reagent gives orange-red or reddish-brown amorphous and barely crystalline precipitates with solutions of alkaloid sulphates and chlorides. Dragendorff reagent was developed for detecting heterocyclic nitrogen compounds, alkaloids and quaternary amines. At least six different Dragendorff reagents are known each containing potassium iodide (16)..

By using high performance liquid chromatography, the qualitative identifications was made by a comparison of the retention times that obtained at identical chromatographic conditions of the analyzed samples and standards. By comparing the retention time of the isolated from isolated alkaloids of P. harmala seeds using different methods of extraction with the standard; it was found that the retention times of the extractions matched with the retention time of standard one peak in the alkaloid rich fraction (19)

In earlier experimental work by Hamid and his group about testing the presence of harmine in P. harmala seed methanol extraction using HPLC, the alkaloids concentrations were found to be haramine 0.465g, harmaline 0.355g at 7.52 min and 15.57 min respectively (20).

In general, the high performance liquid chromatography could be useful in both qualitative and quantitative compound identifications. It was very useful for the analysis of alkaloids mixtures, because of several reasons: 1- the alkaloids have absorption near 220 nm, 2- the ultraviolet that is used in HPLC devices, 3- small amount of impurities with large extension coefficient could results misleading profiles, 4- the choice of solvents was limited , 5- the strong adsorption of alkaloids to the solid phase (reverse phase HPLC) (21).

Conclusion

The results presented here was established the best way in extraction of P. harmala seeds and the presence of harmine alkaloid in the alkaloidal extraction method. Qualitative analysis showed and confirmed the presence of harmine which have an important medicinal activity.

References:

1. Bruno E. T. O. Research in clinical phytopharmacology to develop health care in developing countries: state of the art and perspectives. (2013). Phytopharmacology; 4(2): 194-205.
2. Rates S. M.K. Plants as source of drugs. (2001). Toxicon; 39: 603-613.
3. Sudhinaraset M., Ingram M., Lofthous H. K., Montagu D. What is the role of informal health care providers in developing countries? A systematic review. (2013). Plos One; 8: P. e54978.
4. WHO world health organization. WHO traditional medical strategy 2014-2023 (2013).
5. Goldstein M. S. (2000). The culture of fitness and the growth of CAM. In: Kelner M., Wellrunan B., Pescosolido B., Saks M. complementary and alternative medicine: challenging and change-Amsterdam, The Netherlands: Harwood Academic Publishers. Pp: 27-38.
6. Judd W. S, Campbell C. S., Kellogg E. A., Stevens P.F., Donoghue M. J. (1999). Plant systematics: A phylogenetic approach 1st edition. Publisher: Sinauer Assoc. Inc. Sundl and MA. Systematic Biology, vol. 48, issue 4, pp: 826-828.
7. Astulla A., Zaima K., Mastsuno Y., Hirasawa Y., Ekasari W., Widyawaruyanti A., Zainin N. C. and Morita H. Alkaloids from the seeds of P. haramala showing antiplasmoidal and vasorelaxant activities. (2008). J. Nat. Med.; 62 (4): 470-472.
8. Milad Moloudi Zargari, Peyman Mikaili and Jalal Shayegh. Pharmacological and therapeutic effects of P. harmala and its main alkaloids. (2013). Pharmacognosy Reviews; 7(14):199-212.
9. Ramawat KG, Doss S, Mathura M. (2009). The chemical diversity of bioactive molecules and Therapeutic potential of medicinal plants. In: Ramawat KG, editor. Herbal Drugs Ethnomedicine to Modern Medicine. Berlin Heidelberg: Springer-Verlag: pp. 7–31.
10. Bhat AR, Azam A, Choi I and Athar F. (2011). 3-(1,3,4- Thiadiazole-2-yl) quinoline derivatives: synthesis, characterization and anti-microbial activity. Eur J Med Chem; 46 (7):3158–3166.
11. Melentyeva, G. and Antonova, L. (1988). Pharmaceutical Chemistry, Mir Publishers, Moscow, pp. 375-393.
12. Saleem A., Mia E., Siegfried W., Juha-Matti S. and Kalevi P. (2002). Interaction of folk medicine plant extracts with human α2- Adrenoceptor subtypes. Z. Naturforsch. C. J. Biosci.; 57 (3-4):332-8.
13. Manske RHF. (2001). The Alkaloids: Chemistry and Physiology. New York: academic press. Vol. 2.
14. Rashan, L.J.; Adaim, H.; Husein, A.T. Aubi, M.T. and Rashan, F.j. (1985). Chemical, biological, an seriological studies on Peganum harmala. Report by Baghdad University. Pharmacology College.
15. Glasby JS. Encyclopedia of the alkaloids. London: Plenum Press (1978). p. 58-661.
16. Wagner, H.; Bladt, S.; Zgainsk, E.M. (1985). Plant drug analysis, thin layer chromatography atlas. Berlin, Springer. pp. 241.

17. Molyneux, R.J.; James, L.F.; Panter, K.E. and Ralphs, M.H. Analysis and distribution of swainsonine and related polyhydroxyindolizidine alkaloids by thin layer chromatography. Phytochem. Anal. (1991); 2: 125-129.
18. Waldi D. (1965) Spray Reagents for Thin-Layer Chromatography. In: Stahl E. (eds) Thin-Layer Chromatography. Springer, Berlin, Heidelberg.
19. Skoog, D.A.; Crouch, S.R. and Holler, F.J. (2007). Principles of instrumental analysis. Belmont, CA: Thomson Brooks/Cole.
20. Hamid, R.M-Esfahani; Mohammad, A. F.; Venus, M.; Mohsen, A.; and Mohammad, R.R. (2008). Detection of harmine and harmaline in P. harmala seeds by HPLC. J. of applied sciences; 8(9): 1761-1765.
21. Hussein K. H. and Enas J. K. (2016). Extraction, isolation and characterization of pyrrolizidine alkaloids present in Senecio vilgaris Linn grown in Iraq. Journal of pharmacognosy and phytochemistry; 5 (6):28-37.