

Phytochemicals Screening by GC/MS and Determination of Some Flavonol in Cultivated Iraqi *Eruca sativa* Dried Leaves Extract and its Biological Activity as Antioxidant

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ABSTRACT

Eruca sativa is considered as one of the most important edible plants, which is used widely in many countries as aphrodisiac herbal medicines and as an ingredient for salad and soup, this study concentrate on extraction of bioactive constituents in cultivated Iraqi *Eruca sativa* dried leaves, phytochemical screening by chemical tests and GC/MS, qualitative and quantitative estimation of four biological important flavonol (Quercetin, Kampferol, Rutin and Myricetin) and evaluating the antioxidant activity of methanolic-ethanolic extract. Extraction of bioactive constituents was carried out using 90% methanol and 85% ethanol. Phytochemical screening exposes the presence of glycosides, fixed oils, alkaloids, terpenoids, tannin, phenolic compounds and flavonoids. Qualitative and quantitative estimation of four flavonol (Quercetin, Kampferol, Rutin and Myricetin) was done by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) revealing that Kampferol had the highest concentration followed by Myricetin, Rutin while Quercetin had the lowest, while the antioxidant activity of methanolic-ethanolic extract was evaluated by using DPPH free radical scavenging protocol which showed that the extract was more reactive in a conc. of 20 µg/ml, & less active in a conc. of 2.5 µg/ml (IC₅₀ 33 and 47 µg/ml respectively).

Keywords: *Eruca sativa*, phytochemical screening, Quercetin, Kampferol, Rutin, Myricetin

INTRODUCTION

Eruca sativa (mill), is an edible annual plant, locally known as Al Jarjeer, commonly called Rocket salad, Arugula, Rucolar¹. It is herbaceous plant belongs in Brassicaceae family (Crucifereae) and it is greatly used as vegetable and spice². Since roman time, it has been grown in Mediterranean area but nowadays, it is cultivated and used in various places like in Iraq and used for salad and soup ingredient as shown in figure 1³. Al Jarjeer has been used traditionally as diuretic, tonic, laxative, astringent, digestive, rubefacient and stimulant⁴. It is used mainly as herbal medicine for eye infection, aphrodisiac and kidney problems¹. many research proved that it has a therapeutic property like anti-ulcer activity and cytoprotective⁵, antigenotoxic effect⁶, antibacterial activity⁷, anti-inflammatory⁸, antihyperlipidemic and antihyperglycemic⁹, antioxidant^{10,11} and anticancer¹². Al Jarjeer is considered as a rich source of antioxidant compounds like vitamins (ascorbic acid), carotenoid as well as poly phenolic compounds¹³. One of the most characteristic class of natural occurring polyphenolic compounds is flavonoid which has a wide range of therapeutic action (antimicrobial, antiallergic, anti-inflammatory, antioxidant and anticancer). Among different classes of flavonoid, flavonol class is the most important and widely spread flavonoid, in which quercetin, kampferol, Rutin and Myricetin are the best examples and

the most potent flavonol class¹⁴. The main target of the present study is to investigate the phytochemical compounds in Iraqi origin *Eruca sativa* dried leaves extract by chemical tests and GC/MS also determine the presence of some flavonol (Quercetin, Kampferol, Rutin and Myricetin) by TLC and HPLC, along with their antioxidant effect.

MATERIAL AND METHODS

Plant material

From vegetable market; fresh leaves of *Eruca sativa* (Mill) 500 gram; Iraqi origin were purchased. Then these fresh leaves were identified by the department of Pharmacognosy, college of pharmacy/Baghdad University



Figure 1: *Eruca sativa* (mill)

Table 1: Preliminary phytochemical chemical screening tests¹⁶⁻¹⁸

Active constituent	Chemical Test	Indicated positive test
Glycoside	Baljet's test	Orange color
	Keller-kiallian's test	Junction ring between two layers
Fixed oil	Brontrager's test	Pink color
	Filter paper	Permanent spot oil
Saponnin	Foam test	Persist foam
Alkaloid	Mayer's reagent	Creamy ppt
	Dargendroff's reagent	Orange-brown ppt
	Wagner's reagent	Reddish-brown ppt
Terpenoids	Salkowski test	Reddish brown color
Tannin and phenolic compounds	5% ferric chloride	Deep green or deep blue
	10% lead acetate	White ppt
	1% potassium dichromate	Orange ppt
Flavonoids	aqueous sodium hydroxide	Yellow color
	concentrated sulphuric acid	Yellow-orange color

Table 2: HPLC conditions¹⁴

HPLC parameters	HPLC conditions
Mobile phase	Gradient: solvent A=4% acetic acid in water, solvent B= acetonitrile
Column	Phenomenex C ₁₈ 250 X 4.6 mm, 5µm particle size
Temperature	Room temperature
Flow rate	0.8 ml/ min
Injection volume	20 µl
Detection	UV-VIS detector at λ=280 nm

and by herbarium of Baghdad university authentication was done.

Preparation of plant extract

500 gram of fresh leaves of *Eruca sativa* were washed to be free from extraneous material, dried in shade, powdered using electric blender and then weighted for extraction procedure. 100 gram of the powdered dried leaves of *Eruca sativa* were extracted by using Soxhlet apparatus in which powdered leaves were packed in a thimble of it and extracted with 500 ml of 90% methanol for 18 hours to extract all possible compounds then by using Whatmann no.1 filter paper methanolic extract (fraction 1) was obtained. The extraction procedure was continued by drying the residual plant material (dried leaves) of *Eruca sativa* over night at room temperature. Then extracted with 500 ml of 85% ethanol, placed in around bottom of the flask fitted with the reflux condenser and the remaining compounds, so fraction 2 was obtained by filtration of

ethanolic extract from *Eruca sativa* powdered leaves. Later on, Soxhlet methanolic extract F1 and reflux ethanolic extract F2 of powdered dried leaves of *Eruca sativa* were mixed together to give methanolic-ethanolic extract (Fraction 3). Then F3 concentrated under reduced pressure by using rotary evaporator at temperature not exceeding 40 C⁰, the greenish colored residue dried extract was weighted and subjected for different phytochemical screening¹⁵.

Eruca sativa leaves extracts phytochemical screening

Phytochemical screening by chemical tests

The presence of glycosides, fixed oils, alkaloids, terpenoids, tannin, phenolic compounds and flavonoids had been identified by making general test as preliminary phytochemical screening of *Eruca sativa* dried leaves extract as illustrated in the table (1)¹⁶⁻¹⁸.

Phytochemical screening by GC-MS

Preparation of sample for GC-MS

0.5 g of the methanolic-ethanolic extract (F3) was dissolved 95% methanol. The extract was filtered through microfilter 0.45 µm, then 2 µl of this solution was employed for GC/MS screening¹⁹⁻²¹.

GC-MS screening

GC-MS screening was carried out on a Shimadzu GCMS-QP2010Ultra system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30x0.25 mm IDx1EM df, composed of 100% Dimethyl poly siloxane), helium (99.999%) was used as carrier gas at a Flow Control Mode :Pressure:100.0 kPa, Total Flow :17.6 mL/min, Column Flow :1.33 mL/min, Linear Velocity :43.0 cm/sec, Purge Flow :3.0 mL/min, Split Ratio :10.0, injector temperature 220°C; ion-source temperature 200°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 220°C, ending with a 9 min isothermal at 220°C. Mass spectra were taken at 70 eV, then the time required for sample chromatography was 20 minutes¹⁹⁻²¹.

Phytochemical screening of some flavonols

Al-Jarjeer is loaded with flavonols which act as antioxidant, determination (qualitative and quantitative) of some flavonol was performed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

A- TLC

Qualitative estimation of flavonol (Quercetin, Kampferol, Rutin and Myricetin) in dried leaves extract of Iraqi *Eruca sativa* was carried out by thin layer chromatography using readymade silica gel aluminum plates GF 254, detection by UV light detector at 254nm wave length, standard flavonol quercetin (fluka austia), kampferol (sigma-aldrich, USA), Rutin (fluka austia) and Myricetin (fluka austia). Three different solvent systems for free flavonol²².

S1 = Toluene: Chloroform: Acetone (40:25:35).

S2 = Chloroform: Methanol (90:10)

S3 = Chloroform: Acetone: Formic acid (75:16.5:8.5)

And three different solvent systems for glycosidic flavonol²³

S4 = Ethanol: acetic acid (85:15)

Table 3: Results of preliminary phytochemical chemical screening tests

Active constituent	Glycoside	Fixed oil	Saponnin	Alkaloid	Terpenoids	Tannin and phenolic compounds	Flavon oids
Result	+	+	+	+	+	+	+

Where + =present - = absent

Table 4: Phytocomponents identified in the methanolic ethanolic extract of the dried leaves of *Eruca sativa* by GC-MS.

Number of peaks	Retention time	Peak area %	Formula	Molecular weight	Compound name
1	3.56	20.43	C ₁₄ H ₃₀ O	214	2-Hexyl-1-octanol
2	3.64	2.18	C ₈ H ₁₆ O ₂	144	2,5-Dimethyl-4-hydroxy-3-hexanone
7	4.06	0.56	C ₉ H ₁₆ O ₃	172	Ethyl 2-formyl-4-methylpentanoate
8	4.09	2.36	C ₁₀ H ₂₀ O	156	Cyclohexanol,5 methyl-2-(1-methylethyl)
9	4.19	6.29	C ₁₇ H ₂₇ NO ₂	277	Phenol,2,6-bis(1,1dimethylethyl) -4-methyl-, methylcarbamate
10	4.32	4.07	C ₄ H ₃ N ₅ S	153	6-amino-8-thiapurine
17	4.90	35.27	C ₂₀ H ₄₀ O	296	Trans-phytol
23	5.78	18.23	C ₁₀ H ₂₀ O	156	D, L-Citronellol
44	8.83	2.43	C ₂₃ H ₄₂ O ₄	382	Oxalic acid, allyl octadecyl ester
53	10.55	1.71	C ₇ H ₁₂ O ₂	128	Isobutyric acid, allyl ester

Table 5: Activity of major important phyto-components identified in *Eruca sativa* dried leaf extract by GC-MS.

Number of peak	Retention time	Class of compounds	Name of compounds	Uses
9	4.19	Phenolic compound	phenol	Phenol is used as a buccal anesthetic/analgesic, commonly used as antiseptic, especially as Carbolic soap, from the early 1900s through the 1970s.
17	4.90	Terpenoids compound	Trans Phytol	Phytol can be used as a precursor for synthesis of vitamin E and vitamin K1.
23	5.78		D, L-Citronellol	Citronellol is used in perfumes and insect repellents
44	8.83	Fatty acid compounds	Oxalic acid, allyl octadecyl ester	Oxalic acid's main applications include bleaching, especially for the removal of rust
53	10.55		Isobutyric acid, allyl ester	Isobutyric acid used as food and perfume additives. It is also used as an animal feed supplement to reduce pathogenic bacterial colonization

Table 6: R_f values of some flavonols and their standards in various developing solvent systems.

Flavonol	TLC R _f standard			TLC R _f in F3		
	S1	S2	S3	S1	S2	S3
Quercetin	0.46	0.41	0.71	0.44	0.4	0.72
Kampferol	0.61	0.55	0.81	0.6	0.56	0.8
Myricetin	0.4	0.36	0.55	0.39	0.35	0.56
Rutin	S4	S5	S6	S4	S5	S6
	0.88	0.39	0.87	0.89	0.4	0.86

S5 = Chloroform: methanol: water (40:10:1)

S6 = N-butanol: glacial acetic acid: water (30:10:50)

B- HPLC

Further qualitative estimation of flavonol was done by using SHIMADZU LC-2010AHT high performance liquid chromatography at which identical chromatographic condition, identification was made by comparison the retention time of plant extract (F3) and authenticated flavonol standard. Then quantitative estimation of flavonol was done by using calibration curve in which four

referenced flavonol standards were constructed by preparing series of diluted solutions from stock solutions
Table 7: Retention time of four flavonols in F3 extract with that of reference standard.

Flavonols	R _t value of standard	R _t value of flavonol in F3 extract
Quercetin	4.626	4.631
Kampferol	3.976	3.953
Myricetin	3.588	3.459
Rutin	7.007	7.355

Table 8: Quantitative concentration (ppm) of four flavonols in Iraqi *Eruca sativa* dried leaves methanolic-ethanolic extract F3

Flavonol	Concentration (ppm)	Area under the curve
Kampferol	0.363	4033039
Myricetin	0.305	1214976
Rutin	0.171	5338630
Quercetin	0.022	864009

(100 ppm). The concentrations of each standard were gained after plotting them against the area under the curve²⁴.

RESULT AND DISCUSSION

Methanolic-ethanolic extract (F3) was obtained from the experimental work, in which methanolic extract F1

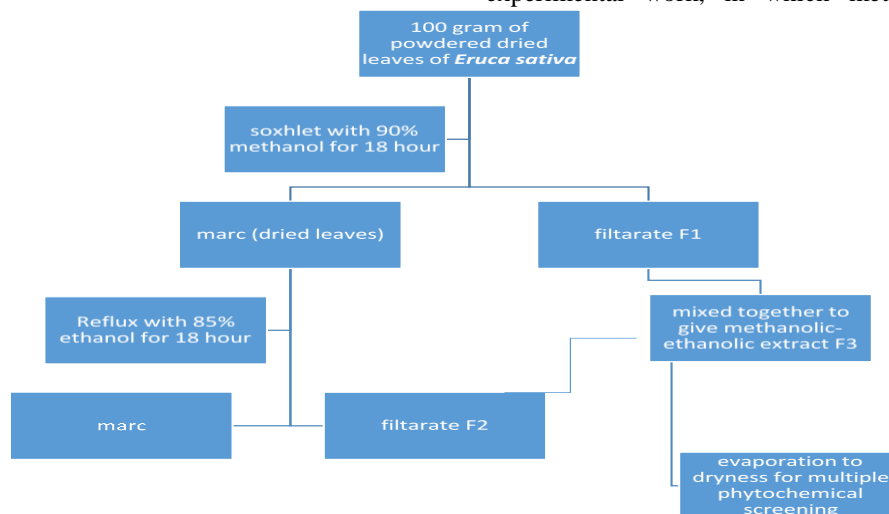


Figure 2: *Eruca sativa* dried leaves extraction schematic procedure

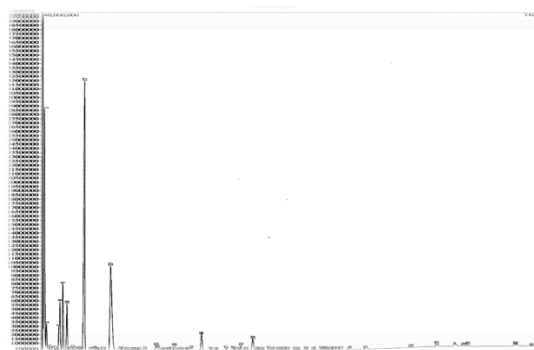


Figure 3: GC-MS Chromatogram of *Eruca sativa* dried leaves.

Free radical scavenging activities (antioxidant) assessment

Reagent: 1, 1-Diphenyl-2-picrylhydrazyl Sigma-Aldrich (Schnelldorf, Germany)

Free radical: The free radical scavenging activities of F3 extract was assessed by using the DPPH radical. The experiment was slightly modified by making 0.5 m. mole /L solution of DPPH in methanol and again diluted four times (4X) in order to showed absorbance below 1. The solution of DPPH (1000µl) was mixed with different concentration of sample (2.5-20µg/ml, 500µl) and the absorbance change at 517 nm was measured 30 min later. The reaction solution without DPPH was used as a blank test and ascorbic acid (2.5-15µg/ml, 500µl) was used a positive control. The experiment conducted on Shimadzu spectrophotometer (UV-120-01), by using low lens. Mean value of triplicate was plotted in graph in order to calculate the concentration required for 50% reduction (50%inhibition concentration, IC50) of DPPH radical. GraphPad Prism version 4 (GraphPad Prim Software, Inc., San Diego, CA, USA) was used for calculating the concentration²⁵⁻²⁷.

extracted by Soxhlet apparatus while ethanolic extract F2 extracted by reflux apparatus, later then by mixing F1 and F2 methanolic-ethanolic F3 gained with 38.73%.

Phytochemical screening by chemical tests

The results of preliminary phytochemical chemical screening tests shown in table 3 indicate the presence of glycosides, fixed oils, Saponin, alkaloids, terpenoids, tannin, phenolic compounds and flavonoids in methanolic-ethanolic extract of Iraqi *Eruca sativa* dried leaves.

Phytochemical screening by GC-MS

Eruca sativa, dried leaves extract revealed the presence of medicinal active constituents. In the GC-MS analysis, 81 bioactive photochemical compounds were identified in the methanolic-ethanolic extract of *Eruca sativa*. The identification of photochemical compounds is based on peak area, molecular weight and molecular formula, some of the GC-MS peaks remained unidentified, because of lack of authentic samples and library data of corresponding compounds. The major ten compounds have been identified for the first time in sample of *Eruca sativa* dried leaves as illustrated in the table 4 and figures 3.

Phytochemical screening of some flavonols

A- TLC

Thin layer chromatography of methanolic-ethanolic extract F3 obtained from dried leaves of Iraqi *Eruca sativa*, confirms the following:

The presence of free flavonols (Quercetin, Kampferol and Myricetin) in F3 extract, as compared with standards.

The presence of glycosidic flavonol (Rutin) in the same fraction when comparison was made with standard.

As illustrated in table 6 and figure 9.

B- HPLC

The result shows that HPLC method was efficient for qualitative estimation and quantitative screening of some flavonol (quercetin, kampferol, Rutin and Myricetin) in Iraqi *Eruca sativa* dried leaves extract, in which both of flavonol standard and F3 extract retention time were identical as shown in figure 10, 11 and illustrated in table

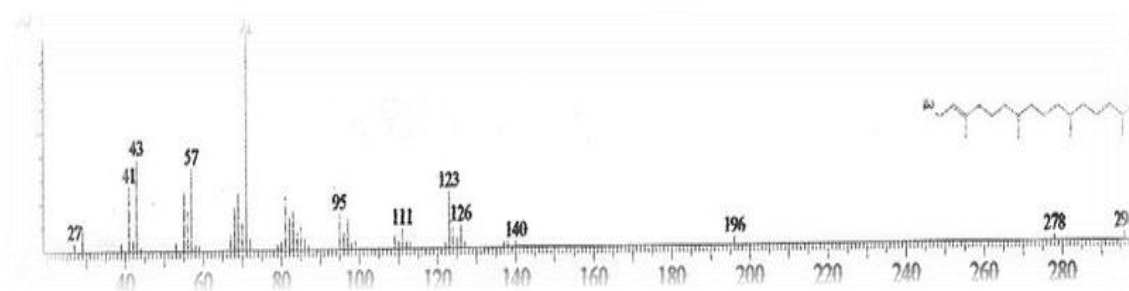


Figure 4: GC-MS Spectrum of phytol

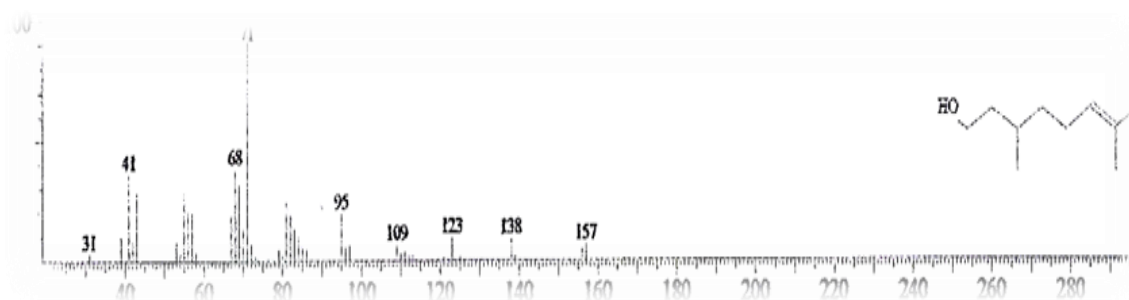


Figure 5: GC-MS Spectrum of D, L-Citronellol

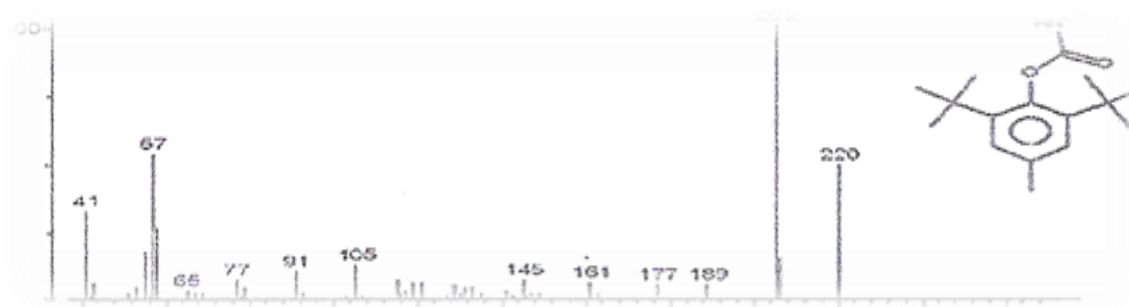


Figure 6: GC-MS Spectrum of phenol

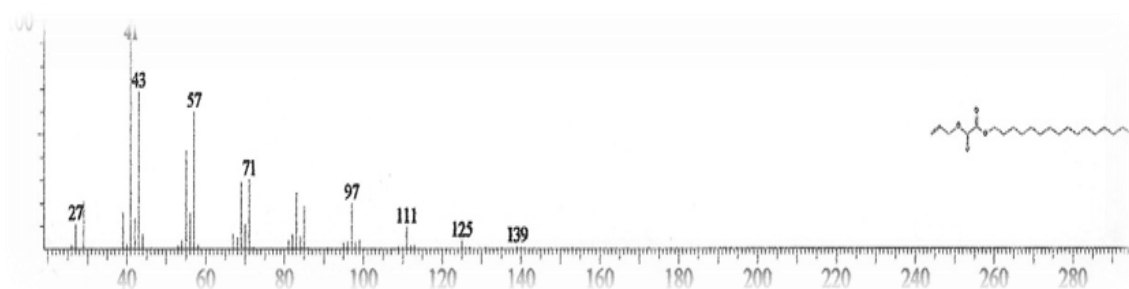


Figure 7: GC-MS Spectrum of Oxalic acid, allyl octadecyl ester

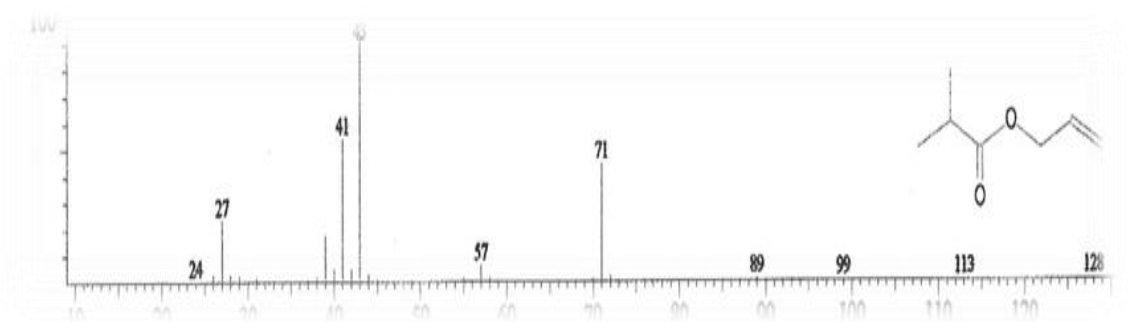


Figure 8: GC-MS Spectrum of Isobutyric acid, allyl ester

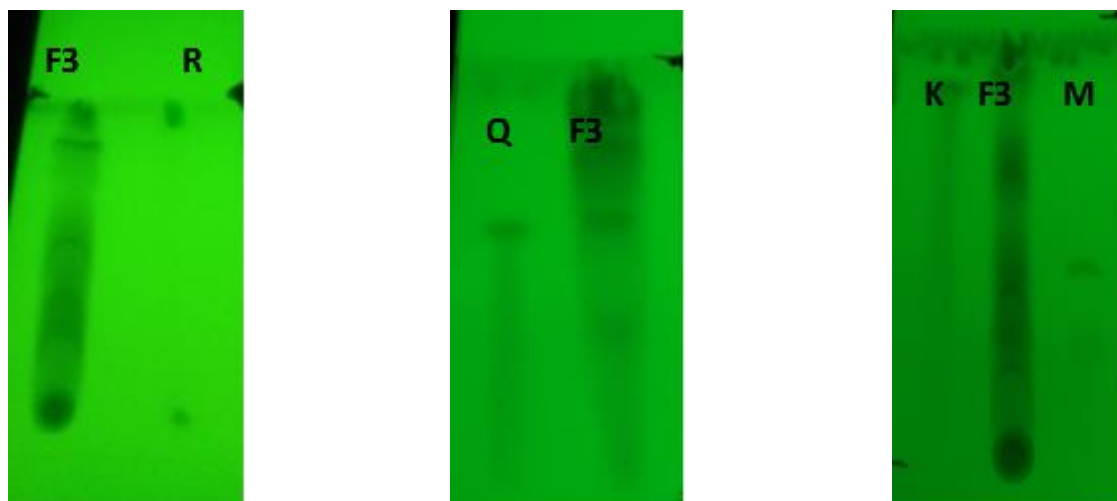


Figure 9: TLC of F3 with four flavonol standards on silica gel GF254 developed in S3 solvent system for quercetin, kampferol and Myricetin and S4 solvent system for Rutin, detected by UV light at 254nm. (Q=Quercetin, K=kampferol, M=Myricetin and R= Rutin)

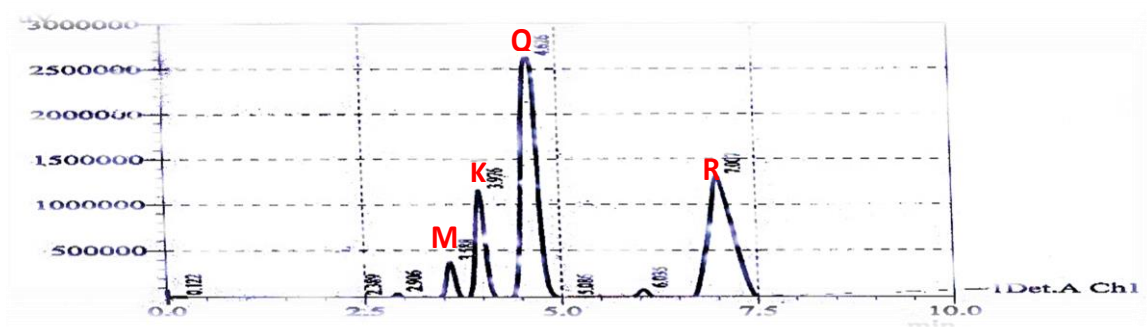


Figure 10: HPLC of standard flavonol (M=Myricetin, K= kampferol, Q=quercetin and R=Rutin).

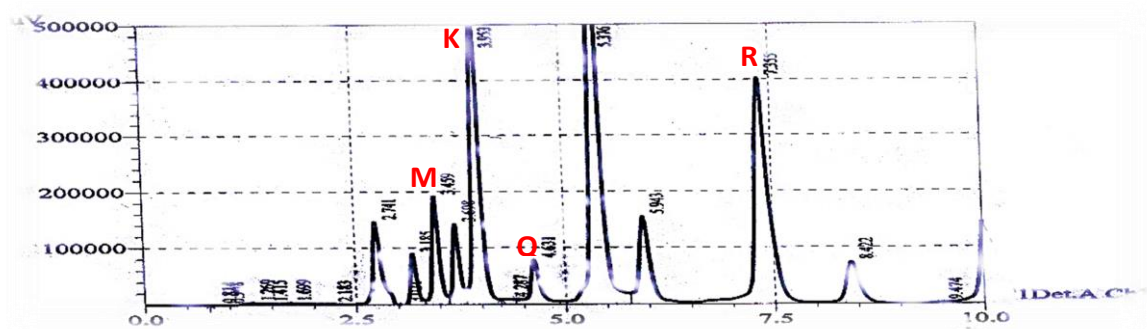


Figure 11: HPLC of four flavonols in F3 extract (M=Myricetin, K= kampferol, Q=quercetin and R=Rutin).

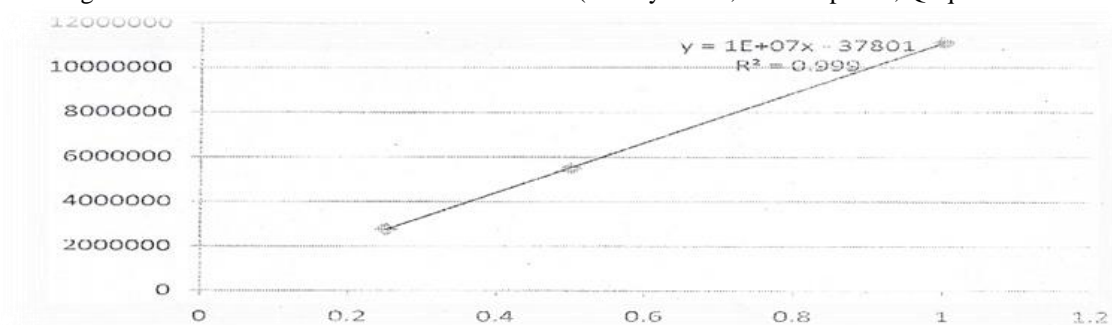


Figure 12: Calibration curve of Kampferol.

7. For quantitative measurements, the calibration curves were plotted using the area under the curve versus the concentration of serial dilutions of each flavonol standard; a straight line were obtained as shown in figures 12-15. The results of quantitative concentration (ppm) of four flavonol in Iraqi *Eruca sativa* dried leaves methanolic-ethanolic extract F3 reveals that Kampferol had the highest concentration followed by Myricetin, Rutin while Quercetin had the lowest, as illustrated in table 8.

Free radical scavenging activities (antioxidant) assessment

F3 was studied for Free radical scavenging activities. Ascorbic acid was used as a standard antioxidant compound (IC₅₀= 5.75 µg/ml), Separate antioxidant cure was established for concentration of F3. Results showed that F3 was more active in scavenging 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Free radical (I/C₅₀= 2.5) with 20 µg/ml concentration

Free radical scavenging activities

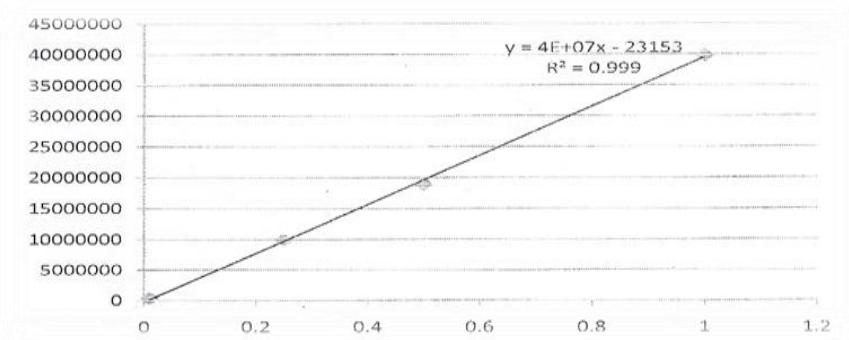


Figure 13: Calibration curve of Quercetin.

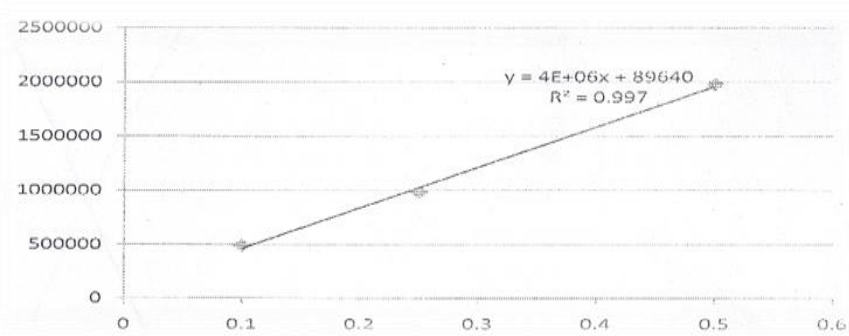


Figure 14: Calibration curve of Myricetin.

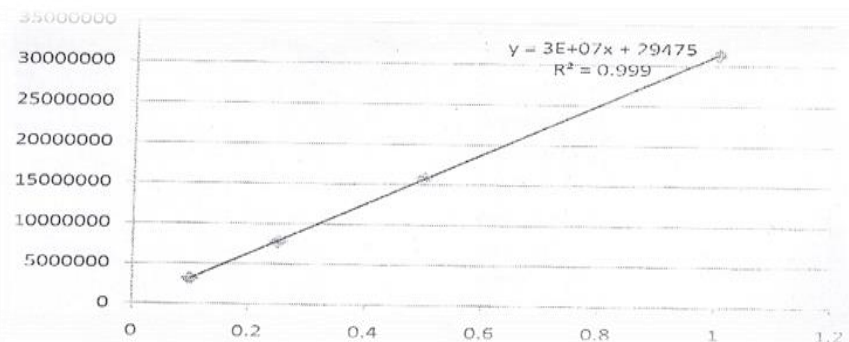


Figure 15: Calibration curve of Rutin.

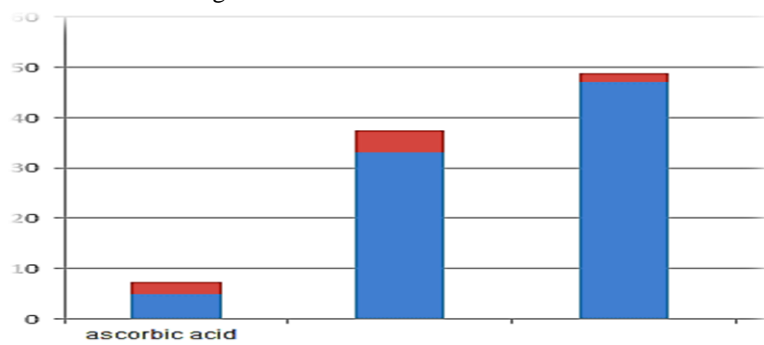


Figure 16: Free radical scavenging activity of F3

F3 was investigated for its free radical scavenging capacity. DPPH (1, 1-Diphenyl-2-picrylhydrazyl) was used in the experiment. The bleaching of DPPH color in the experiment by F3 extract represents the scavenging capacity of this extracts. Results shows that F3 extract was more reactive in a conc. of 20 µg/ml, & less active in a conc. of 2.5 µg/ml (IC₅₀ 33 and 47 µg/ml respectively), Ascorbic acid was used as standard antioxidant (IC₅₀ = 5.75 µg/ml).

CONCLUSION

Phytochemical screening of cultivated Iraqi plant used widely in many countries as aphrodisiac herbal medicine named *Eruca sativa* was done and the results show the presence of bioactive constituents (glycosides, fixed oils, Saponnin, alkaloids, terpenoids, tannin, phenolic compounds and flavonoids) in methanolic-ethanolic extract F3 of Iraqi *Eruca sativa* dried leaves investigated by chemical tests and GC/MS. Also chromatographic HPLC analysis was carried out to qualify and quantify four types of flavonol (Quercetin, Kampferol, Rutin and Myricetin) in F3 and the results reveal that Kampferol had the highest concentration followed by Myricetin, Rutin while Quercetin had the lowest, while assessing the antioxidant activity of F3; the DPPH scavenging protocol was used; the result show that F3 extract was more reactive in a conc. of 20 µg/ml, & less active in a conc. of 2.5 µg/ml (IC₅₀ 33 and 47 µg/ml respectively).

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