Isolation and Identification of Phenolic Compounds from *Dianthus orientalis* Wildly Grown in Iraq.

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**Abstract**

The plant *Dianthus Orientalis* that belongs to the Caryophyllaceae family is one of the useful plants in Iraq. Its seeds are commonly used for toothache. This project provides the first comprehensive research done in Iraq and the world to study the phytochemicals and the methods of extraction and isolation of active constituents from *Dianthus orientalis* wildly grown in Iraq. The plant was harvested from Penjwin in AL-Sulaymaniyah city, Iraq in September 2019. The whole plant was washed carefully, dried in shade area for two weeks, and milled in a mechanical grinder to a coarse powder. The plant was defatted by maceration with hexane for 7 days and dried after that extracted by cold extraction methods using 80% methanol solvent for 9 days then fractionation with chloroform, ethyl acetate and n-butanol to separate the active constituents according to the change in polarities.

The chloroform, ethyl acetate fractions were used for identification and isolation of phenolic compounds by TLC, PTLC, HPLC and LC/mass. FTIR. Results of the phytochemical screening exposed the presence of, phenols in the plant extract. The phenolic compound (vanillic acid, coumaric acid, cinnamic acid, genistein, oleuropein) were separated and purified by PTLC. The isolated compounds were subjected to several chemical, chromatographic and spectral analytical techniques for their identification such as TLC, HPLC, FTIR and LC/mass.

**Keywords:** Vanillic acid, Coumaric acid, Cinnamic acid, Genistein, Oleuropein, HPLC, LC/Mass.

**أعلاه**

دِيناثوس* ديانيثوس أو القرنفل البري النبات الذي ينمو بشكل طبيعي في العراق. النبات يتم جمعه من منطقة بنجوين في محافظة السليمانية في شهر أيلول من سنة 2019. النبات كاملاً جُمَّع وُجِّه بعناية، وُجِّه في مكان مشمس لفترة أسبوعين، ثم طاحنه. النبات تم استخلاص أحماض الفينولية والمواد الدابغة بطرق متعددة تشمل كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا الطبقة التحضيرية، وكروماتوغرافيا الطبقة الارتفاعية. نتائج الكشف الكيميائي على النبات أظهرت пр пр пр пр presence of phenols in the plant extract. The phenolic compounds (vanillic acid, coumaric acid, cinnamic acid, genistein, oleuropein) were separated and purified by PTLC. The isolated compounds were subjected to several chemical, chromatographic and spectral analytical techniques for their identification such as TLC, HPLC, FTIR and LC/mass.

** ключевые слова:** Vanillic acid, Coumaric acid, Cinnamic acid, Genistein, Oleuropein, HPLC, LC/Mass.

**التخلص**

بعد نبات القرنفل البري في العراق، النبات الذي ينمو بشكل طبيعي في العراق. النبات يتم جمعه من منطقة بنجوين في محافظة السليمانية في شهر أيلول من سنة 2019. النبات كاملاً جُمَّع وُجِّه بعناية، وُجِّه في مكان مشمس لفترة أسبوعين، ثم طاحنه. النبات تم استخلاص أحماض الفينولية والمواد الدابغة بطرق متعددة تشمل كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا الطبقة التحضيرية، وكروماتوغرافيا الطبقة الارتفاعية. نتائج الكشف الكيميائي على النبات أظهرت presence of phenols in the plant extract. The phenolic compounds (vanillic acid, coumaric acid, cinnamic acid, genistein, oleuropein) were separated and purified by PTLC. The isolated compounds were subjected to several chemical, chromatographic and spectral analytical techniques for their identification such as TLC, HPLC, FTIR and LC/mass.

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Dianthus caryophyllus is a very important species in Caryophyllaceae family. It has been used traditionally in the treatment of throat and gum infections, the treatment of wounds, as a cardio-tonic, diaphoretic, vermifuge and for the treatment of gastro-intestinal disorder. The plant traditionally used in China, Japan and Korea in the treatment of wounds and gastro-intestinal disorder and various other ailments (8-11). The chemical composition and the essential oil of the carnation flowers (Dianthus caryophyllus) were studied. Phytochemical tests showed that of Dianthus caryophyllus contained triterpenes, alkaloids, coumarins and cyanogenic glycosides (12). The chemical composition and the essential oil of the carnation flowers (Dianthus caryophyllus) was studied. Twelve volatiles were identified by gas chromatography-mass spectrometry (GC-MS) as the main components of carnation flower oil. The major components were phenyl ethyl alcohol, eugenol, hexyl benzoate, hexenyl benzoate, benzoin, nootkatone, benzyl salicylate, m-cresyl phenyl acetate, hexadecanoic acid and eicosene (13). Three flavonoids including apigenin-C-glycoside, kaempferol 3-O-β-d-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-d-gluco-pyranoside and kaempferol 3-O-[α-L-rhamnopyranosyl-(1→6)]-β-d-gluco-pyranoside (14-15). Two benzoic acid derivatives, protocatechuic acid and vanillic acid, flavonol glycoside peltatoside and flavone dactis celtein were isolated from the plant (16).

Material and Method

Collection of plant materials

Dianthus orientalis L. were obtained from Penjwin in AL-Sulaymaniyah city, Iraq in September 2019. The plant was identified and authenticated by Dr. Karzan Aumar Kadir /Department of Biology /College of Sciences/ University of Sulaimani. The plant was washed thoroughly, dried under shade, and ground in a mechanical grinder to a coarse powder.

Equipment and chemical

The instruments used were rotary evaporator (BUCHI Rotavapor R-205, Swiss), sonicator (Branson Sonifier, USA), high-performance liquid chromatography (HPLC) (Knuera, Germany). All chemicals and solvents used were of analytical grade and obtained from Riedel-de Haen, Germany, except methanol, which is HPLC grade was purchased from Sigma-Aldrich, Germany. The standard vanillic acid, coumaric acid, were purchased from Chengdu Bio purify Phytochemicals, China (purity >97). TLC aluminum plates pre-coated with silica gel (20 cmx20cm, 0.2 mm thick) used were obtained from MACHELEY-NAGEL-Germany.

Extraction

The whole plant coarse powder 250gram was macerated with normal hexane for one week in conical flask 2000ml with shaking many times in the shade and then filter it, and then the organic layer was taken and dried in the shade the defatted powdered plant material was soaked in 1500ml methanol, with occasional shaking, at room temperature. After 3 days, the methanol soluble materials were filtered off and this method is repeated for three times (extraction will done in 9 days). The filtrate was evaporated to dryness under vacuum using rotary evaporator. A dark brown-greenish residue was obtained. The residue twenty grams was suspended in 500ml water and partitioned successively with chloroform, ethyl acetate, and n-butanol (3x500ml) for each fraction. The first two fractions dried over anhydrous sodium sulfate, filtered, and evaporated to dryness.

Preliminary phytochemical investigation

Alkaloids, saponin, phenolic compounds investigation were carried out with: Alkaloids test Test for saponins Tests for phenols: A: Ferric chloride Test, B: NaOH test. Isolation of phenolic compounds from the ethyl acetate fraction and chloroform fraction by preparative layer chromatography (PLC):

One gram of each fraction dissolve in 3 ml of methanol and applied on 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 solvent system (chloroform: acetone:formic acid)(75:16:1). The band had been scrapped off, eluted with methanol and then filtered; the filtrate evaporated to dryness, the band that separated from ethyl acetate fraction was symbolized as E1. Isolation from the chloroform fraction by preparative layer chromatography (PLC):

Four bands were isolated from chloroform fraction utilizing the same procedure applied to the ethyl acetate fraction and using the same mobile phase (chloroform:acetone:formic acid) (75:16:1). The compounds were isolated from chloroform fraction were symbolized as C1, C2, C3, C4.
Identification of the isolated phenolic derivatives from ethyl acetate and chloroform fraction of Dianthus orientalis

The compound that symbolized as E1, C1, C2, C3, C4 was identified by several methods including chemical, chromatographic, and spectral methods as:

Spraying with 5% ethanolic KOH on TLC plate

HPLC analysis

HPLC technique (Knuaer, Germany) was applied for the detection of different constituents found in the ethyl acetate, chloroform fractions as flavonoids and phenolic acids, and for identification of the isolated compounds from Dianthus orientalis. The mobile phase contains 1% aq. Acetic acid solution (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 1 ml/min, the column was thermostatically controlled at 280 °C and the injection volume was kept at 20 μl. A gradient elution was performed by varying the proportion of solvent B to solvent A as shown in the table (1). The HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) flow rate 1 ml/min.

Table 1. The gradient elution changing A and B proportion with time

| Time | Mobile A % | Mobile B % |
|------|------------|------------|
| 0    | 90         | 10         |
| 28   | 60         | 40         |
| 59   | 40         | 60         |
| 60   | 10         | 90         |

FTIR

Identified chemical bands in molecules. IR spectra range of scanning was 4000-400 cm⁻¹

LC/MS: Analytical LC-MS was performed using Agilent/box system joined to an Applied Biosystems API 2000 mass spectrometer. 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 10001, 200K resolution, and dynamic exclusion set to 1 with a limit of 90 seconds.

Result and Discussion

Table 2. Phytochemical Screening of Dianthus Orientalis plant

| Chemical test for phenols          | Results                                      |
|------------------------------------|----------------------------------------------|
| A.Ferric chloride test             | Positive due to formation of dark brown color |
| B.NaOH                             | Positive due to formation of yellow color .  |
| 3.Saponin test                     | Positive due to froth formation.             |
| 4.alkaloid test: Dragendorff’s reagent | Positive due to formation of orange-brown precipitate |

High-performance liquid chromatography (HPLC) examination of ethyl acetate fraction and chloroform fraction

Figure 1. HPLC chromatogram for ethyl acetate fraction
**Identification of E1**

Spraying with 5% ethanolic KOH on TLC plate gives a yellow colored spot.

**HPLC of isolated E1**: The HPLC chromatogram of standard vanillic acid and isolated cpd E1 were shown in figure (4), spectrum of std vanillic acid and isolated cpd E1 and as shown in figures (5).

**FTIR**

IR spectrum of isolated cpd E1 was showed in the figure (6) and interpretation of the bands in the table (3).

**LC/mass**

Analytical LC-MS was performed using an Agilent System joined to an Applied Biosystems API 2000 mass spectrometer. The LC-MS chromatogram of the isolated compounds E1 as in figure (7).

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Figure 2. HPLC chromatogram for chloroform fraction.

Figure 3. TLC chromatogram for isolated cpd A: E1 and standard vanillic acid B: C4 and standard coumaric acid developed in the (chloroform acetone: formic acid) (75:16:1) solvent system, Detect under UV light at 254.

Figure 4. HPLC chromatogram of standard vanillic acid and isolated cpd E1.
Figure 5. UV spectrum of standard vanillic acid and isolated cpdE1.

Figure 6. IR spectrum of isolated cpdE1

Table 3. Interpretation of the IR bands for E1 are shown below

| IR band of isolated cpdE1 | Interpretation                  |
|---------------------------|---------------------------------|
| 3290                      | OH stretch vibrations band       |
| 2939                      | C-H asymmetric stretching        |
| 2828                      | C-H symmetric stretching         |
| 1663                      | C=O stretching                   |
| 1448                      | C=C Aromatic stretching          |
| 1266                      | In plane C-H bending             |
| 1110                      | C-O-C stretching                 |
| 809                       | Out of plane C-H Aromatic bending|
| 679                       | Out of plane C=C Aromatic bending|
All these data coincide with that reported for vanillic acid therefore compound E1 could be vanillic acid with mwt 168.1 gram/mol.

**Identification of C4**
Spraying with 5% ethanolic KOH on TLC plate give yellow colored spot.

**HPLC for isolated cpd C4:** The HPLC chromatogram of standard coumaric acid and isolated cpd C4 are shown in figure (8), spectrum of std coumaric acid and cpd C4 and as shown in figure (9).

**FTIR:** IR spectrum of isolated cpd C4 was showed in the figure (10) and interpretation of the bands in the table (4)
Figure 8. HPLC chromatogram of standard coumaric acid and isolated cpd C4

Figure 9. UV spectrum of standard coumaric acid and isolated cpd C4

FTIR

Figure 10. IR spectrum of isolated cpd C4
Table 4. Interpretation of the IR bands for C4 are shown below.

| IR band of isolated cpd C4 | Interpretation                              |
|-----------------------------|---------------------------------------------|
| 3307                        | OH stretch vibrations band                   |
| 2972                        | C-H asymmetric stretching                    |
| 2877                        | C-H symmetric stretching                     |
| 2360                        | C=C stretching                               |
| 1645                        | C=O stretching                               |
| 1448                        | C=C Aromatic stretching                      |
| 1375                        | O-H bending                                  |
| 1085                        | C-O stretching                               |
| 879                         | Out of plane C-H Aromatic bending            |
| 524                         | C-H stretching                               |

All these data coincide with that reported for coumaric acid therefore compound C4 could be coumaric acid.

Identification of C1
Spraying with 5% ethanolic KOH on TLC plate give yellow colored spot.

HPLC of isolated C1: The HPLC chromatogram of standard oleuropien and isolated cpd C1, spectrum of oleuropien standard and isolated cpd C1 were shown in the figures (11, 12) respectively.

Figure 11. HPLC chromatogram of standard oleuropien and isolate cpd C1

Figure 12. UV spectrum of standard oleuropien and isolate cpd C1

FTIR: IR spectrum of isolated cpd C1 is showed in the figure (13) and interpretation of the bands in the table (5)
Table 5. Interpretation of the IR bands for C1 are shown below.

| IR band of isolated cpdC1 | Interpretation                      |
|---------------------------|------------------------------------|
| 3294                      | OH stretch vibrations band          |
| 2972                      | C-H asymmetric stretching           |
| 2875                      | C-H symmetric stretching            |
| 1683                      | C=O stretching                      |
| 1492                      | C=C Aromatic stretching             |
| 1379                      | O-H bending                         |
| 1139                      | C-O stretching                      |
| 1087                      | in plane C-H Aromatic bending       |

All these data coincide with that reported for oleuropien therefore compound C1 could be oleuropien.

Identification of C2
Spraying with 5%ethanolic KOH on TLC plate give yellow colored spot.

HPLC of isolated C2: The HPLC chromatogram of standard genistein and isolated cpd C2 was shown in figure (14), spectrum of standard genistein and isolated cpd C2 was shown in figures (15).
FTIR: IR spectrum of isolated cpd C2 was showed in the figure (16) and interpretation of the bands in the table (6).

### Table 6. interpretation of the IR bands for C2 were shown below.

| IR band of isolated cpdC2 | Interpretation                  |
|---------------------------|--------------------------------|
| 3204                      | OH stretch vibrations band      |
| 2941                      | C-H asymmetric stretching      |
| 2808                      | C-H symmetric stretching       |
| 2337                      | C=C Aromatic stretching        |
| 1417                      | C=C Aromatic stretching        |
| 1122                      | C-O stretching                 |

All these data coincide with that reported for genistein therefore compound C2 could be genistein.

**Identification of C3**
Sprayng with 5%ethanolic KOH give yellow colored spot.

**HPLC of isolated C3:** The HPLC chromatogram of standard cinammic acid and isolated cpdC3, spectrum of cinammic acid std and isolated cpd C3, were shown in figures (17, 18).
Figure 17. HPLC chromatogram of standard cinamic acid and isolated cpd C3.

Figure 18. UV spectrum of cinamic acid std and isolated cpdC3

FTIR: IR spectrum of isolated cpd C3 was showed in the figure (19) and interpretation of the bands in the table (7)

Figure 19. IR spectrum of isolated cpdC3
Table 7. Interpretation of the IR bands for C3 were shown below.

| IR band of isolated cpdC3 | Interpretation          |
|---------------------------|-------------------------|
| 3385                      | OH stretch vibrations   |
| 2970                      | C-H asymmetric stretching|
| 2883                      | C-H symmetric stretching|
| 1683                      | C=O stretching          |
| 1446                      | C=C Aromatic stretching |
| 1389                      | O-H bending             |
| 1087                      | C=O stretching          |
| 1033                      | in plane C-H Aromatic bending|
| 879                       | Out of plane C-H Aromatic bending|

All these data coincide with that reported for cinamnic acid therefore C3cpd could be cinamnic acid.

**Conclusion**

The following points were pinched based on prior findings:
1. Phytochemical screening of *Dianthus orintalis* widely grown in Iraq demonstrates the presence of various phytochemicals, which were separated from plant according to differences in their chemical nature.
2. The phenolic compounds: vanillic acid, coumaric acid, genistein, cinamnic acid, and oleuropein were isolated from the plant.
3. isolated phenolic acids were identified by TLC, preparative TLC, HPLC, IR LC/Mass.

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