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Chromatographic separation and identification of fatty acids and phenolic compounds from the seeds of *Citrullus colocynthis* L. schrad plant growing in Iraq

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Abstract

The current research was presented the phytochemical composition of *Citrullus colocynthis* schrad seed extracts and it was aimed of the separation and identification of fatty acids from this plant by using a continuous soxhlet apparatus and sequence solvent systems depending on the polarity , the extracts hexane (CI_1) ,chIoroform (CI_2), ethyl acetate (CI_3), ethanol (CI_4) and hot aqueous (CI_5) were obtained from this seeds and saponification process was done to obtain the free fatty acid compounds (palmatic, stearic, oleic, linoleic and elaidic acids). The increasing of concentrations of fatty acid compounds was appeared from CI_5 to CI_1 because of decreasing of the polarity. Also, the extracts (CI_3, CI_4 and CI_5) were carried out by the acid hydrolysis process to get the free phenolic compounds, which were identified by HPLC technique. The phenolic compounds, which were appeared in *C. colocynthis* Schrad seed extracts (Rutin , Caffeic acid , Ellagic acid , Gallic acid , Quercetin , Myricetin , Luteolin). Rutin and ellagic acid were appeared in the extracts (CI_3,CI_4 and CI_5) .Also Caffeic acid and Gallic acid was identified in CI_3 and CI_4 , while Quercetin and Myricetin were showed in CI4 and CI5. Finally Luteolin was only appeared in CI_5.

Keywords: *Citrullus colocynthis* (L.) Schrad , fatty acids , acid hydrolysis , phenolic compounds , saponification

Introduction

Citrullus colocynthis (L.) Schrad belongs to the family cucurbitaceae , common names for this plant include colocynth, bitter gourd, bitter apple, and bitter cucumber [1], the Cucurbitaceae family is a great plant family which composed of about 120 genera and 825 species , and also is one of the most genetic diversity groups of food plants , Plants of this family are usually drought-tolerant, intolerant to wet and poorly drained soils and also frost-sensitive , this species is a perennial herbaceous creeping plant and it is commonly characterized by its angular and rough stems, rough, deeply (3–7) lobed leaves of (5-10) cm and solitary flowers with pale yellow colour, each plant produces about (15–30) globular fruits having a diameter of nearly (7 -10)cm , green skin having yellow stripes [2], and the seed are smooth, compressed , ovoid- shaped ,the sizes are around 6 mm and they are light yellowish –

orange to dark brown in color[3], As this plant is cultivated in the Mediterranean basin and tropical countries like a traditional medicinal plant , and It is distributed in the saharo-arabian area of Africa, the mediterranean basin and some regions of tropical Asia[4].

Many bioactive compounds of fruit are arranged like alkaloids, flavonoids, carbohydrates glycosides, fatty acids and essential oils [5], also this species have showed to have several active chemical constituents as colocynthin, colocynthetin, α-elaterin, cucurbitacines, cucurbitacin glycosides, flavonoids and flavone glycosides, the famous bioactive components of fruit are cucurbitacins; cucurbitacins E (richly got from pulp), Phenolic compounds, Flavonoids, Fatty Acids, Alcoholic as well as Ketonic alkyl chains, these metabolites as phenols, tannins and flavonoids have very important role in defense mechanism against diseases caused by different bacteria and fungi, the main bioactive components imparting medicinal values are group of Cucurbitacins i.e cucurbitacin (A, B, C, D, E, J and L) and some other compounds such as alkaloids , terpenoids , tannins , saponins , anthranol, caffeic acid, cardic glycoside [6].

The medicinal uses of various parts of this plant over the years have stimulated interest in researching its pharmacological activities and analysing its extracts and oils for the key active component responsible for the medicinal feature of this species , this has led to the use of this plant in the expansion of new drugs, as the fruits of C. colocynthis are used to treatment constipation, ulcers , dyspepsia, joints pain and it is purgative, anthelmintic, antipyretic [7],*Citrullus colocynthis* was found that he has antidiabetic[8], hypolipidemic [9], antioxidant [10],anti-inflammatory[11], antimicrobial [12,13],pesticidal and immunostimulant activity[5].

Materials and Methods

Collection of the seeds

The seeds of *C. colocynthis* (L.) Schrad were collected from the Mosul Dam region and classified in the Directorate of the Midicinal plants Development project in the Mosul Dam of the Iraqi Minstry of Agriculture and Agriculture Reform . After the seeds were cleaned from the dust , they were grinded and put in a paper batch and kept in conditions away from moisture until use.

Preparation of some plant Extracts by using continuous soxhlet apparatus:

After the seeds of *Citrullus colocynthis* (L.) Schrad were dried and also crushed by an electric mill, where 25 gm of the well- ground powder was placed in the soxhlet batch system using 200 ml of hexane was added to the flax seeds extracted oil , the extraction process continued at a rate of 6 hours per day until the solvent in the device became colorless , finally, the extract was concentrated by a rotary vacuum evaporator (RVE) [14] .

Four solvents were utilized in the soxhlet apparatus by sequence solvent system concept; Hexane (CI_1) , Chloroform (CI_2) , Ethyl acetate (CI_3) and Ethanol (CI_4) . Hot aqueous extract (CI_5) was carried out using Grand method [15].

Saponification

In this process it was taken 10 ml of each the using crude extracts of Hexane, Chloroform, Ethyl acetate, Ethanol and Hot aqueous and added 100 ml of 7.5M KOH, by using a reflex for 90 min. at 100°C, then added 100 ml of distilled water and 50 ml ether solvent and put this mixture in the separating funnel and took the aqueous layer and added the concentrated H2SO4, until pH=2. In the end added 50 ml of ether and put again in the separating funnel and take the organic layer that contained a free fatty acids. [16,17].

Identification of fatty acids by using GLC-analysis:

The separated fatty acid compounds were identified in the laboratories of the Minstry of science and technology / Dept. of Environment and water by GLC model shimanezo, Japanese, 2010 using ionized flame detector and using the poetic column type (SE-30) ,with length (30m) with different diameters (0.25mm,0.5mm).As well as, the temperature was in the injection area and detector (280 and 330)^{o} C,while the column temperature starts from (120-280) ^{0}C in at a rate of 8 ^{o}/min. using passive nitrogen gas as carrier at pressure rate of 100 kp.

Acid hydrolysis process :

A 10 ml of each the using crude extracts of Ethyl acetate , Ethanol and Hot aqueous were taken separately and 25 ml of (1N) HCl were added to it , after which the reflux was done at 100 ℃ for a period of one hour , then the solution was placed in the separating funnel after cooling down and 50 ml of ethyl acetate was added to it twice with continues shaking , then two layers were formed , the upper layer (organic layer) of ethyl acetate and bottom layer. the top layer was taken and 3g MgSO4 was added to it .the samples were kept in tightly covered glass bottles and placed in the refrigerator until they were identified by the HPLC device [18,19].

The phenolic compounds detected depending on the area of the compound and as the percentage ratio of the separated compound, or they were converted to concentrations (mg.g^{-1}) according to the previously approved equation [20].

Identification of phenolic compounds using HPLC--UV device

The identification of phenolic compounds carried out in the in the laboratories of the Minstry of science and technology/Dept. of Environment and water resources after conducting the acid hydrolysis process. According to the method presented before [21] .By using high –performance liquid chromatography device (HPLC)type sykamn of German origin with a flow rate of 1.3 (ml min.$^{-1}$) .The mobile phase is (A) which include (Methanol: D.W:Formic acid ,(70:25:5) with the column (18-

ODS)has dimensions (25 cm * 4.6 mm) and the responses were detected at the UV-280 nm wavelength .

Results and Discussion

The identification of fatty acid compounds of *citrullus colocynthis* (L.) Schra seeds by using GLC-analysis

The identification of the compounds that presented in the hexane (CI_1), chloroform (CI_2), Ethyl acetate (CI_3), Ethanol (CI_4) and hot aqueous (CI_5) extracts after saponification process and we identified of five fatty acids; (Palmatic, Stearic, Oleic, Linoleic, and Elaidic acids)Table(1),which showed that Palmatic acid of (5.1%) and the highest concentration in the hexane extract (CI_1) as according to non-polarity of this mentioned solvent and this concept give us the reasone of increasing concentration of it ,the lowest concentration (1.25%) in hot aqueous extract (CI_5) as polar solvent .The Stearic acid compound has the highest concentration (5.2) in also hexane extract (CI_1) and the lowest concentration (1.22) in the hot aqueous extract (CI_5) . Oleic acid has the highest concentration (18.55) in the hexane extract (CI_1) and the lowest concentration (4.25) in hot aqueous extract (CI_5).

Linoleic acid was also appeared in the hexane extract (39.25) as a highest concentration and the lowest concentration (13.68) in hot aqueous extract (CI_5). Elaidic acid was presented with highest concentration (9.25) in the hexane extract , the lowest concentration (1.08) in hot aqueous extract (CI_5).

This result is accordance with a previous study (Ismael and Khorsheed , 2021) and indicated that the sequence of solvents system in the extraction showed the same result. A chromatographic chart was obtained that showed that the retention time for each compound was determined by using a standard sample of fatty acid compound table (1) fig. (1,2,3,4,5,6,7,8,9,10).

Table (1): The percentage ratio of concentration of fatty acid compounds presented in various extracts of *Citrullus colocynthis* (L.) Schrad

No.	Name %	Palmatic	Stearic	Oleic	Linoleic	Elaidic
1	**CI_1**	5.1	5.2	18.55	39.25	9.25
2	**CI_2**	4.00	3.89	14.28	30.25	5.44
3	**CI_3**	4.75	4.22	16.25	35.69	7.11
4	**CI_4**	3.02	2.14	9.25	22.56	2.56
5	**CI_5**	1.25	1.22	4.25	13.68	1.08

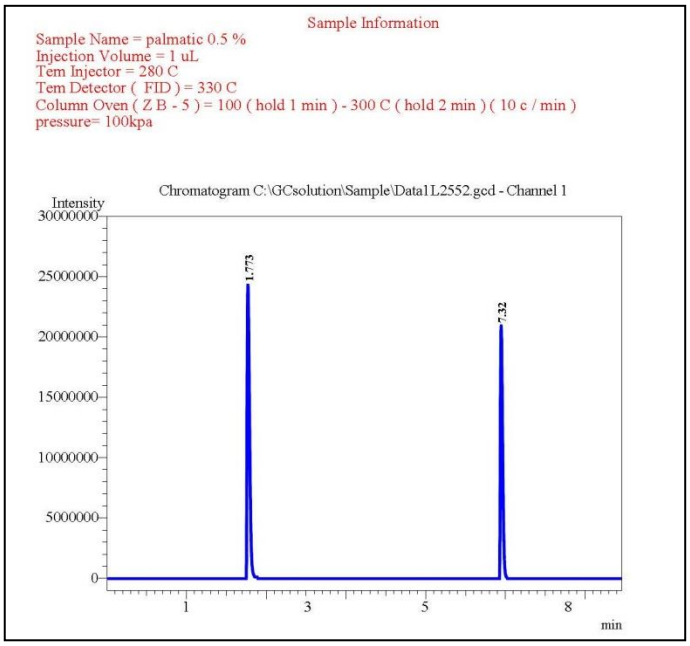

Figure (1): The standard curve of palmatic

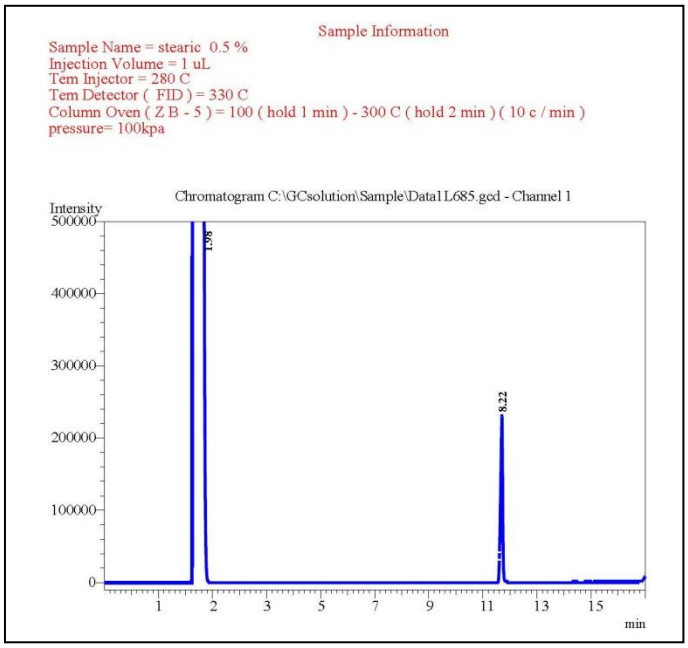

Figure (2): The standard curve of stearic

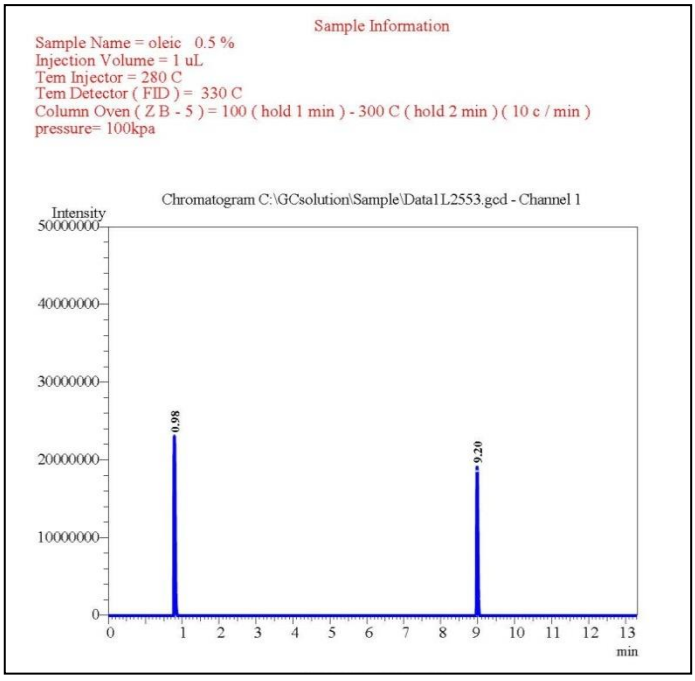

Figure (3): The standard curve of Oleic

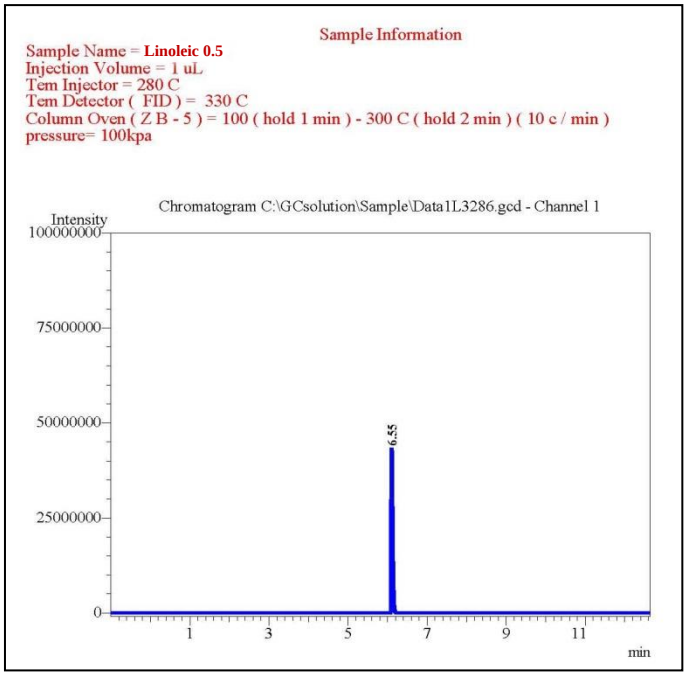

Figure (4): The standard curve of Linoleic

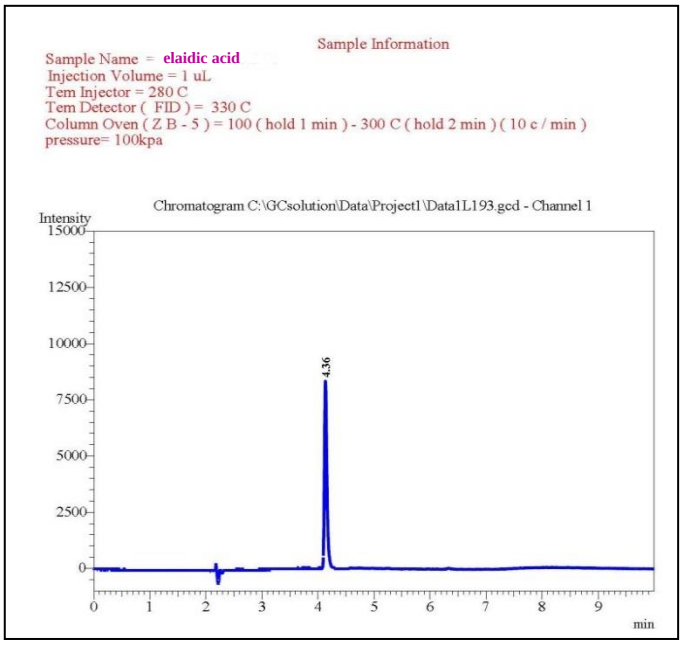

Figure (5): The standard curve of elaidic acid

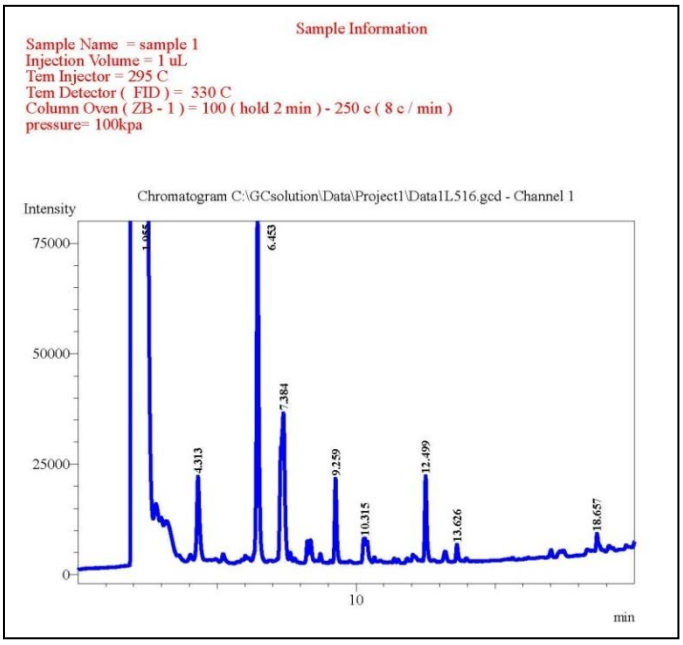

Figure (6): The fatty acid compounds form the saponified hexane extract (CI_1)

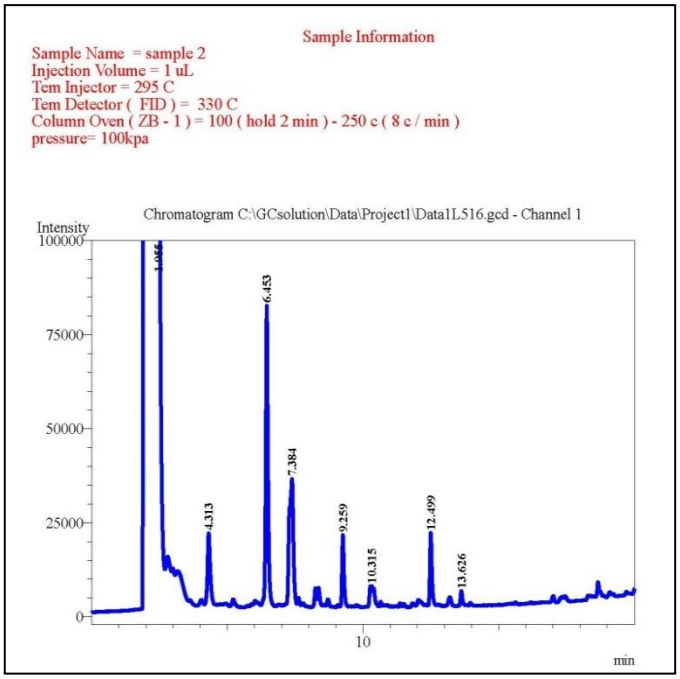

Figure (7): The fatty acid compounds form the saponified chloroform extract

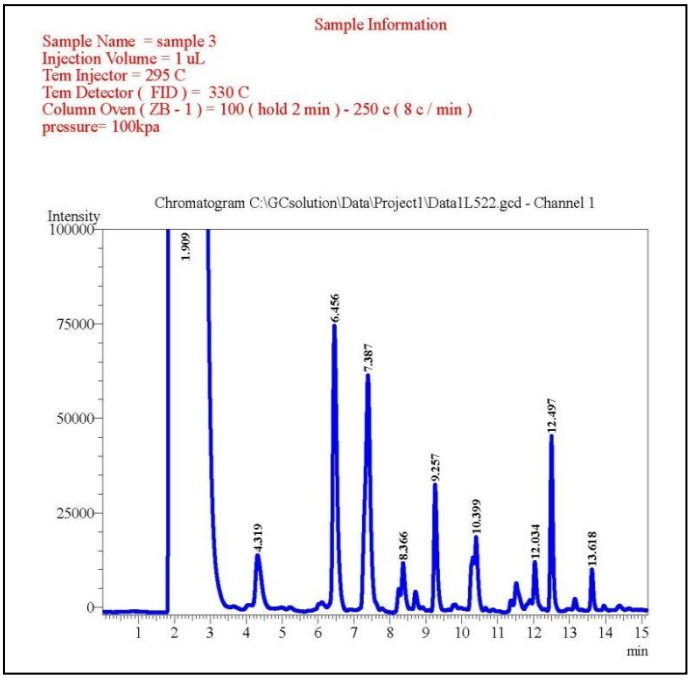

Figure (8): The fatty acid compounds form the saponified athyl acetate extract

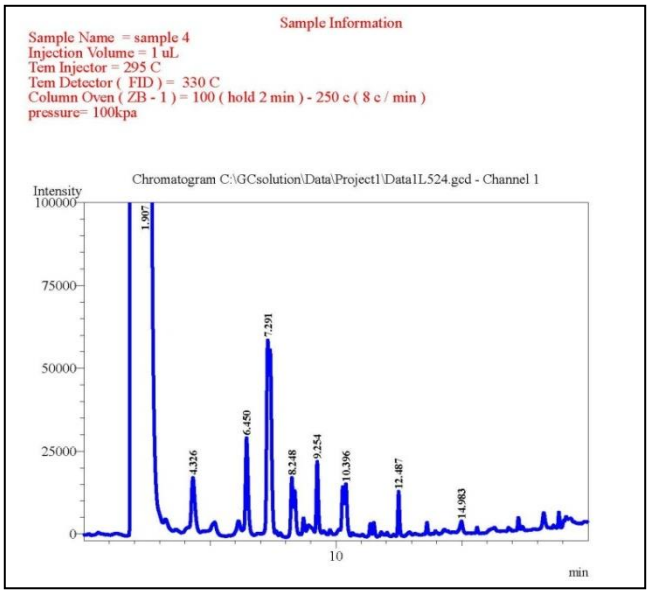

Figure (9): The fatty acid compounds form the saponified ethanol extract (CI_4)

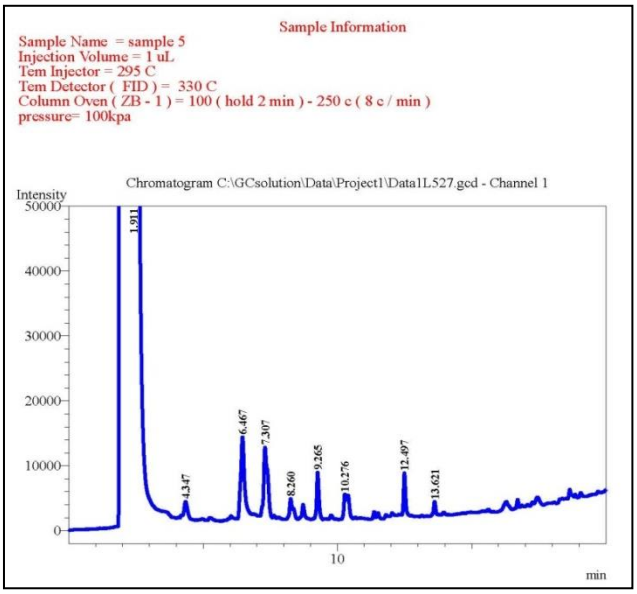

Figure (10): The fatty acid compounds form the saponified hot aqueous extract

Identification of number of phenolic compounds of *C. colocynthis* Schrad seeds by using HPLC--UV device

The chart of analysis obtained shows that the retention time of each sample was obtained and compared with standard.The time for Rutin (6.560 min), Caffeic acid (5.927 min), Ellagic acid (8.367 min), Gallic acid (9.10 min),Quercetin (3.040min), Myricetin (11.20 min),Luteolin(13.513min) . Table(2), fig. (11,12, 13,14, 15, 16,17).This indicates the presence of the phenolic compounds in the seeds of *Citrullus colocynthis* (L.) Schrad .

Rutin was showed in three extracts (CI_3 ,CI_4 ,CI_5) after acid hydrolysis process .The concentrations of Rutin were (0.185 , 0.147 , 0.279) ($mg.g^{-1}$) . Caffeic acid was appeared in two extracts (CI_3,CI_4) with the concentrations (0.046, 0.133) ($mg.g^{-1}$). Ellagic acid was also detected in three extracts (CI_3 ,CI_4 ,CI_5) with the concentrations(0.167,0.181,0.491) ($mg.g^{-1}$). Gallic acid was appeared in two extracts (CI_3,CI_4) with the concentrations (0.133, 0.130) ($mg.g^{-1}$) .

While Quercetin was appeared at (3.040 min) and with concentration of (0.016 ($mg.g^{-1}$)) in (CI_4) and was showed at (0.026 ($mg.g^{-1}$)) in (CI_5) , but it was not detected in (CI_3) .Myricetin was also showed in two extracts (CI_4,CI_5) with concentration of (0.113 ,0.261) ($mg.g^{-1}$) .Finally, Luteolin (13.513min) was detected only in the hot aqueous extract (CI_5) at concentration (0.041($mg.g^{-1}$)) ,but it was not detected in (CI_3) and (CI_4).Table (2) and fig.(11, 12,13,14,15,16,17,18,19,20).

Table (2): Indicated the standard retention times and the concentration of some phenolic compounds by using HPLC technique of *C. colocynthis* Schrad.

No.	Standard phenolic compounds	Standard retention times (Rt. min.)	Ethyl acetate CI_3		Ethanolic extract CI_4		Hot aqueous extract CI_5	
			Conc. (mg.g-1)	Rt. min.	Conc. (mg.g-1)	Rt. min.	Conc. (mg.g-1)	Rt. min.
1.	Rutin	6.560	0.185736	6.45	0.147672	6.45	0.279296	6.48
2.	Caffeic acid	5.927	0.04608	5.90	0.133704	5.90	--------	----
3.	Ellagic acid	8.367	0.167808	8.58	0.181448	8.58	0.491984	8.51
4.	Gallic acid	9.10	0.133432	9.15	0.130384	9.15	---------	----
5	Quercetin	3.040	---------	---	0.016456	3.12	0.02636	3.18
6.	Myricetin	11.20	----------	---	0.113816	11.25	0.261224	11.25
7.	Luteolin	13.513	----------	---	----------	----	0.041312	13.58

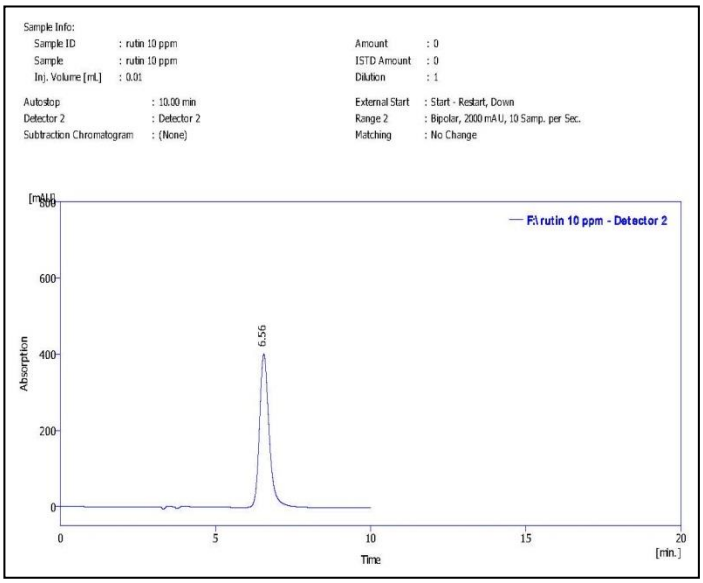

Figure (11): The standard curve of rutin

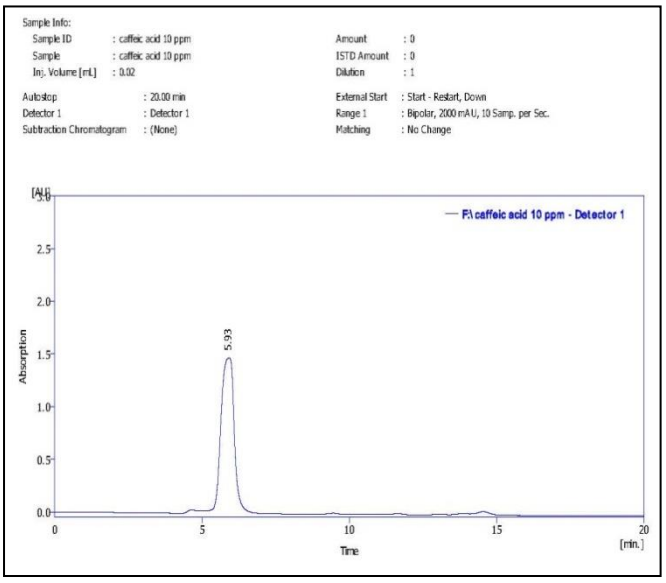

Figure (12): The standard curve of caffeic acid

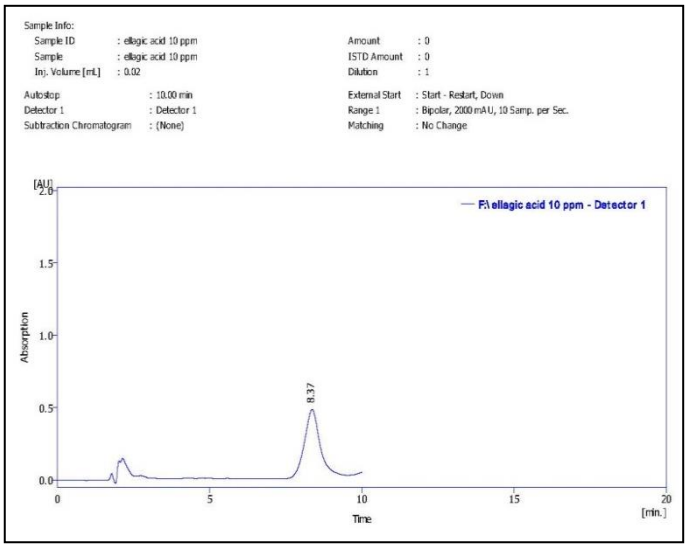

Figure (13): The standard curve of ellagic

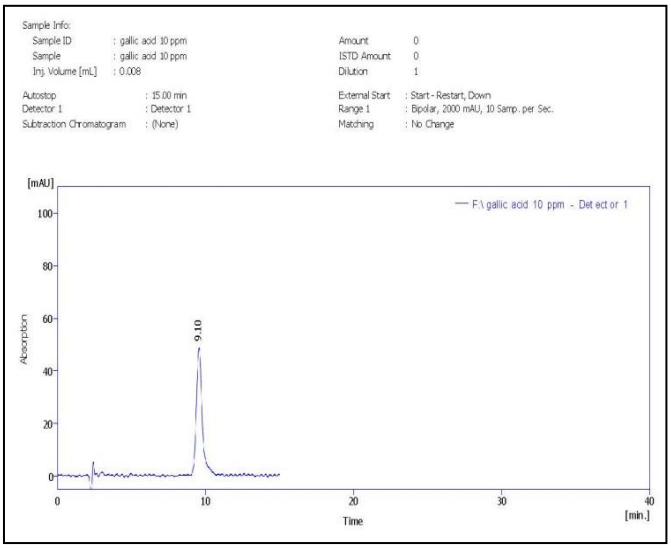

Figure (14): The standard curve of gallic acid

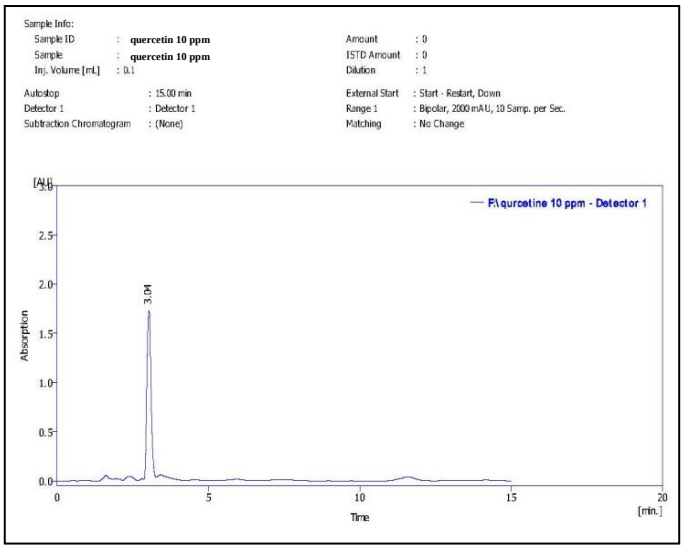

Figure (15): The standard curve of quercetin

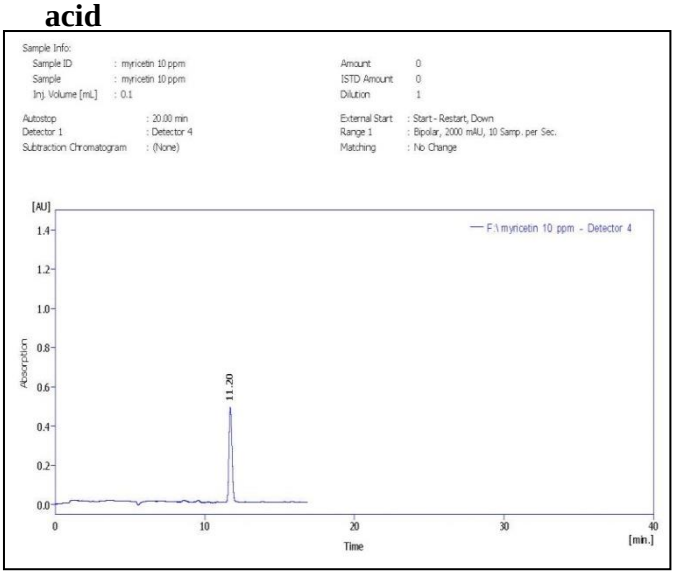

Figure (16): The standard curve of myricetin

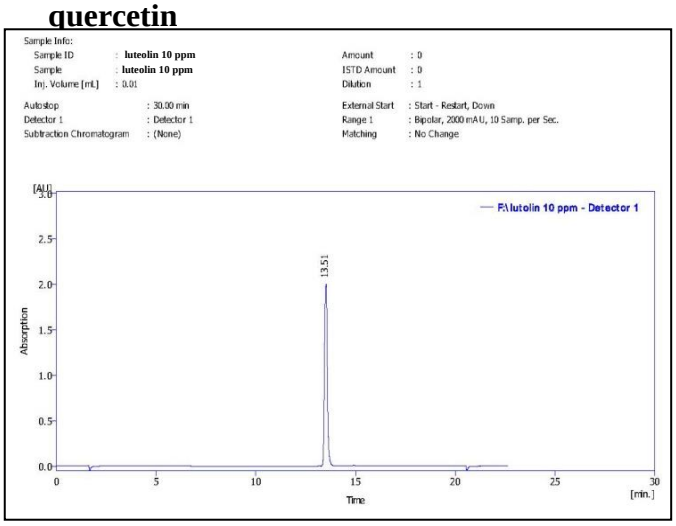

Figure (17): The standard curve of luteolin

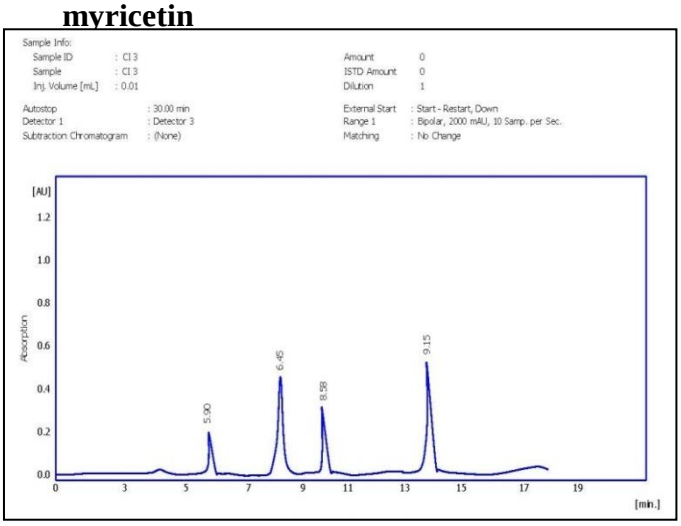

Figure (18): The ephenolic compounds form the acid hydrolysis athyl acetate

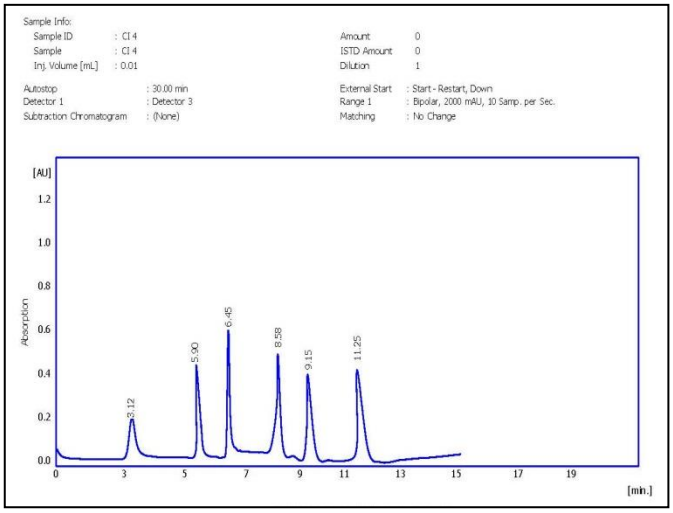

Figure (19): The phenolic compounds form the acid hydrolysis ethanol extract (CI_4)

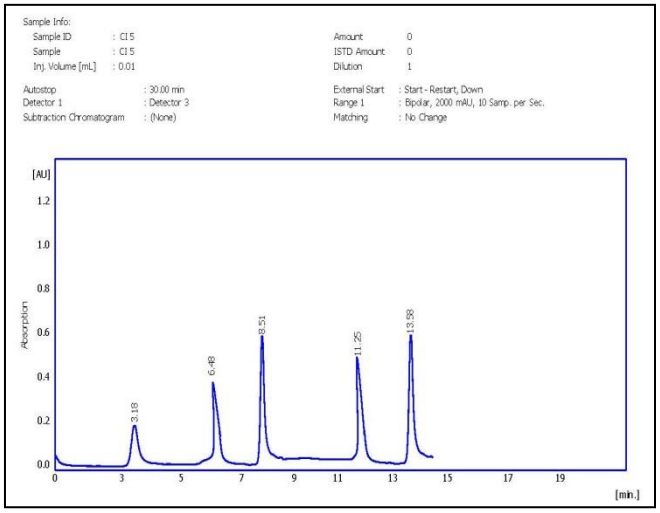

Figure (20): The phenolic compounds form the acid hydrolysis aqueous extract (CI_5)

From the results that involved (tables and figures) , it was confirmed which *C. colocynthis* (L.) Schrad seeds were among the seeds of plants, which are rich with fatty acids and phenolic compounds because of the seeds include materials which belong to the secondary metabolites.

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