Separation and Identification of Phenolic Acid from Borago officinalis (F: Boraginaceae) Cultivated in Iraq

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Abstract

The plant Borago officinalis, which belongs to the Boraginaceae family and Celebrated as borage, is one of the useful medicinal plants cultivated in Iraq. It was used in old medicine in Iraq, Syria and Europe for management of various diseases. It is commonly used as a tonic, tranquilliser, management of cough, sore throat, pneumonia, urinary tract infection, rheumatoid arthritis, antioxidant, and anticancer. This project provides the first comprehensive research done in Iraq to study the phytochemicals and the methods of extraction and isolation of active constituents from Borago officinalis cultivated in Iraq. The plant was harvested in spring from AL-Rifai, Nassiriyah city/IRAQ in February 2019. The aerial parts were washed carefully, dried under dark for two weeks and milled in a mechanical grinder to a fine powder. The plant was extracted by cold extraction methods using 85% methanol solvent for three days then Fractionation with petroleum ether, chloroform, ethyl acetate and n-butanol(n.b) to separate the active constituents according to the change in polarities. The ethyle acetate fraction and n-butanol fraction were used for identification and isolation of phenolic compounds by TLC, HPLC, HPLC, and LC/mass. Results of the phytochemical screening exposed the presence of, phenols, tannins, fatty acid, in the plant extract. The phenolic acid (Sinapic acid, Rosmarinic acid, Caffeic acid) were separated and purified by PLC. The isolated compounds were subjected to several chemical, chromatographic and spectral analytical techniques for their identification such as TLC, HPLC, UV and LC/mass.

Keywords: Sinapic acid, Rosmarinic acid, Caffeic acid, HPLC, LC/Mass.

Fصل وتحديد الاحماض الفينولية الموجودة في نبات لسان الثور المستزرع في العراق

*فرع العقاقير والنباتات الطبية بكلية الصيدلة، جامعة بغداد، بغداد، العراق

غفر العقاقير والنباتات الطبية

Borago officinalis L., المعروف ب بوراجيناكة، (Boraginaceae) نبات لسان الثور المستزرع في العراق. ويستخدم النبات في الطب الشعبي في العراق. النبات يستخدم في علاج العديد من الأمراض ككراهية ومضادات للالتهابات والالتهابات البولية وغيرها. تم استخرج النبات بطرق شبه البرودة للكشف عن الأحماض الفينولية الموجودة في النبات. حيث تم فصل النبات عن طريق الكلوروформ والثنائي أцeten والبتانول (n.b) للكشف عن الأحماض الفينولية الموجودة في النبات. تم استخدام الاملاح العاكسة والخلاصة العاكسة للكشف عن الأحماض الفينولية الموجودة في النبات. النتائج تشير إلى وجود احماض فينولية وحمض روزماريكي وحمض السينابيك. كلامات المفتاحية: احماض الفينولية، احماض السينابيك، احماض الفينولية، هرمونات الدم، الالتهابات الرئوية وENCEPHALITIS.

Introduction

Borage (Borago officinalis L.) is an annual plant belonging to the family, Boraginaceae. It originates from Western regions of Mediterranean area and grows nearly in whole America, Europe, Canada and Iran (1,2). The plant grows during November to January and reaches a height of 70 to 100 cm (3). Its stem is shielded with hairs that secrete a strong smell nearly the aroma of fresh cucumbers while on the tops of the shoots there are the star-shaped inflorescences which initially are pink, later turn blue, seldom white (5,6). Aerial parts have been used in old medicine in Iraq as tonic, tranquilizer, management of cough, pneumonia, sore throat, swelling and inflammatory diseases. The leaves and flower possess biological activities for cancer and heart diseases prevention (7) and have antibiotic properties (8), condense cardiovascular diseases (9) and provide benefits for improving health due to their various biological events (10).

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Results reported by previous studies shown the presence of phenolics acid, flavonoids (Quercetin, Myricetin, Luteolin and Rutin) and isoflavonoid besides, the dominant individual fatty acids of methanolic extract as Oleic acid which is an unsaturated fatty acid (omega-9), linoleic fatty acids (omega-6) and Hexadecanoic acid. The methanolic extract was more biologically active than ethanolic extract.

Material and Method

Plant collection

*Borago officinalis* plant was harvested from AL-Rifai, Nassiriyah city/IRAQ in February 2019. The aerial plant was dried in the shadow for two weeks and powdered. The plant was identified and authenticated by Prof. Dr Israa Mohammed Department of Biology /College of Sciences/ University of Baghdad.

Extraction and fractionation of the different active constituent

Two hundred and fifty grams of the powdered plant material was soaked in 1500ml, with 85% methanol and shaking, at room of temperature. After three days, the methanol soluble materials was filtered off. The filtrate was evaporated until dryness under vacuum using a rotatory evaporator. A dark greenish residue was obtained. Then suspended in 500ml water and partitioned successively with petroleum ether (B.p. 30-60 °C), chloroform, ethyl acetate, and n-butanol (3×500ml) for each fraction the first three fractions dried over sodium sulfate anhydrous, filtered, and evaporated to dryness. The scheme of fractionation is shown in (Figure 2).
Identification of phenolic compounds in *Borago officinalis* plant extract

1. Preliminary phytochemical showing of the phenolic compound using a methanolic extract from the plant using a NaOH test, lead acetate, and ferric chloride test.\(^{(15,16)}\)

2. Isolation and purification of phenolic compounds from the fractions ethyl acetate and n-butanol by preparative layer chromatography. Isolation of phenols was done by using preparative TLC; 2 gram of each fraction dissolve in 10 ml of methanol and applied to the number of PLC plates as a semi concentrated solution in streak using a capillary tube on each plate, then the plate placed inside glass tank which contained the S1 solvent system. The band had been scrapped off, eluted with methanol and then filtered, the filtrate evaporated to dryness, in a vacuum as shown in Figure 3 and 4.

3. Thin-layer chromatography. In this qualitative identification, a ready-made aluminium plates of silica gel of 245 with developing solvent systems were used for detection the plant phenols in fractions ethyl acetate and n-butanol with standards, as listed in Table (1).

4. Qualitative estimate of ethyl acetate and n-butanol fractions by high-performance liquid chromatography (HPLC) the expected phenols in fractions were separated by HPLC method and detected in comparison with standard compounds. The mobile phase consisted 1 % aq. Acetic acid solution (A) and acetonitrile (B) solvents, the flow rate was adjusted to 0.7 ml/min, the column was thermostatically controlled at 280°C, and the injection volume was kept at 20 μl. Gradient elution was accomplished by varying the proportion of B to A solvents. The gradient elution was changed from 10 % to 40% B in a linear fashion for 28 min, from (40 to 60) % B in 39 min, after that from (60 to 90) % B solution in 50 min. The mobile phase mixture back to the initial state of solvents (B: A: 10: 90) in 55 min and permitted to run for another ten min.

5. LC-MS Analytical was done using the Agilent System joined to an Applied Biosystems API 2000 mass spectrometry. Mobile phase solvents acetonitrile and water A column of 0.19mm external diameter (75μm I.D.) and 200mm length was packed with Thermo Scientific Hypersil Gold C18 with 5μm particle size. Samples were run under the following conditions: m/z range was 250 to 1000 , 200K resolution, top 5 configurations with one MS scan and five MS/MS scans, and dynamic exclusion set to 1 with a limit of 90 seconds. A 2.5 hour LCMS separation was used for all blank and standard samples.

Results and Discussion

*Borago officinalis* plant active constituents

In this study, cold extraction method was done by 85% absolute methanol to extract the active constituent depend on the nature of these active constituents. Each 250 g of plant extract yielded 32 g residue

Preliminary qualitative phytochemical analysis

The results of the phytochemical analysis of polyphenol in methanolic crude extract given in (Table 2).

| Active group | Test          | Reaction        | Result  |
|--------------|---------------|-----------------|---------|
| Polyphenol   | Lead acetate  | White ppt       | positive|
| Polyphenol   | Ferric chloride test | Dark green colour | positive|
| Polyphenol   | NaOH test     | Yellow colour   | positive|
|              | after addition NaOH solution |             |         |

The isolation of phenols were done by using preparative TLC, in jar contained the S1 solvent system. As in Figure 3 and 4.

Table 2. Results of the phytochemical analysis of polyphenol in the methanolic crude extract

Figure 3. Preparative thin layer chromatography plate for ethyl acetate fraction on silica gel GF254 developed in the S1 system, detection by UV light at 254nm.
Figure 4. Preparative thin-layer chromatography plate for n.b fraction on silica gel GF254 developed in the S1 system, detection by UV light at 366nm.

Table 3. TLC profile of isolated compound (cpd) number 1 compared with standard rosmarinic acid using the mobile phase solvents (S1, S2, S3).

| Solvent system | Rf of standard Rosmarinic acid | Rf of isolated Phenolic acid number 1 |
|----------------|-------------------------------|--------------------------------------|
| S1             | 0.247                         | 0.246                                |
| S2             | 0.13                          | 0.13                                 |
| S3             | 0.2                           | 0.2                                  |

Table 4. TLC profile of isolated compound number 2 compared with sinapic acid standard using the mobile phase solvents (S1, S2, S3).

| Solvent system | Rf of standard Sinapic acid | Rf of isolated Phenolic acid number 2 |
|----------------|----------------------------|-------------------------------------|
| S1             | 0.71                       | 0.71                                |
| S2             | 0.47                       | 0.47                                |
| S3             | 0.5                        | 0.51                                |

Table 5. TLC profile of isolated compound number 3 compared with caffeic acid standard using the mobile phase solvents (S1, S2, S3).

| Solvent system | Rf of standard caffeic acid | Rf of isolated Phenolic acid number 3 |
|----------------|----------------------------|--------------------------------------|
| S1             | 0.517                      | 0.517                                |
| S2             | 0.182                      | 0.182                                |
| S3             | 0.294                      | 0.293                                |

Figure 5. TLC chromatography (A) for separated compound number (1) from ethyl acetate fraction and Rosmarinic acid STD using silica gel Gf254 as adsorbant and S1 as mobile phase and (B) separated compound after detected by spraying with five % ferric chlorides.

Figure 6. TLC chromatography for separated compound number (1) from ethyl acetate fraction and R.A standard using silica gel Gf254 as adsorbant and mobile phase S2.
Figure 7. TLC chromatography for isolated compound number (1) from ethyl acetate fraction and Rosmarinic acid standard using silica gel Gf254 as adsorbant and mobile phase S3.

Figure 8. TLC chromatography (A) for isolated compound number (2) from ethyl acetate fraction and Sinapic acid standard using silica gel Gf254 as adsorbant and mobile phase S1 (B) separated compound after detected by spraying with five %ferric chlorides.

Figure 9. TLC chromatography for isolated compound number (2) from ethyl acetate fraction and standard S.A using silica gel Gf254 as adsorbant and mobile phase S2.

Figure 10. TLC chromatography for isolated compound number (2) from ethyl acetate fraction and S.A standard using silica gel Gf254 as adsorbant and S3 as mobile phase.
Figure 11. TLC chromatography (A) for separated compound number (3) from ethyl acetate fraction and Caffeic acid STD using silica gel Gf254 as adsorbant and S1 as mobile phase and (B) separated compound number 3 after detected by spraying with five % ferric chloride.

Figure 12. TLC chromatography for isolated compound number (3) from ethyl acetate fraction and caffeic acid standard using silica gel Gf254 as adsorbant and mobile phase S2.

Figure 13. TLC chromatography for isolated compound number (3) from ethyl acetate fraction and caffeic acid standard using silica gel Gf254 as adsorbant and S3 as mobile phase.

**HPLC analysis**

The result gained from HPLC analysis method.

1. The retention time of Rosmarinic acid standard match with a retention time of isolated compound number 1 and UV spectrum of separated compound number 1 match with UVs spectrum of Rosmarinic acid standard as in Figure 14 and 15 and Table 6.

2. The retention time of the isolated compound number 2 match with a retention time of Sinapic standard and UV spectrum of the separated compound number 2 match with standard Sinapic acid as in Figure 16 and 17 and Table 6.

3. The retention time of the isolated compound number 3 match with a retention time of Caffeic acid standard and UV spectrum of the separated compound number 3 match with Caffeic acid standard as in Figure 18 and 19 and Table 6.

Figure 14. HPLC chromatogram of isolated compound number 1 and Rosmarinic acid standard.
Figure 15. The UV spectrum of isolated compound number 1 and Rosmarinic acid standard.

Figure 16. HPLC chromatogram of the isolated compound number 2 and Sinapic acid standard.

Figure 17. The UV spectrum of isolated compound number 2 and Sinapic acid standard.
Figure 18. HPLC chromatogram of Caffeic acid standard and isolated compound number 3.

Figure 19. UV spectrum of isolated compound number 3 and caffeic acid standard.

Table 6. Retention times in minutes for standards (rosmarinic acid, sinapic acid, caffeic acid) and isolated cpd 1,2,3.

| Retention time of Rosmarinic acid standard. | Retention time for isolated cpd number 1. |
|--------------------------------------------|------------------------------------------|
| 20                                        | 20                                       |
| Retention time of Sinapic acid standard.   | Retention time for isolated cpd number 2. |
| 15                                        | 15                                       |
| Retention time of Caffeic acid standard.   | Retention time for isolated cpd number3.  |
| 10                                        | 10                                       |

**LC/mass**
The result gained from LC/mass.
1. LC/mass chromatogram as shown in (Figure 20) the isolated compound number 1 is Rosmarinic acid with molecular weight 360.31 Gram/mol as shown in Figure (21).
2. LC/mass chromatogram as shown in (Figure 22) the isolated compound number 2 is Sinapic acid with molecular weight 224.21 Gram/mol as shown in Figure (23).
3. LC/mass chromatogram as shown in (Figure 24) the isolated compound number 3 is Caffeic acid with molecular weight 180.16 Gram/mol as shown in Figure (25).
Figure 20. LC/MS chromatogram of isolated compound number 1

Molecular ion peak at m/z 360 that correspond to a molecular formula of C18H16O8(3,4-dihydroxyphenyllactic acid) Rosmarinic acid and Molecular ion peak at m/z359[M-H] of Rosmarinic acid.

Figure 21. Chemical structure of Rosmarinic acid with molecular weight 360.31 Gram/mol \(^{(18)}\)
Figure 22. LC/MS chromatogram of isolated compound number 2.

Molecular ion peak at m/z 223 that correspond to the molecular formula of C11H12O5 (3,5-dimethoxy-4-hydroxycinnamic acid) sinapic acid.

Figure 23. Chemical structure of Sinapic acid with molecular weight 224 Gram/mol\(^{19}\).
Figure 24. LC/MS chromatogram of isolated compound number 3.

Molecular ion peak at m/z 181 that correspond to the isotope of a molecular formula of C9H8O4 (3,4-dihydroxycinnamic) caffeic acid, and molecular ion peak m/z 179.3 [M-H] for caffeic acid.

Figure 25. Chemical structure of Caffeic acid with molecular weight 180 Gram/mol. \(^{(20)}\)
Conclusion

In the light of the results obtained, the study concluded the following:
1. Phytochemical screening of new Iraqi plant *Borago officinalis* was done for aerial part of the plant nearly two hundred and fifty grams of the powdered plant material, and the results include the separation and identification of phenolic acids (Rosmarinic acid, caffeic acid and Sinapic acid).
2. Rosmarinic acid with Mwt 360 gram/mol isolated from ethyle acetate and n.butanol fraction with high quantity nearly 13.3 mg.
3. Sinapic acid with molecular weight 224 Gram/mol isolated from ethyle acetate fraction with small quantity nearly 6 mg.
4. Caffeic acid with molecular weight 180 Gram/mol isolated from ethyle acetate fraction with high quantity nearly 9 mg.
5. All isolated phenolic acids were identified by TLC, preparative TLC, HPLC, UV, LC/Mass.

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