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

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**Abstract**

*Loranthus europaeus* 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 25°c. Chemical detection of crude 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. europaeus* methanolic extract then quantitative and qualitative estimation of the total flavonoids isolated from the plant fruits.

**Key words:** *Loranthus europaeus*, HPLC technique, Rutin, TLC, Luetolin

**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 “Druide” (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, toxic 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 25°c. The extract solution was filtered by Buchner funnel, and then concentrated at 40°c by rotary evaporator, finally dried by lyophilizer, and the resultant crude powder extract was kept at -20°c until use [8].

**Collection and Extraction**

Fifty grams of dried fruits were extracted overnight in 250ml of 80% methanol by maceration, using shaker incubator 25°c. The extract solution was filtered by Buchner funnel, and the resultant crude powder extract was kept at -20°c 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

<table>
<thead>
<tr>
<th>Secondary Metabolites</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s reagent</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ethanol with KOH</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Benedict reagent after hydrolysis</td>
</tr>
<tr>
<td>Saponins</td>
<td>Shaking Extract</td>
</tr>
<tr>
<td>Terpenes and steroids</td>
<td>ferric chloride</td>
</tr>
</tbody>
</table>

C. Extraction of total flavonoids from L. eurpaeus
Six gram from dried methanol extract was refluxed 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 40°C in 20min. 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% AlCl₃ 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

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Ratio</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane: Ethyl acetate: Glacial acetic acid</td>
<td>31:14:5</td>
<td>A</td>
</tr>
<tr>
<td>Chloroform: Glacial acetic acid: Formic acid</td>
<td>44:3.5:2.5</td>
<td>B</td>
</tr>
<tr>
<td>n-Butanol: Glacial acetic acid: Distilled water</td>
<td>20:5:25</td>
<td>C</td>
</tr>
</tbody>
</table>

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).

![Graph](image)

**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).

![TLC Chromatogram](image)

**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. europaeus dried fruits flavonoid extract indicated the presence of:

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

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

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

iii. Retention 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 respectily, figure (7 A and B), in comparsion with retention 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

\[ \text{Total flavonoid} = \frac{\text{Peak area of extracts}}{\text{Peak area of standard}} \times \frac{\text{Standard solution concentration}}{\text{total volume of extract}} \times \text{peak area of standard} \]

- **Rutin**: 16419597 \( \frac{\text{mg}}{\text{ml}} \) \( \times \) \( 1 \frac{\text{mg}}{\text{ml}} \) \( \times \) \( 100 \text{ml} \) = 70.5 mg

- **Lueteolin**: 9679273 \( \frac{\text{mg}}{\text{ml}} \) \( \times \) \( 0.5 \frac{\text{mg}}{\text{ml}} \) \( \times \) \( 100 \text{ml} \) = 18.2 mg

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

Fig. (5): HPLC analysis for test flavonoid

Fig. (6): HPLC analysis for [A] Rutin and [B] Lueteolin 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 rich with flavonoids (2.545 mg/g) 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


