Purification and Identification of Total Flavonoids Extracted from *Moringa oleifera* Leaves in Iraq

تنقية وكشف الفلافونويدات المستخلصة من اوراق نبات شجرة البان في العراق

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Abstract

***Moringa oleifera* is an important medicinal plant, which contains a lot of bioactive compounds. The dried plant leaves were extracted in 70% methanol by maceration using shaker incubator at 40°C. Chemical detection of bioactive compounds in crud plant extracts was performed. The total flavonoid was isolated from the extract using reflux, The use of thin layer chromatography technique that assisted in the detection of Qurciten, Rutin and Luteoline of flavonoids isolated from the extract, the existence of qurciten and rutin confirmed by the use of high performance liquid chromatography technique. The calculation of total flavonoid of *M. oleifera* leaf extracts by using Spectrophotometric technique was done. The aglycon moiety was extracted by ethyl acetate, and then evaporated to dryness. The dried residue then redissolved in 50% ethanol.**

Key words: *Moringa oleifera*, HPLC technique, Rutin, TLC technique, Lueteolin, Quareciten.

الملخص

يعد نبات شجرة البان من اهم النباتات الطبية التي تحتوي على كمية من المواد الفعالة. استخلصت هذه المواد من اوراق النبات الجافة عن طريق النقع بالميثانول 70% باستخدام الحاضنة الهزازه بدرجة حرارة 40 م. اجري الكشف الكيميائي عن المواد الفعالة في المستخلص النباتي الخام. تم عزل الفلافينويد الكلي من المستخلص باستخدام جهاز الارتداد. بعدها خضع الفلافينويد للكروموتوكرافيا الفصل بالطبقة الرقيقة باستخدام مختلف المذيبات كوسيط ناقل. ساعد استخدام تقنية الكروماتوكرافي الطبقة الرقيقة في الكشف عن وجود الكوارستين, الروتين واليوتيولين من الفلافينويد المعزول من المستخلص وقد عززت هذه النتائج عن طريق استخدام تقنية الكروماتوكرافي سائل عالي الجودة في الكشف عن وجود الكوارستين و الروتين من الفلافينويد المعزول من المستخلص. تم حساب كمية الفلافينويد الكلية في مستخلص اوراق شجرة البان باستخدام تقنية التحليل الطيفي. استخلص الجزء غير السكري للفلافونويدات بالأثيل اسيتيت ثم تبخيره حتى الجفاف، ثم اعادة اذابته بالإيثانول 50%.

الكلمات الدالة: شجرة البان, الاستشراب السائل فائق الأداء, كرموتوغرافيا الفصل بالطبقة الرقيقة, لوتيولين, الروتين, الكوارستين.

Introduction

M. oleifera is one of the best plants with a wide range of medicinal application [1]. It is a tropical plant excessively known to be of great medicinal values [2,3]. Different parts of it have been scientifically found to possess some medicinal properties such as anti-hypertensive (flower and seed), hypolipidemic (flower), anti-inflammatory (root and flower), and anti-ulcer (bark) [4]. The plant *M. oleifera* is widely used as food product and in the treatment of different diseases. Its parts were used to treat cancer whether in powdered form, alcoholic or aqueous extract [5]. As a member of the Moringaceae family *M. oleifera* also known as Horseradish based on the taste of leaves, or Drumstick Tree based on the appearance of its unripe seed pods. It is one of the most useful trees currently found throughout the tropics of the world [6].While less frequently referred to as 'The Tree of Life' or 'Miracle Tree' due to its economic importance and versatility [7]. Or Ben Oil Tree [8]. It is one of 14 species in the genus Moringa, which is the genus in the family Moringaceae, its name derived from the Malayalam word 'muringo' from southern India [9]. *M. oleifera*, native of the western and sub-Himalayan tracts, Pakistan, Asia, Africa India, and Arabia [10,11] distributed in the Cambodia, Philippines, Caribbean Islands and the Central America, North and South America [12]. *M. oleifera*, is a small or medium sized tree Ranging in height from 5 to 12m with an open, umbrella-shaped crown, straight trunk and with thick, soft, corky, deeply fissured bark. The tree produces a tuberous tap root. The evergreen or deciduous leaves (depending on climate) have leaflets 1 to 2 cm in diameter. The flowers are white or creamy color. The fruits

(pods) are initially light green, slim, eventually becoming dark green, firm and long, depending on the variety. Fully mature, dried seeds are round or triangular, the kernel being surrounded by a lightly wooded shell with three papery wings [13].

Materials and Methods

A. Plant Collection and Extraction

M. oleifera was obtained from a local plantation in Baghdad and identified by Professor Dr. Ali Al- Mosawy (Department of Biology, College of Science, Baghdad University) the plant leaves were air dried at room temperature and thereafter reduced to powder form. The dried leaves of the plant was powdered using a blinder for 10 minutes, and then extracted with methanol (70%), 50 grams of the processed plant were extracted in 250 ml of the solvent and left in shaking incubator (40ºC) for 24hrs. Extract was then filtered with gauze and then with filter paper .The obtained extract was then evaporated at (45ºC) using a rotary evaporator and the resultant crude extract was dried using lyophilizer. Dried extract was collected, weighed and kept in freeze at (-20ºC) until use [14].

B. Detection of active compounds

Phytochemical tests were carried out to detect the presence of some secondary metabolites in the crude extract.

Table (1): Active compounds and reagents that used in chemical detection with references

active compounds	Reagents	References
Alkaloids	Mayer's reagent	[15]
Flavonoids	Ethanol with KOH	[16]
Glycosides	Benedict reagent	[17]
Steroids	chloroform, acetic anhydride, sulphuric acid	[18]
Saponins	Shaking Extract ferric chloride	[19]
Tannins	Ferric chloride	[20]
Terpenes	chloroform, acetic anhydride, sulphuric acid	[18]

C. Extraction of Flavonoids

Two grams, from dried methanol extract was reflected for 8hrs. using 200 ml of 2M HCl solution, filtered and the filtrate was cooled then transferred to a separator funnel. The aglycon moiety was extracted by 50 ml ethyl acetate for three times. The collected ethyl acetate layers were washed with distilled water to remove the excess acid, then evaporated to dryness by rotary evaporator at 40ºC. The dried residue then re-dissolved in 30 ml 50% ethanol [20].

D. Determination of Total Flavonoids:

1. Quantitative Assay

Rutin standard stock solution was prepared in 50%ethanol (1mg/ml) from which serial dilutions were made to get rutin standard solutions with concentration of (0.2, 0.5, 1, 2.5 and5) mg/ml. Amount of 1ml was transferred from each standard solution and from the extracted flavonoid into a glass tubes, then 0.75 ml of 5% sodium nitrite solution was added and mixed well to be left to stand at room temperature for 5 minutes. About 1.5 ml of 10% AlCl3 in 50% ethanol was added to all tubes, then shacked and left to stand at room temperature for another 5 minutes. At last 5ml of 1N NaOH solution was added to all tubes [21]. The absorbance was read at 510nm, and a standard curve was plotted between the absorbance and the concentration, the amount of total flavonoids was calculated as rutin from the equation of straight line that obtained from the plotted curve.

2. Qualitative Assay [22]

The thin-layer chromatography (TLC) is commonly used because it is simple. It used to separate the extracted flavonoids into the different flavonoids. Table (1) showing the solvent systems that used as a mobile phase to select the most proper one that separate the extracted *Moringa oleifera* flavonoids efficiently.

Table (2): Solvent systems were used in this study as a mobile phase of TLC and their Ratios.

Solvent System	Symbol	Ratio
Glacial acetic acid: *n*-Hexane: Ethyl acetate	a	1:6.2:2.8
Glacial acetic acid: Chloroform: Formic acid	b	0.7:8.8: 0.5
n-Butanol : Distilled water: Glacial acetic acid	c	4:5:1

Standard solutions was prepared 0.1mg/ml in 50%ethanol from rutin, kaempferol, quercetin, luteolin, then mixed of standard solutions well and put one spot from each sample (the extracted plant flavonoid) and standards on a thin layer chromatography (TLC) , TLC was activated at 100°C for 30 minutes in an oven and cooled at room temperature before use.

E. Detection of Flavonoids Compound by HPLC[23]

HPLC application for flavonoids standards rutin and quercetin, and for flavonoid of the plant leaf extracts which was used to detection quality of the flavonoids. The conditions for detection of rutin and quercetin were as follow:

Mobile phase: Methanol: Water (70:30)
Column: C18 (25cm)
Flow rate: 0.5ml/min.
Injected volume: 10µl.
Wave length: 280nm.
Instrument: waters/487 USA

Results

1- Plant Extracts (Methanolic Extract)

Fifty grams of *M. oleifera* dried leaves were used in this study. The weight of residue of *M. oleifera* leaves extract after extraction with methanol 70% and lyophilized was 8g which represents 16% of the original leaves sample weight. The appearance of the residue was dark green in color.

2-Active Compounds Detected in the Plant Extract

Results of chemical detections of active compounds in the leaves of *M. oleifera* crude methanolic extract were shown in Table (2)

Table (3): Chemical detection of some active compounds in *M. oleifera* methanolic extract.

Active compounds	Results
Alkaloids	+
Flavonoids	+
Glycosides	+
Steroids	+
Saponins	+
Tannins	+
Terpenes	+

Note: + indicates the presence of the active compound

3- Determination of Total Flavonoids

The dried leaves powdered yielded about 8g residue 2 g of which was reflected with acidic solvent (2M HCl) to break down the glyosidic linkage. The non-aqueous (aglycon) residue was dissolved in 30 ml of 50% ethanol, the yielded residue was (2.52 g) of 50 g dried leaves for following probes.

A-Quantitative Assay

Results in Figure (1) and Table (4) indicate that total flavonoid in one gram of *M, oliefera* dried leaves was 22.5 mg/g represent 2.25% (w/w) determined as rutin according to straight line equation [24] Figure (1).

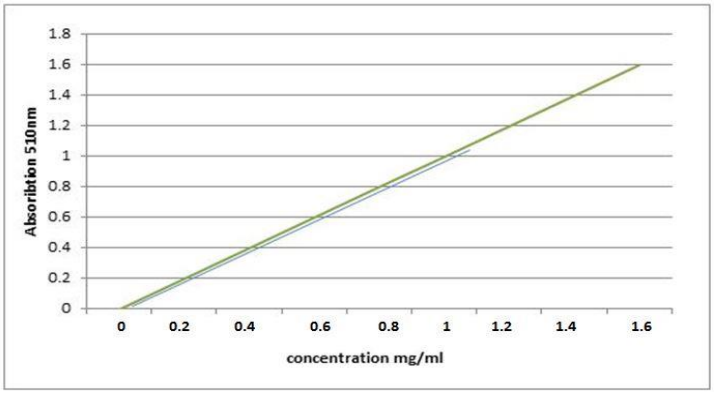

Fig. (1): Standard curve for rutin as determined spectrophotometrically at 510 nm.

The absorbance of the spectrophotometric analysis for *M. oleifera* total flavonoids and rutin standard solutions at 510nm illustrated Table (4).

Table (4): Spectrophotometric analysis of *M. oleifera* total flavonoids and rutin standard solutions.

Solutions	Concentration (mg/ml)	Absorbance (at 510nm)
Rutin standard solutions	0.01	0.006
	0.1	0.062
	0.2	0.172
	0.25	0.266
	0.5	0.55
	1	1.263
The extracted solution	0.90	1.08666

B. Qualitative Assay

The flavonoid (sample) of *M. oleifera* leaves extract was subjected to thin layer chromatography analysis (TLC) to determine the flavonoids existent in sample. The results in Figure (3) showed the presence of Rutin, Quareciten and Luteoline in plant extract by using TLC method. Flavonoids were determined by virtue of comparison with Rf (Retardation factor) values of the standards. Rf values of the standards are given in Table (5). Table (5) showing that mobile phase (b) was the best one because it gave good separation of the flavonoids, By comparison with (a) and (c) mobile phases that used in this study. The following equation used to calculate the Rf value] 25].

$$\text{Rf Value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent (Solvent Front)}}$$

Table (5): Detection of Flavonoids in *M. oleifera* methanolic extract by TLC.

Flavonoids	mobile phase (b)	RF Values of standard	Number of sample spots	RF Values of sample	Test
Querciten	Glacial acetic acid: Chloroform: Formic acid	0.07	4	0.07	Under UV light
				0.09	
Rutin		0.09		0.12	
Luteoline		0.12		0.2	

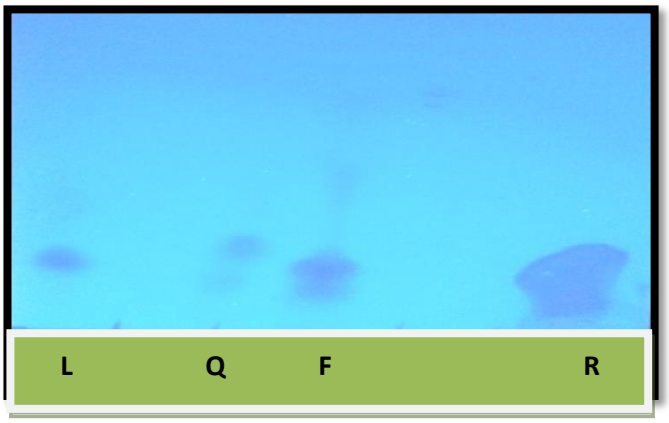

Fig. (2): TLC chromatography for the mobil Phase (a). *M. oliefera* flavonoids extract (F), Luteoline standard (L), Querciten standard (Q), Rutin standard (R)

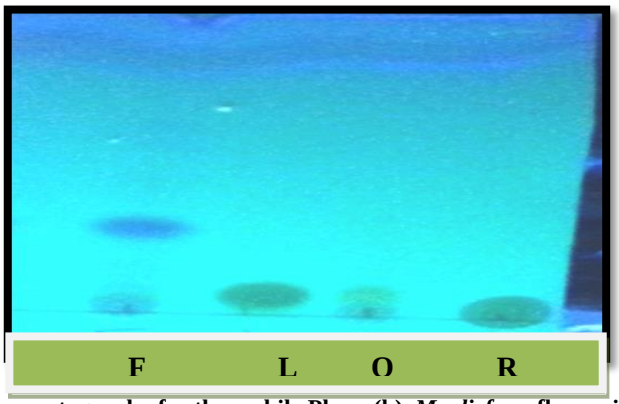

Fig. (3): TLC chromatography for the mobile Phase (b). *M. oliefera* flavonoids extract (F), Luteoline standard (L), Querciten standard (Q), Rutin standard (R)

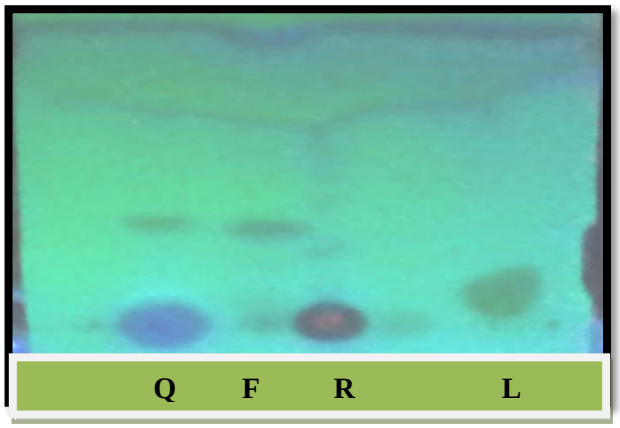

Fig. (4): TLC chromatography for the mobile Phase (c). *M. oliefera* flavonoids extract (F), Luteoline standard (L), Querciten standard (Q), Rutin standard (R)

4-Qualitative estimation of flavonoid using HPLC technique

HPLC analysis of the methanolic extract for *M. oleifera* obtained from dried leaves flavonoid extract indicated the presence of:

- **A.** Quarecetin, with retention time (4.612) minutes, Figure (5) in comparison with quarecetin standard (4.693) as Figure (6).
- **B.** Rutin, with retention time (3.486) minutes, Figure (5) in comparison with Rutin standard (3.388) as Figure (7).

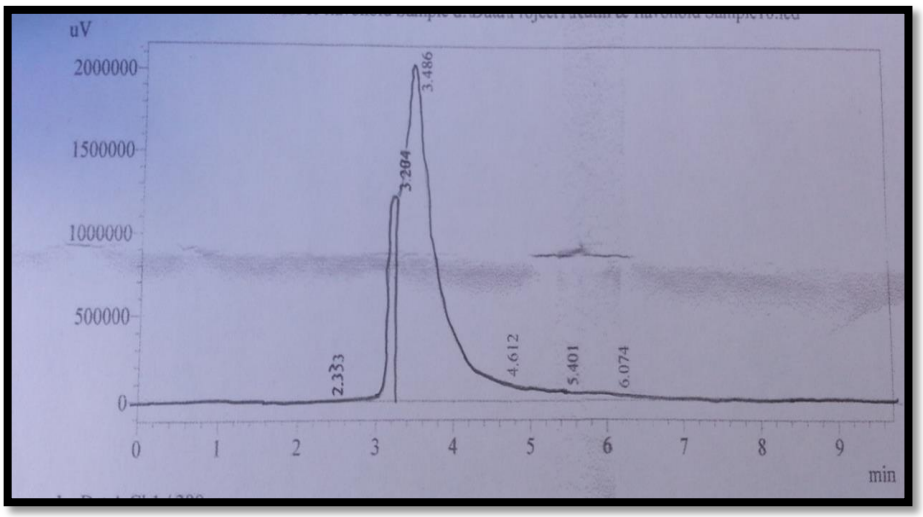

Fig. (5): HPLC analysis of the *M. oleifera* dried leaves flavonoid extract.

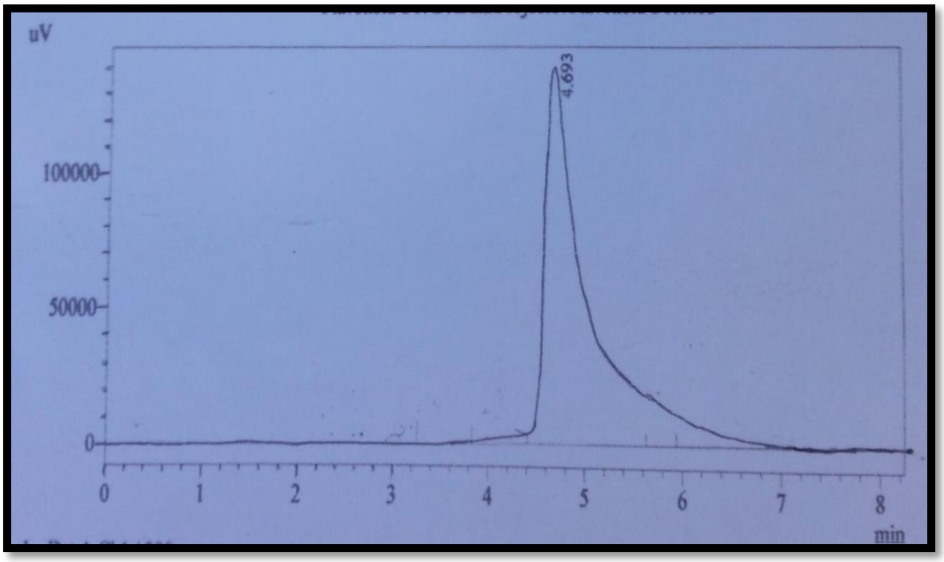

Fig. (6): HPLC analysis for qurecetine standard

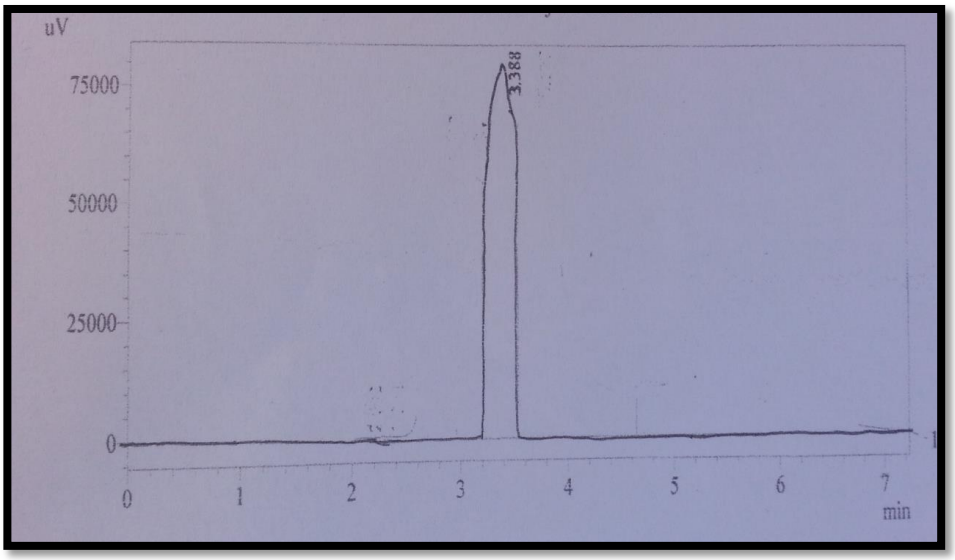

Fig. (7): HPLC analysis for Rutin standard

Discussion

The present study focuses on the determination the quality and quantity of total flavonoid in *M. oleifera* leaf which is the first estimate in Iraq. In this study chemical detections of active compounds in the leaves of *M. oleifera* using methanolic extract showed presence of flavonoids, alkaloids, glycosides, saponins, tannins, terpenes and steroids. Previous pharmacological and chemical studies on *M. oliefera* leaves extract have indicated the presence of several chemical compounds, including tannins, alkaloids, saponins, reducing sugars, carbohydrates, eugenol and glycosides [26,27]. Flavonoids compounds such as myricetin, quercetin and kaempferol [28]. It was clear that the *M. oleifera* leaf rich with flavonoids 22.5 mg/g represent 2.25% (w/w) dried powder leaves] that might give an emphasis on the role of the plant in it is pharmacological action. The quantitative phytochemical screening of *M. oleifera* leaves extract revealed that the plant contains 1.643 % flavonoids, 0.148 % alkaloids [29]. The TLC indicates the presence of querciten, rutin and lueteolin. Previous study indicated the presence of flavonoids in *M. oleifera* leaves in particular, quercetin and kaempferol glycosides that broken down to yield the natural antioxidant flavonoids, querciten and kaempferol, [30,31].

Conclusion

Different classes of active compounds were detected in *M. oleifera* leaves methanolic extract including alkaloids, flavonoids, saponins terpenes, steroids glycosides, and tannins. Leaves of *M. oleifera* were rich with flavonoid, total estimated flavonoid 22.5 mg\g represent 2.25% (w/w) including qurecetin, lueteolin and rutin.

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