Identification and Quantitative Estimation of Lutein in Iraqi *Spinacia oleracea* Family *Chenopodiaceae* by Using Chromatographic Methods

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Abstract:
Lutein is an antioxidant carotenoid present in the highest quantities in dark, leafy green vegetables such as spinach. In this study, and for the first time we try to separate and calculate the quantity of lutein in Iraqi spinach to know the dietary requirements from this active drug to avoid age macula degeneration caused by lutein depletion.

Extraction and fractionation of lutein from Iraqi spinach leaves were carried out by soxhlet apparatus using petroleum ether: acetone (1:1) as a solvent system, then lutein was isolated by preparative thin layer chromatography and identified by thin layer chromatography using two different solvents system: (petroleum ether: diethyl ether:acetic acid) and (petroleum ether: acetonitrile: methanol) compared with standard, melting point, mixed melting point and high performance liquid chromatography (HPLC). 50gm of Iraqi spinach gives about 32mg of lutein.

Key words: Lutein, carotenoid, spinacia plant.

Introduction:
The chenopodiaceae family consists of 100 species of evergreen or semievergreen annuals, perennials, and shrubs. Spinach is one of them; the leaves are the most frequently used parts of spinach[1,2]. spinach contains a number of antioxidant including carotenoids, polyphenols[3,4] and flavonoids (quercetin)[5]. The carotenoids are composed of 2 main classes, carotenes (betacarotene) and xanthophyllus (lutein)[6]. lutein (Fig1) is a powerful antioxidant that protect macula of the retina against damage by filtering blue light before it can damage the macula. If sunglasses are the first line of defense against blue light, lutein is the last[7,8]

![Lutein structure](image)

Lutein;trans-lutein;4-[18-(4-hydroxy-2,6,6-trimethyl-1-cyclohexenyl)-3,7,12,16-tetramethyl octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl- cyclohex-2-en-1-ol[9]

Fig-1 structure of Lutein

Lutein provides nutritional support to our eyes and skin- the only organs of the body directly exposed to the outside environment. Lutein has been linked to promoting healthy of eye and skin through reducing the risk of...
macular degeneration[10,11], be good at protecting the eyes, the arteries and the lungs from damaging free radicals[12], support normal eye function and protect the retina by blocking harmful blue light[13] reduce the risk of heart diseases and cancers[14].

The amount of lutein in the eye may be depleting with age, and since our body doesn’t make lutein, we must constantly replace it through the food we eat. Dark leafy green vegetables like spinach are especially good sources.

Many researches have suggested a minimum of 6-10mg per day of lutein is necessary to realize lutein’s benefits[15] many studies around the countries investigate and calculate the amount of lutein in their spinach leaves (example in USA 58gm of fresh spinach contain about 6mg of lutein)[16,17], so it is our privilege to present this work to be the first phytochemical work that separate this active drug and calculate it’s quantities in the Iraqi species.

Materials and Methods:
The plant material (leaves) of Iraqi Spinacia oleracea Family Chenopodiaceae was collected during the months of November and December from the garden of Pharmacy college and identified by department of pharmacognosy, college of pharmacy, University of Baghdad, and authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib. The plant was dried at room temperature in the shade and was pulverized by mechanical mills and weighed.

50gm of the spinach leaves were extracted with 100ml of organic solvent (Petroleum ether : Acetone) (1:1) using soxhlet apparatus(Koyota, Japan) for 8 hours , then extract was evaporated to dryness under reduced pressure at a temperature not exceeding 40°C to give greenish colored residue (16.032gm)kept in closed dark container.

1. Thin Layer Chromatography (TLC):

The greenish residue was examined by TLC using the following system:

Silica gel GF: a slurry was prepared by mixing 30gm silica gel GF (MERCK) with 60 ml distilled water. The slurry was spread out in a layer of 0.25mm thickness on 20X20 cm glass plates using "Jobling Laboratory Division TLC coater". The plates were activated at 110°C for one hour before use.

Developing solvent systems: the solvent mixture was placed in a glass tank (22.5cmX22cmX7.0cm) lined with Whatman No.1 filter paper. A 100ml of solvent mixture was placed in each tank and covered with a glass lid and allowed to stand for 45 minutes before use. Different solvent systems were used for carotenoid compounds[18]:

S1 Petroleum ether: Diethyl ether: Acetic acid[18] 80:20:1
S2 Petroleum ether: Acetonitrile: Methanol[18] 20:40:40

Standard References compound: pure Lutein compound (National Vitamin Company "Casa Grande")

A) Detection method: the chromatogram was examined by eyes and under Ultra violet light (DESAGA HEIDELBERG) at wave length UV 445nm.
2. High Pressure Liquid Chromatography (HPLC)

HPLC equipped with a variable wave length detector, azorbax ODS 150mmX4.6mm id reverse phase column or equivalent, and a suitable 10-μl injection value. mobile phase : ethyl acetate: acetonitrile (12:88) 1.6 ml/min, injection volume 10μl; detector wave length 450nm.

Results and Discussion:

Preliminary investigation indicated that Iraqi spinach leaves have lutein in a good amount, extraction done using mixture of two organic solvents since lutein is carotenoid pigment soluble in organic solvent. Initial identification of active compound (lutein) is done by thin layer chromatography using two different solvent systems, further purification and separation of Lutein from crude extract is done by preparative TLC. TLC plates of extract and standard showed the following characteristic spots: (Fig-2&3)

![TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S1 as a mobile phase under 445nm wave length](image1)

![TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S2 as a mobile phase under 445nm wave length](image2)
From the previous figures we calculate the Retardation factor ($R_f$) value which is defined as the distance moved or traveled by the compound to the distance moved by the solvent and it is constant for each compound when the chromatography is carried out using the same technique, mobile phase, and the same conditions. Usually the $R_f$ value is used for the identification of the separated compound by comparison with the $R_f$ value of a standard.

**Table 1:** The $R_f$ values of the extract and standard are tabulated below.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>standard</th>
<th>extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>0.53</td>
<td>0.51</td>
</tr>
<tr>
<td>S2</td>
<td>0.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>

**Separation and purification of lutein by preparative TLC**

Preparative TLC plates were prepared by mixing of 75g silica gel with 150ml distilled water. The slurry was spread out in a layer of 2mm thickness on five 20X20cm glass plates. The plates were allowed to dry over night at room temperature and then activated by heating at 110°C for one hour before use.

The residue was dissolved in a minimum quantity of acetone and applied with standard reference lutein on a number of preparative TLC plates using (S1) solvent system. The solvent was allowed to rise to a height of 15cm from the base line. The band of lutein in the extract and standard lutein were observed under UV light at 445 wave length (fig-4) both of them have the same $R_f$ value.

Therefore lutein band had been scratched out, eluted with acetone, and then filtered. The filtrate evaporated to dryness, in vacuo, to give yellow crystals (32mg), upon re-crystallization out of boiling ethanol 95% a fluffy yellow crystals were obtained having a sharp melting point of 176-177°C.

**Fig 4:** preparative TLC plate for the standard (lutein) and sample (spinach extract) using silica gel GF as a stationary phase and S1 as a mobile phase under 445nm wave length

Furthermore, the identity was approved by HPLC the retention time was also authenticated with standard reference as shown in (Fig-5&6).

Potentiation of the isolated Lutein together with the standard compound (Fig-7), show a sharp peak with higher intensity.
Fig 5: HPLC of standard Lutein

Fig 6: HPLC of extract
Lutein was separated from crude extract by preparative TLC as yellow pure crystals in a weight of 32mg from 50gm plant leaves, this result indicates that Iraqi spinach content of lutein is higher than that in other countries, since the American Optometric Association (AOA) reminds peoples that caring for eyes includes paying attention to nutrition and based on this research we can protect our eye from age-related eye diseases by eating this type of foods which is rich in lutein content specially Iraqi species which contain good quantity of lutein. (percentage of Lutein in Iraqi spinach:
50gm contain 32mg (0.032gm) so percentage will be 0.032x100 = 0.064w/w
50
While percentage in a certain type of USA spinach
58gm contain 6mg (16,17) (0.006gm) so
Percentage will be 0.006x100 = 0.01w/w
58

Conclusion:
Above result indicate that the Iraqi spinach contain lutein more than the other type and since the daily requirement from this important substance is 6-10mg per day (15) so Iraqi species provide a good source for lutein to protect our eyes from age macula degeneration.

References:
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التشخيص والتقييم الكمي لللوتين في السبانخ العراقي باستعمال الطرق الكروماتوغرافية

ابنات جواد كاظم

جامعة بغداد/ كلية الصيدلة / قسم العقاقير والنباتات الطبية

الخلاصة:
اللوتين هو صبحة كاروتينويدية مماثلة للأكسدة وموجودة بكميات كبيرة في الخضروات الداكنة الحضرية مثل السبانخ اللوتين ومركبات المشتقة منه مثل زيت اللوتيك تركز في شبكية عين الإنسان لحماية العين من الأذى الخارجي لكن تقدم عمر الإنسان قد تصاب العين بما فيهما (هرموشية الشبكية) فيبدأ تكاثر اللوتيك بالانخفاض وفي هذه المرحلة يكون الإنسان معرضة إلى هذه المادة أما عن طريق الغذاء أو عن طريق الدواء لذا لازمية هذه المادة للإنسان فقد حارتنا في هذه الدراسة لأول مرة في العراق فضلا وصوب كمية اللوتيك في السبانخ العراقى ومقارنتهما مع الانتجات الأخرى من السبانخ في انخفاض أخرى من العالم استخلاص وبعض اللوتيك من أوراق السبانخ العراقي قد تم استخدام جهاز سوكليليت وباستخدام مزيج من البلورولوم ايلSHORT الأسباب كمديمات عضوية. وكانت النتائج تناول حمض المستخلص بطريقة كروماتوغرافيا الطبقيه والظافرية عن وجود مركب اللوتيك الذي تم فصله عن بقايا اللوتيك الموجوده في هذه الدراسة على شكل بلورات صفراء تشبه وذات درجة حرارة انصهار بلغ 207-208م. ومع حساب كمية اللوتيك الموجودة في جنس النبات العراقى تنتج النتائج ان تفوق الكميه الموجودة في اجنس اخرى لنفس النبات.