Purification and Identification of Flavonoids Extracted from *Loranthus Eurpaeus* Fruits

التنقية والكشف عن الفلافونويدات المستخلصة من ثمار نبات بلوط الهدال

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Abstract

***Loranthus eurpaeus*is an important medicinal plant, which contains a lot of bioactive compounds. The dried plant fruits were extracted overnight with 80% methanol by maceration using shaker incubator 25c°. Chemical detection of crud plant extracts was performed. The total flavonoids was isolated from the extract using reflux, and subjected to thin layer chromatography (TLC) using different mobile systems. The purified material was augmented by using high performance liquid chromatography (HPLC). The aglycon moiety was extracted by ethyl acetate, and then evaporated to dryness. The dried residue then redissolved in 50% ethanol. Purification procedures of flavonoid were fully described in this study. The aims of this study was to detect the active compounds present in *L. eurpaeus* methanolic extract then quantitative and qualitative estimation of the total flavonoids isolated from the plant fruits.**

Key words: *Loranthus eurpaeus*, HPLC technique, Rutin, TLC, Lueteolin

الملخص

يُعد نبات بلوط الهدال من النباتات الطبية المهمة ويحتوي على مجموعة من المركبات ذات الفعالية الحيوية. اجري الاستخلاص باستخدام الميثانول بتركيز80 بالتنقيع وباستخدام الهزاز في25° وثم الكشف الكيمائي لمكونات المستخلص الخام. تم استخلاص الفلافونويدات من ثمار نبات الهدال باستخدام الـ Reflex و تقدير المحتوى الكلي لها, ثم الكشف عن الفلافينويدات للمستخلص المنقى باستخدام تقنية كروماتوغرافيا الطبقة الرقيقة وباستخدام مذيبات مختلفة كوسيط ناقل. ولزيادة التأكد للفلافينويدات النقية تم كشفها بتقنية الـ HPLC. استخلص الجزء غير السكري للفلافونويدات بالأثيل اسيتيت ثم تبخيره حتى الجفاف. ثم اعادة اذابته بالإيثانول 50%. الهدف من هذه الدراسة هو الكشف عن المركبات الفعالة الموجودة في مستخلص نبات بلوط الهدال الميثانولي، اضافة للتقدير الكمي والنوعي لمجموع المركبات الفلافينويدية من ثمار نبات بلوط الهدال.

الكلمات المفتاحية: بلوط الهدال, الاستشراب السائل فائق الأداء, الروتين, كرموتوغرافيا الفصل بالطبقة الرقيقة, لوتيولين.

Introduction

Loranthus europaeus Jacq. (Loranthaceae) is hemiparasitic mistletoe of South-Eastern Europe, Anatolia and South Russia [1]. *L. europaeus* has a similar branching pattern to the evergreen mistletoe *Viscum album* L., but it is deciduous, yellow-berried mistletoe, with dull brown twigs, with flowers located in stipulate inflorescences and respectively berries [2]. *L. europaeus* grows mostly on branches of *Quercus* species and occasionally of chestnuts [3] as host trees. The alliance of oaks and mistletoes became a symbol of knowledge and strength, and it was aptly rendered in the word "Druid" (i.e. the oak-knower), which is derived from the Greek word for oak [4]. Mistletoes on oaks have a symbolism and a healing status that is very interesting, because both species were highly prized by ancient people, all chemists and herbalists [5]. The fruits are berries, usually containing a single seed, that are dispersed by birds [6]. Historically, the intentions of mistletoe uses were manifold and conflicting in several cases (i.e., swellings or tumours, epilepsy, hysteria, delirium, vertigo, antispasmodic, tonic and narcotic, diseases of spleen and liver, labor-pains, weakness of the heart' and oedema, eczema, ulcers of the feet, burns, and granulating wounds) [7].

Materials and methods

A. Plant Collection and Extraction

The dried fruit was bought from Iraqi market and authenticated by Dr. Ali AL-Mossawy, Biology Department, College of Science, Baghdad University .The plant fruits were air-dried at room temperature and crashed by blender to be extracted. Fifty grams of dried fruits were extracted overnight in 250ml of 80% methanol by maceration, using shaker incubator 25c°. The extract solution was filtered by Buchner funnel, and then concentrated at 40c° by rotary evaporator, finally dried by lyophilizer, and the resultant crude powder extract was kept at -20c° until use [8].

B. Phytochemical tests

Phytochemical tests were carried out to detect the presence of some secondary metabolites in the crude extract according to the procedures outlined Table (1) [9].

Table (1): Phytochemical detection

Secondary Metabolites	Reagents
Alkaloids	Mayer's reagent
Flavonoids	Ethanol with KOH
Glycosides	Benedict reagent after hydrolysis
Saponins	Shaking Extract ferric chloride
Terpenes and steroids	chloroform, acetic anhydride, sulphuric acid

C. Extraction of total flavonoids from *L. eurpaeus*

Six gram from dried methanol extract was reflected for 8hr using 200 ml of 2M HCl solution. The filtrate was cooled and transferred to a separator funnel. The aglycon moiety was extracted by 50 ml X3 ethyl acetate. The collected ethyl acetate layers were washed with distilled water to eliminate the excess acid then evaporated to dryness by rotary evaporator at 40ºC in 20minut.The dried residue was weighted then re-dissolved in 30 ml 50% ethanol [9].

D. Determination of Total Flavonoids.

1. *Quantitative Assay*

Rutin standard stock solution was prepared (1mg/ml in 50%ethanol), from which serial dilutions were made to get rutin standard solutions with concentration of (0.01, 0.1, 0.2, 0.25, 0.5 and 1) mg/ml in 50% ethanol. Amount of 1ml was transferred from each standard solution and from the extracted flavonoid into a test tube, and 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. To all tubes 1.5 ml of 10% $AlCl_3$ in 50% ethanol was added, shakes well and left to stand at room temperature for another 5 minutes. At last 5ml of 1N NaOH solution was added to all tubes. The absorbance was read at 510nm, and a standard curve was plotted between each concentration and the absorbance, then the amount of total flavonoid was calculated from the equation of straight line that obtained from the plotted curve [10].

2. *Qualitative Assay*

For determining the extracted flavonoids three different solvent systems were used as shown in Table (2) [11].

Table (2): Different TLC Solvent System and their Ratios

Solvent System	Ratio	Symbol
***n*-Hexane: Ethyl acetate: Glacial acetic acid**	**31:14:5**	**A**
Chloroform: Glacial acetic acid: Formic acid	**44:3.5:2.5**	**B**
***n*-Butanol: Glacial acetic acid: Distilled water**	**20:5:25**	**C**

Standard solutions was prepared 0.1mg/mlin 50% ethanol from rutin, kaempferol, quercetin, luteoline, and mixed of standard solutions, a thin layer chromatography was performed on silica gel Gf254 aluminum plates which activated at 100°C for 30 minutes in an oven and cooled at room temperature before use [11].

E. Qualitative and quantitative estimation of flavonoid using HPLC technique

HPLC application for flavonoids standards solutions rutin, kaempferol, quercetin and luteolin, and the total flavonoids of the dried fruit extract was used to measure the concentration of the extract. The condition for this assay as follows[12]:

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

Results

A. Plant Collection and Extraction: Fifty gram of *L. eurpaeus* powdered fruits yielded residue weighted 6g which represents 12% of the original fruits sample. The appearance of the extract was brown in color.

B. Phytochemical tests: Results of chemical detections of active compounds in the fruits of *L. eurpaeus* crude methanolic extract were: Alkaloids, Flavonoids, Glycosides**,** Saponins,Terpenes and Steroids**.**

C. Determination of Total Flavonoids.

1. *Quantitative Assay*

The absorbance of the spectrophotometric analysis for *L. eurpaeus* total flavonoids and rutin standard solutions at 510nm illustrated in figure (1).

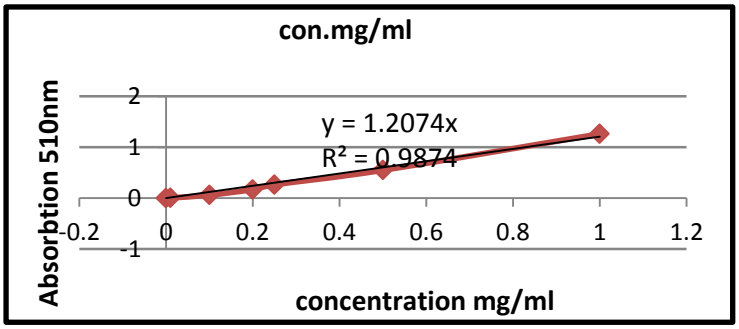


Fig. (1): Standard curve for Rutin as determined spectrophotometrically at 510 nm.R2=0.9874.

The plotting process between varying standard rutin concentrations and the equation of a straight line in fig. (1). Results indicated that total flavonoid in (1g) *L. eurpaeus* dried fruits was 2.545 mg determined as rutin according to straight line equation.

2. Qualitative Assay

During comparison of different mobile phases figure [2, 3, 4], it was found that mobile phase (B) was the proper one as long as it gave good separation of the components figure (4).

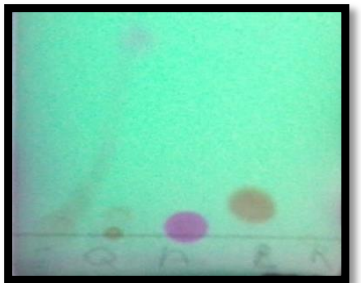


Fig. (2): TLC Chromatogram for the mobilephase [A]. *L. eurpaeus* dried fruits flavonoids extract (1), Quareciten (2), Luteoline (3), .(Kaempferol (4) Rutin (5

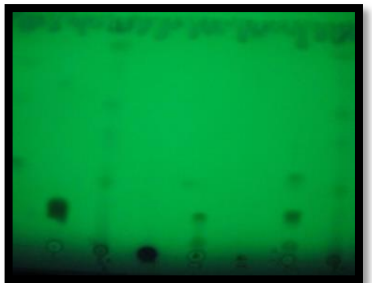


Fig. (3): TLC Chromatogram for the mobile phase [C]. *L. eurpaeus* dried fruits flavonoids extract (1), Kaempferol (2), Quareciten (3), Luteoline (4),

Fig.(4):TLC Chromatogram for the mobile phase [B]. Luteoline (1), *L. eurpaeus* dried fruits flavonoids extract (2), Quareciten (3), Kaempferol (4) Rutin (5). Mixed standers (6).

E.Qualitative and quantitative estimation of flavonoid using HPLC technique

From figure (5) HPLC analysis of *L. eurpaeus* dried fruits flavonoid extract indicated the presence of**:**

i. Rutin, with retantion time (1.894)minutes.

ii. Lueteolin, with retantion time (2.470)minutes.

All results were obtained in compound with rutin standard retantion time (1.866) and Lueteolin standard retantion time (2.458).

iii. Retantion time of two flavonoid standards: Quareciten and Kaempferol (the showed of spots of the two type in TLC) are (2.317 and 2.798) minutes respectly, figure (7 A and B), in comparsion with retantion time of tested flavonoid (2.470) minutes.

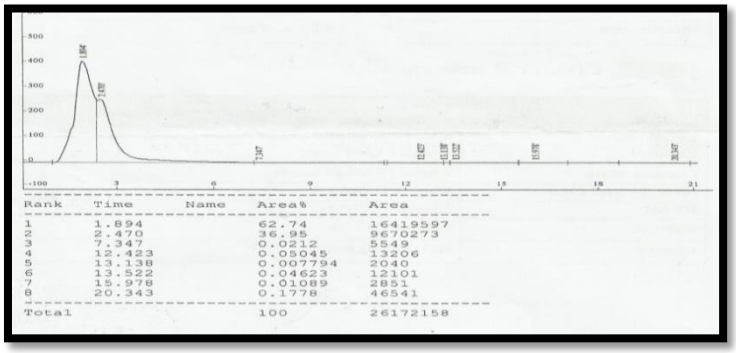
When data applied for peak height or area under the curve at retention time of the standard and extracted flavonoid type, the concentration for total flavonoid type was calculated as follow:

Total flavonoid (mg) in 1g dried fruit powder

$$= \frac{\textbf{\textit{Peak area of extracts}}}{\textbf{\textit{Peak area of standard}}} \times \textbf{\textit{Standard solution concentration}} \times \textbf{\textit{total volume of extract}}$$

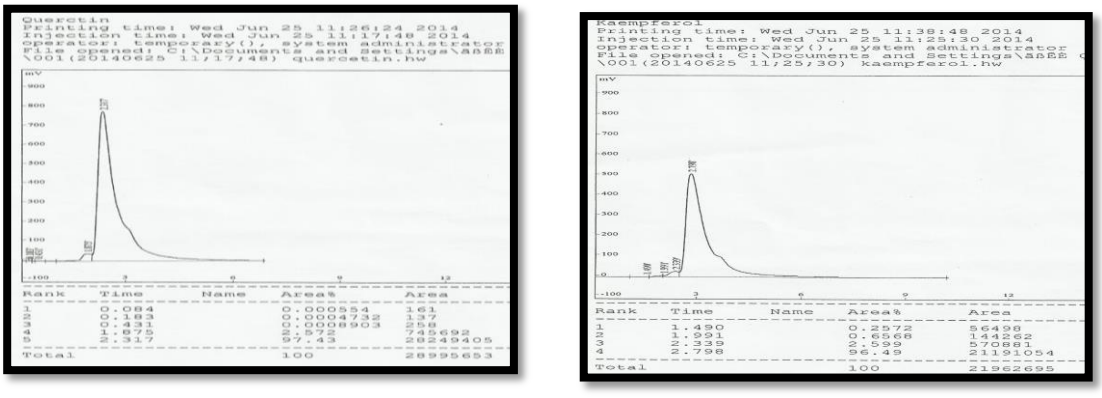
- **Rutin**=$\frac{16419597}{23273538} \times 1\frac{mg}{ml} \times 100ml = 70.5\ mg$
- **Lueteolin**=$\frac{9670273}{26534042} \times 0.5\frac{mg}{ml} \times 100ml = 18.2\ mg$

- One gram of dried fruit contains 1.8 mg of Rutin + 0.46 of Lueteolin.
- The rest quantities 0.28 mg may be suggested as Quareciten and Kaempferol.



Rank	Time	Name	Area%	Area
1	1.894		62.74	16419597
2	2.470		36.95	9670273
3	7.347		0.0212	5549
4	12.423		0.05045	13206
5	13.138		0.007794	2040
6	13.522		0.04623	12101
7	15.978		0.01089	2851
8	20.343		0.1778	46541
Total			100	26172158

Fig. (5): HPLC analysis for test flavonoid

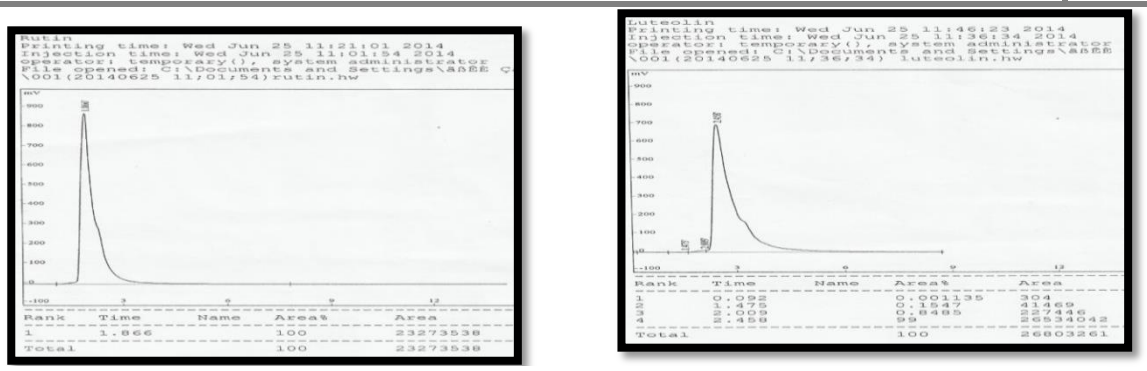


Rank	Time	Name	Area%	Area
1	0.084		0.000554	161
2	0.183		0.0004732	137
3	0.431		0.0008903	258
4	1.675		2.572	745692
5	2.317		97.43	28249405
Total			100	28995653

Rank	Time	Name	Area%	Area
1	1.490		0.2572	56498
2	1.991		0.6568	144282
3	2.339		2.599	570881
4	2.798		96.49	21191054
Total			100	21962695

[A] **[B]**

Fig. (6): HPLC analysis for [A] Rutin and [B] Lueteolin stander



[A] [B]

Fig. (7): HPLC analysis for [A] Quareciten and [B] Kaempferol stander

Discussion

The present study focuses on the presence of total flavonoid in this *L. europaeus* fruits which haven't been estimated before in Iraq. It was clear that the *L. europaeus*is rich with flavonoids (2.545 mg\1g dried powder fruit) that might give an emphasis on the role of the plant in it is pharmacological action. As shown by TLC Chromatogram, there is trace amount of Quareciten and Kaempferol both play a role in biological action as Rutin and Lueteolin. It has been reported that flavonoids and phenolic acids are the sources of antioxidants in plants [13]. Previous pharmacological and chemical studies on some species of the Loranthaceae have indicated the presence of several chemical compounds, including flavonoids, alkaloids [14] and polysaccharides-Glycosides [15]. Many chemical components such as tannin, terpenoids, phenols, flavonoids, Glycosides, Triterpenoids and resins present in *L. micranthus* Linn in methanolic extract [16]. Also *L. bengwensis* was indicated presence of alkaloids, flavonoids, tannins, cardiac glycoside, terpenes and steroids [17]. Study in the 2012 [18], showed the determination of total flavonoids content in selected plants belonging to family Loranthaceae, in the three plants *P. acacia*, *P. curviflorus* and *P. austro Arabica*, were found to be 5.39, 5.82, 6.2 g /100 g of dry plant weight respectively. Total flavonoid content was found to be 22.5 mg/g for methanolic extracts of Macrosolen parasiticus L. Danser a parasitic plant belongs to the family Loranthaceae [19]. According to HPLC results, in previous study flavonoids, were identified the present of (Kaempferol, quercetin and rutin) [20]. Rutin which match the preliminary phytochemical investigations was present as flavonoids in methanol fractions and HPLC [21]. In TLC Chromatogram, there is trace amount of Quareciten and Kaempferol both play a role in biological action as Rutin and Lueteolin (This chromatogram can be used as fingerprint for the compound obtained from this plant). This agree with that show the concentration of quercetin varied in selected plants belonging to family Loranthaceae, with 0.157 (*P. austro arabica*) to 0.062 g% (*P. acacia*) and *P. curviflorus* contained 0.115 g% w/w quercetin [18].

Conclusion

The study indicated that the *L. europaeus*dried fruits rich with flavonoid. Total flavonoid estimated the major component rutin and lueteolin and trace of quareciten and kaempferol.

References

1. Balle, S., Dandy, J.E., Gilmour, J. S., Holltum, R.E., Stearn, W. T. and Thoday, D. (1960). *Loranthus*, *Taxon.* 9:208-210.
2. Briggs, J. (2009). *Loranthus europaeus*, New to Britain, and notes on *Viscum album*, both new to Kew Gardens, Haustorium. 56:2-3.
3. Harvala, E., Exner, J. and Becker, H. (1984). Flavonoids of *Loranthus europaeus*, J. Nat. Prod. 47: 1054- 1055.
4. Barnhart, R. K. and Steinmetz, S. (2008). Chambers dictionary of etymology. Chambers Harrap Publishers Ltd., Edinburgh.
5. De Cleene, M. and Lejeune, C. (2003). In: Compendium of symbolic land ritual plants in Europe, De Cleene, M. and C. Lejeune (*eds.*), Man and Culture Publishers, Ghent. Pp. 411-425.
6. Fleming, T. (2002). Physicians' Desk Reference (PDR) for Herbal Medicines. Medical Economics Company, New Jersey.
7. Steiner, R. (1989). Discussions with practicing physicians, In Physiologically-therapeutic based on spiritual science, Dornach: Rudolf Steiner Verlag. Pp: 285-301.
8. Arokiyaraj, S., Perinbam, K., Agastian, P. and Balaraju, K. (2007). Immunosuppressive effect of medicinal plants of kolli hills on mitogen-stimulated proliferation of the human peripheral blood mononuclear. Indian J. Pharmacol. 39: 180-183.

9. Harborne, J.B. (1984). Phytochemical Methods: A guide to modern techniques of plant analysis, second edition, Chapman and Hall, London

10. Wang, C., Chang, S., Stephen, B., and Chen, B. (2009). Isolation of carotenoids, flavonoids and polysaccharides from *Lycium barbarum* and evaluation of antioxidant activity. Food chemistry. 120:184-192.

11. Marcica, M.S., Vesna, R., Mirza, B. and Zeljan, M. (2009). From functional food to medicinal product: Systematic approach in analysis of polyphenolics from popolis and wine. Nutrition Journal. 8:33.

12. Hamad, M., Hassan, A., Numan, I. (2013). Evaluation of Anti-Inflammatory Effect of Ethyl Acetate and Methanol Extracts of *Loranthus europaeus* in Experimental Models of Acute Inflammation in Rats. Int. J. Pharm. Sci. Rev. Res. 23(2): 170-174.

13. Cook, N. C. and Samman, S. (1996). Flavonoids-Chemistry, metabolism, cardioprotective effects, and dietary sources.7 (2):66-76.

14. Fernandez, T., Wagner, M., Varela, B., Ricco, R., Hajos, S. and Gurni, A. (1998). Study of argentine mistletoe: The hemiparasiteLigariacuneifolia (Loranthaceae). J. Ethnopharmacol. 62:25-34.

15. Sinha, A., Taylor, W., Khan, I., McDaniel, S. and Essko, J. (1999). Glycoside primers of *Psittacanthus cucullaris*. J. Nat. Prod. 62:1036-1038.

16. Egbuonu, A. and Nwankwo, N. (2011). Phytochemical properties of some solvent fractions of petroleum ether extract of the African mistletoe (*Loranthus micranthus* Linn) leaves and their antimicrobial activity, Afr. J. Biotechnol. 11(62): 12595-12599.

17. Olatunde, A. and Dikwa, M. A. (2014). Qualitative and Quantitative Analysis of Phytochemicals of *Loranthus bengwensis* Leaf .IntR J. Pharm. Sci. 5(1): 10.

18. Bamane, F. H., Badr, J. M. and Amin, O.A. (2012). Antioxidant activities and flavonoid contents of selected plants belonging to Family Loranthaceae. (Plicosepalus acacia, P. curviflorus and Phragman thera austroarabica showed comparable antioxidant activity, apparently associated with their quercetin content). African Journal of Biotechnology. 11(78): 14380-14385.

19. Lobo, R., Sodde, V., Dashora, N., Gupta, N. and Prabhu, K. (2011). Quantification of flavonoid and Phenol content from *Macrosolen parasiticus* (L.) Danser. J. Nat. Prod. Plant Resour. 1 (4):96-99.

20. Carroll, K.K., Guthrie, N., So, F.V. and Chambers, A.F. (1998). Anticancer properties of flavonoids, with emphasis on citrus flavonoids, in Flavonoids in Health and Disease (Rice-Evans CA and Packer L (eds), Marcel Dekker Inc., New York. 437–446.