PHYTOCHEMICAL STUDY OF THE IRAQI BETA VULGARIS LEAVES AND ITS CLINICAL APPLICATIONS FOR THE TREATMENT OF DIFFERENT DERMATOLOGICAL DISEASES

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ABSTRACT

In view of increasing resistance to existing anti-microbial agents, side effects and sometimes high cost of treatment, interest in medicinal herbs has been progressively increased. Beta vulgaris var. cicla belongs to the family Chenopodiaceae locally known in Iraq as salk is ranked among the ten most powerful vegetables with respect to antioxidant capacity, anti-inflammatory, hepatoprotective, and anti-tumor properties. It contains a significant amount of phenolics, catechin hydrate, epica-techin, ferulic, protocatechuic, vanillic, p-coumaric, p-hydroxybenzoic, caffeic and syringic acids. The plant extract suggested that they might be used as an important natural antioxidant source due to its high levels of phenolics, flavonoids and proline. Beta vulgaris species is an herbaceous biennial leafy vegetable cultivated in many parts of the world for its year round availability, low cost and wide use in many traditional dishes. The leaves can be used in salads or cooked like spinach, and the stems are usually chopped and cooked like celery. Beta vulgaris L. species are used as a popular folk remedy for liver and kidney diseases, for stimulation of the immune and hematopoietic systems, and as a special diet in the treatment of cancer. Literature survey revealed that there was very little research on the health benefit of perpetual Spinach in the world while no study concerned in its flavonoids in Iraq. In this study fresh and dried leaves were extracted using aqueous and methanolic extracts, both extracts were investigated for its phytochemical contents, and it was found that the Iraqi species is rich with flavonoids specially querectin and kaempeferol, in addition to phenolic compounds. From both extracts...
solutions and ointments were prepared with different concentrations and their clinical effects for the treatment of Acne and psoriasis were evaluated using 360 patients. The clinical study showed that the plant extract gave significant healing effect for the treatment of Acne within two weeks using aqueous solutions prepared from extracts of fresh and dried leaves with more predominant effect for the fresh leaves. And the solution dosage form is better than ointment in the treatment of Acne. Same results obtained for psoriasis but ointment dosage form was much more effective, these results related to the antimicrobial and antioxidant activities of the plant extracts which can be applied clinically for treatment of many topical diseases.

Key Words: phytochemicals, topical preparations , Iraqi Beta vulgaris.

INTRODUCTION

Perpetual Spinach is a popular vegetable grown for its leaves, which are used as green or potherb. It does not have a thickened leaf midrib or a thickened petiole (leaf stem). Scientifically it is known as Beta vulgaris var.cicla belong to the family Chenopodiaceae locally known as Salk. \(^{(1,2)}\) Figure (1).

![Beta vulgaris var. cicla (Salk) cultivated in Iraq](image)

Figure 1- Beta vulgaris var. cicla (Salk) cultivated in Iraq

Perpetual Spinach is an excellent source of vitamins (A,C and E), minerals and wide range of antioxidant phytonutrient compounds like phenolic acid, flavonoids, betalains and carotenoids which have been strongly linked to the protection from numerous diseases from heart disease to cancer.\(^{(3,4)}\) Flavonoids are potent antioxidant and reported as having a wide range of biochemical function ( anti-allergic, anti-inflammatory, antimicrobial and anticancer ) among these flavonoids quercetin and kaempferol are the most important and widely spread flavonols class.\(^{(5)}\)
Literature survey revealed that there is very little research on the health benefit of perpetual Spinach in the world while no study concerned in its flavonoids in Iraq. The present study deals with investigation of phytochemicals found in methanolic and aqueous perpetual Spinach leaves (cultivated in Iraq) extract in addition to the formulation of different topical preparations from both plant extracts and evaluate its clinical applications for the treatment of acne and psoriasis.

**MATERIALS AND METHODS**

**I. Plant materials**

Fresh leaves of perpetual Spinach were purchased from vegetable markets of Baghdad; the plant was identified by the department of pharmacognacy, college of pharmacy/ university of Baghdad; and authenticated by the Herbarium of Baghdad University.

100 gram of fresh perpetual Spinach leaves were collected and divided into two parts: the first part, 50 gram of fresh leaves was packed in a thimble of soxhlet extractor. 500 ml of 80% ethanol was used in soxhlet for 24 hours to extract all possible potent compounds. The ethanolic extract was then filtered and 5% of hydrochloric acid was added to separate basic compounds from acidic and neutral compounds, the separation was done by using chloroform solvent and separatory funnel to the chloroform layer; 5% sodium hydroxide was added to separate acidic compounds from neutral compounds by using separatory funnel. The aqueous layer was taken (which contain all possible acidic compounds like phenolic acid and flavonoids) and evaporated under reduced pressure at temperature not exceeding 40°C to give greenish colored residue fraction 1 (F1) as shown in figure 2(6).

The second part, 50 gram of fresh perpetual Spinach leaves collected and dried under shad, powdered and extracted with 500 ml of 50% methanol (v/v) and 1.2 M HCl acid for two hours by using reflex apparatus for extraction all possible potent compounds. Then the extract was filtered and concentrated under reduced pressure at temperature not exceeding 40°C to give greenish colored residue fraction 2 (F2) as shown in figure 2(7).
II- Chemical tests for phytochemical evaluation

Preliminary phytochemical evaluation of perpetual Spinach leaves were done by making general tests for fixed oils, glycosides, alkaloids, phenolic compound, tannins and flavonoids.

1- Test for fixed oils \(^{(8)}\)

Small quantities of various extracts (F1 and F2) were separately pressed between two filter papers; the appearance of permanent spot oil on the paper indicates the presence of fixed oils.

Figure 2: Schematic procedure for first and second parts of perpetual spinach leaves extraction \(^{(6,7)}\)
2- Test for glycosides

A- Baljet’s test
1ml from each extracts (F1 and F2) with 1ml of picric acid makes it alkaline with sodium hydroxide, an orange color developed is considered as a positive result.

B- Keller-Kiallian’s test
1ml from extracts (F1 and F2) with 2ml of glacial acetic acid and 1 drop of 0.1% ferric chloride solution then add 1ml of H2SO4 (drop by drop), the appearance of a ring junction between two liquid layers indicates the presence of the sugar part of glycosides and considered as positive result.

C- Brontrager’s test
1ml of F2 extract with 1ml of diluted ammonia solution, the appearance of pink color is considered as positive result. While F1 was treated with chloroform and then chloroform layer was separated. To this equal amount of dilute ammonia solution added the appearance of pink color, showing the presence of glycosides and considered as positive result.

3- Test for saponins

Each extract (F1 and F2) was diluted with 10ml of distilled water and it was agitated in test tube for 15 seconds and standing for 15 minutes, the formation of not less than 1cm layer of foam shows the presence of saponins and considered as positive result.

4- Test for alkaloids

A small portion of extracts (F1 and F2) were tested with various reagents for the presence of alkaloids.

A- Mayer’s reagent; cream precipitation is considered positive.

B- Dargendroff’s reagent; orange brown precipitation is considered positive.

C- Wagner’s reagent; reddish brown precipitation is considered positive.

5- Test for phenolic compounds and tannins

The tests for phenolic compounds and tannins were carried out with following reagents:

A- 5% ferric chloride solution; deep green or deep blue is considered positive.

B- 10% lead acetate solution; white precipitation is considered positive.
C- 1% potassium dichromate solution; orange precipitate

6-Test for flavonoids\(^{(12)}\)

A- With aqueous sodium hydroxide solution
   Anthrocyanins; blue to violet color is positive.
   Flavones and flavonol; yellow color is positive.
   Flavonones; yellow to orange color is positive.

B- With concentrated sulphuric acid
   Anthrocyanins; yellow orange color is positive.
   Flavones and flavonol; yellow to orange color is positive.
   Flavonones; orange to crimson color is positive.

III-Chromatographic Identification of quercetin and kaempferol flavonoids
Perpetual Spinach is loaded with flavonoids which mainly act as antioxidants; identification of these flavonoids was performed by

1- Identification of flavonoids (quercetin and kaempferol) by TLC\(^{(13)}\)
   Using readymade aluminum plates of silica gel GF254; two different detection methods: first by using ultraviolet light detector at 254 nm and 366nm, second by using iodine vapor in the jar. Standard flavonoids in comparison with three different solvent systems S1, S2 and S3. Standard flavonoids are: quercetin (Fluka-Austria) and kaempferol (Sigma-Aldrich, USA).
   Different developing solvent systems were:-
   S1 = Chloroform: Acetone: Formic acid (75:16.5:8.5)
   S2 = Chloroform: Methanol (90:10)
   S3 = Toluene: Chloroform: Acetone (40:25:35).

2- Identification of flavonoids in HPLC
   Further identification to the flavonoids in plant extracts (F1 and F2) were performed by HPLC. For qualitative estimation comparison of retention time obtained at identical chromatographic conditions of plant extract with authentic standards was done.
Table (1): HPLC Conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mobile phase</th>
<th>Column</th>
<th>Flow rate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>Acetonitrile: methanol : glacial acetic acid (70:30: 0.1)</td>
<td>C\textsubscript{18} 5 mm x 150 mm</td>
<td>0.5 ml / min</td>
<td>UV. Detector at λ 306 nm</td>
</tr>
<tr>
<td>Kaempherol</td>
<td>methanol : water ( 7.5 : 92.5 )</td>
<td>C\textsubscript{18} ODS</td>
<td>1.5 ml / min</td>
<td>UV. detector at λ 308 nm</td>
</tr>
</tbody>
</table>

IV-Formulation of different topical preparations from the plant extract:

Aqueous solutions and ointments were prepared from F1 and F2 extracts as follows:
1- Aqueous solutions: 100 ml of 1 and 2 % w/v aqueous solutions from F1 and F2 extracts were prepared to which 0.18 g methyl paraben and 0.2 g propyl paraben were added as preservatives.

2-Ointments: 100 g of 1 and 2 % w/w ointments from F1 and F2 extracts were prepared by fusion method as follows:
Poly ethylene glycol (4000) 47.5 g
Poly ethylene glycol (400) 47.5 g
Cetyl alcohol 5.0 g
To which 0.18 g methyl paraben and 0.2 g propyl paraben as preservatives

V-Clinical evaluations

For treatment of acne, 160 patients were included in this study where 20 patients received aqueous solution containing 1% of F1 extract and 20 patients received 2% aq. solution of F1, same was applied for F2 extract; to be applied topically twice daily for two weeks. 20 patients received ointment containing 1% of F1 extract and 20 patients received 2% ointment of F1 extract, same was applied for F2 extracts; to be applied topically on the affected area for two weeks.

For treatment of psoriasis, 160 patients were classified in groups; 20 patients were given 1% ointment and 20 patients received 1% solution of F1 extract, same is applied for F2 extract; to be applied topically on the affected area for one month. 20 patients received 2% solution of F1 extract and 20 patients received 2% ointment of F1 extract, same is applied for F2 extract; to be applied topically on the affected area for one month. All these studies were done under medical supervision.
RESULTS AND DISCUSSIONS

The fresh leaves of perpetual Spinach was divided into two parts, the first one extracted with 80% ethanol by soxhlet extractor and fractionation to yield 10.2% w/w aqueous extract (F1) and the second part, the fresh leaves dried, powdered and extracted with 50% methanol and 1.2M HCl by reflux apparatus to yield 10.3% w/w methanolic extract (F2).

The various extract of fresh and dried leaves were subjected for different chemical tests for preliminary screening of F1 and F2 extracts of the plant and the results indicated the presence of different compounds in plant extracts as shown in table (2).

Table (2): Preliminary phytochemical evaluation of fresh and dried leaves of Beta vulgaris.

<table>
<thead>
<tr>
<th>Active constituents</th>
<th>Tests</th>
<th>F1</th>
<th>F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed oil</td>
<td>Spot test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Baljet’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Keller kiallan’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Brontrager’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Foam test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Mayer’s test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff’s test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wagner’s test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic compounds and tannins</td>
<td>FeCl₃ test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Potassium dichromate test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Dil. NaOH solution</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Conc. H₂SO₄</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Where + =present; - =absent

Further investigation on the flavonoids of this plant is carried out due to their antibacterial and the antioxidants activity of flavonoids

TLC of the extract (F1 and F2) obtained from fresh and dried leaves of Iraqi perpetual spinach confirm the presence of quercetin and kaempferol in F1 and F2 using three different solvent systems in comparison was made with standards, as represented in (table 3) and (figure 3,4 and 5).

Table (3): Rf value of plant extracts (F1 and F2), standard quercetin and kaempferol.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf value of quercetin standard</td>
<td>0.45</td>
<td>0.41</td>
<td>0.71</td>
</tr>
<tr>
<td>Rf value of quercetin in F1</td>
<td>0.43</td>
<td>0.39</td>
<td>0.70</td>
</tr>
<tr>
<td>Rf value of quercetin in F2</td>
<td>0.44</td>
<td>0.40</td>
<td>0.72</td>
</tr>
<tr>
<td>Rf value of kaempferol standard</td>
<td>0.55</td>
<td>0.52</td>
<td>0.82</td>
</tr>
<tr>
<td>Rf value of kaempferol F1</td>
<td>0.53</td>
<td>0.50</td>
<td>0.80</td>
</tr>
<tr>
<td>Rf value of kaempferol F2</td>
<td>0.54</td>
<td>0.51</td>
<td>0.81</td>
</tr>
</tbody>
</table>
S1 = Chloroform: Acetone: Formic acid (75:16.5:8.5)  
S2 = Chloroform: Methanol (90:10)  
S3 = Toluene: Chloroform: Acetone (40:25:35).

Figure 3: TLC of leaves extract (F1 and F2) of perpetual spinach obtained by extraction method using silica gel GF 254 as adsorbent and S1 as a mobile phase detected by UV light at 245nm (from right to left: methanolic extract, kaempferol, quercetin standard and aqueous extract)

Figure 4: TLC of leaves extract of perpetual spinach obtained by extraction method using silica gel GF 254 as adsorbent and S1 as a mobile phase detected by UV light at 366nm (from right to left: methanolic extract, kaempferol, quercetin standard and aqueous extract)

Figure 5: TLC of leaves extract of perpetual spinach obtained by extraction method using silica gel GF 254 as adsorbent and S1 as a mobile phase detected by UV light at iodine vapor (from right to left: methanolic extract, kaempferol, quercetin standard and aqueous extract)
Further identification to the quercetin and kaempferol in plant extracts (F1 and F2) were performed by HPLC in which the retention time of both standards (quercetin and kaempferol) and the plant extracts (F1 and F2) were identical as represented in the figures below:

Figure 6: HPLC analysis of quercetin standard

Figure 7: HPLC analysis of quercetin in methanolic extract

Figure 8: HPLC analysis of quercetin in aqueous extract
Figure 9: HPLC analysis of kaempferol standard

Figure 10: HPLC analysis of kaempferol in methanolic extract

Figure 11: HPLC analysis of kaempferol in aqueous extract
Formulation and clinical aspects
The aqueous extracts of the fresh leaves F1 and the methanolic extract of the dried leaves F2 of the Iraqi plant Beta vulgaris var. cicla belong to the family Chenopodiaceae locally known as salk shows the presence of high percent flavonoids (mainly quercetin and kaempferol) and phenolic compounds which are reported to possess several desirable biological activities, including antioxidant, anti-inflammatory, hepatoprotective, and anti-tumor properties \(^{17,18}\). Different topical preparations (solutions and ointments) prepared from F1 and F2 extracts and applied clinically for the treatments of Acne and psoriasis. For treatment of Acne; two groups of patients received prepared solution containing 1% and 2% of F1 extract separately to be applied on the infected area twice daily for two weeks and two groups of patients received prepared solution containing 1% and 2% of F2 extract separately to be applied in the same way. Two groups of patients received the prepared ointments containing 1% and 2% of F1 extract separately to be applied twice daily for two weeks, another 2 groups of patients received prepared ointment containing 1% and 2% of F2 extract separately to be applied in the same way. The study was done under medical supervision and follow up treatment. It was found that solution dosage form showed better response (complete healing) than ointment dosage form for both F1 and F2 extracts, this could be attributed that drug should be released from its ointment base and absorbed though the skin to be effective, while drug in solution is readily available for absorption \(^{19}\). It was found that the prepared solutions from F1 extract (which is the aqueous extract obtained from the fresh leaves) gives 90% complete healing while that for F2 (methanolic extracts of dried leaves) gives 73% complete healings indicating that some of the plant content were lost during drying the leaves.

The healing effect of the plant extracts for Acne could be mainly attributed for the antimicrobial and anti-inflammatory activity of flavonoids which is present in high concentration in the Iraqi plant \(^{20,21}\).

For treatment of psoriasis; 2 groups of patients received the prepared solutions containing 1% and 2% of F1 extract separately, same is applied for F2 extract. It was found that only 30% of the patients show complete healing after twice daily treatment for one month with the solution dosage form. While 80% of the patients show complete healing after using ointments containing 2% of F1 extract in comparison to 48% patients receiving 2% ointment containing F2 extract, indicating that in treatment of psoriasis, the affected area required to be covered and be in contact with the drug for longer time and this is achieved with the ointment dosage
form . The healing effect suggested that the plant might be used as an important natural antioxidant source due to its high levels of phenolics and flavonoids and the aqueous extract of the fresh plant can also be used as an accessible source of natural antioxidants which in many cases are free radical scavengers or quenchers of activated states, comprise a vast number of classes of organic molecules including most prominently the phenolics (22).

CONCLUSION

Preliminary phytochemical studies of the aqueous and methanolic extracts of fresh and dried leaves of Iraqi Beta vulgaris Var.cicla showed the presence of flavonoids mainly quercetin and kaempferol in addition to glycosides, saponin, tannins and phenolic compounds. The clinical study showed that the plant extract gave significant healing effect for the treatment of Acne within two weeks using aqueous solutions prepared from extracts of fresh and dried leaves with more predominant effect for the fresh leaves. And the solution dosage form is better than ointment in the treatment of Acne. Same results obtained for psoriasis but ointment dosage form was much more effective .The study showed that the Iraqi plant possess high amount of flavonoids and phenol compounds that can act as antibacterial and antioxidants and give promising alternatives specially due to the increasing resistance to existing anti-microbial agents, side effects and sometimes high cost of treatment.

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22. Ozlem Sacan1, Refiye Yanardag; Antioxidant and antiacetylcholinesterase activities of chard (Beta vulgaris L. var. cicla), Food and Chemical Toxicology 2010, 48: 1275–1280