UPLC-ESI-MS/MS and Various Chromatographic Technique for Identification of Phytochemicals in *Populus euphratica* Oliv. Leaves Extract

**Abstract**

The aim of this study is to screen the phytochemicals found in *Populus euphratica* leaves since this type of trees are used traditionally by many villagers as treatment for eczema and other skin disease and also this plant is poorly investigated for their phytochemicals especially in Iraq. Phytochemical screening of the extracts obtained from the n-hexane and chloroform fraction of leaves of *Populus euphratica* was done by Thin-layer chromatography and various spraying reagents to test if alkaloids, sterols and other compounds are present. UPLC-electrospray ionization –tandem mass spectroscoery along with GC-MS and HPTLC are used to identify the phytochemicals present in the plant leaves. UPLC-ESI-MS/MS method 20 compounds have been identified in various fractions among which are protopine alkaloids, salicin, salicortin, tremulacin. GC-MS showed that the observed data obtained are matched with that in NIST library and confirmed the presence of a Hexadecanoic acid trimethylsilyl ester in 43.80% beta Sitosterol and Diisooctyl phthalate 11.46%.

**Keywords:** *Populus euphratica*, GC-MS, phytochemical, UPLC-ESI-MS/MS

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Introduction

*Populus euphratica* tree is a member of the Salicaceae family, which is abundant in phenols and its glycosides such as Salicin and populin (1). Previous studies on *Populus euphratica* have reported that some phenolic compounds (2) have been identified and volatile oils (3) have been detected in this plant. W. Wei et al. (2015) undertook a detailed chemical investigation of the leaves of *Populus euphratica* that afforded 13 compounds, among which 6-O-ciscinnamoylsalicin and 6-O-benzoylsalicortin were new compounds. The spectral data of 6-O-trans-cinnamoylsalicin and salicortin were reported for the first time. Meanwhile, nine known compounds were characterized as follows: salicin (4), benzyl-O-b-D-glucopyranoside tremulacin (5), salireposide (6), cinnamrutinoside A (7), and saligenin (8). Rahimi et al. (2008) have concluded that the smoke of burnt leaves of the *Populus euphratica* tree was curative in about 66% of patients with warts and there was a low recurrence rate of about 4%. (9).

*Populus euphratica* extract has been used traditionally by some villagers in some areas of Iraq in the treatment of eczema and various skin conditions. So in this study we will investigate the phytochemicals found in the leaves of this naturally abundant tree found in every city in Iraq and trying to resonate its traditional use.

The goal of this study is investigating and screening the phytochemicals and their proportions in hexane fraction of leaves of *Populus euphratica* tree naturally grown in Iraq since there is no phytochemical study had been done previously in Iraq and trying to resonate its traditional use in the treatment of eczema.

Material and Methods

**Plant material collection**

Leaves of *Populus euphratica* Oliv. was collected from the bank of the Tigris river in the periphery of Tikrit city in April 2018, Authenticated in Iraq Natural History Research Center and Museum by dr. khasaa Rasheed. Leaves were cleaned dried in shade at room temperature and then pulverized and stored for further use.

**Method of work**

100gm of air-dried powder of the leaves is weighted and defatted with petroleum ether overnight to get rid of chlorophyll and waxy material then extracted in soxhelt with 90% methanol for 9hr, the extract is combined and dried by rotary evaporator the dry extract is weighted and the yield of extraction is calculated to be 37gm.

The dry extract is dissolved in 100ml of distilled water. Then partitioned with 100ml of n-hexane using a separatory funnel for three times all the upper layers are combined together and dried using rotary evaporators, the dried hexane fraction was weighted to be 7.5 gm. The aqueous part is partitioned with chloroform 100ml 3 times to get 17.2gm of chloroform extract. Again the aqueous part is partitioned with butanol to get butanol fraction (12g) symbolized B.B fraction. Thin-layer chromatography and the spraying with Liebermann-Burchard reagent were used to identify the hexane extract containing phytosterosols. General identification test for alkaloid has been done with Dragendorff's reagent and Mayer's reagent both gave positive results for alkaloid (10).

B.B fraction is hydrolyzed by reflex for 6 hrs. using 5% HCL to get Butanol after hydrolysis fraction symbolized B.A. Readymade TLC pre-coated plate of 1mm GF254 for isolation and 0.25 mm GF254 was used for purification of isolated compounds.

**Acid-base extraction of alkaloids from the crude extract**

Part of the crude extract is suspended in n-hexane and partitioned with water to remove pigment and other non-polar compounds then the aqueous part is treated with NH4OH to PH10 to liberate free alkaloid then equal volume of chloroform is added to separatory funnel partitioned and the lower organic layer was acidified with 5% H2SO4 to PH2 , and again partitioned with equal volume of water, the aqueous layer now contain all alkaloid as salt, to this layer add NH4OH to PH10 and partitioned with chloroform the chloroform layer now contain free tertiary alkaloids, this fraction is symbolized as chA fraction (11).

**High-performance thin-layer chromatography (HPTLC) examination of chA B.B fraction**

The presence of flavonoids and phenolic glycoside and alkaloids in the analyzed fractions were confirmed by using the modern technique of HPTLC using Eike-Reich/CAMAG-Laborator/Switzerland, by comparing retention factor of analyzed sample with that of standards.

**Method for UPLC-ESI-MS/MS**

ESI-MS positive and negative ion acquisition mode was carried out on a XEVO TQD triple quadrupole instrument Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer Column: ACQUITY UPLC - BEH C18 1.7 μm - 2.1 x 50 mm Column Flow rate: 0.2 mL/min Solvent system: consisted of (A) Water containing 0.1% formic (B) Methanol containing 0.1% formic acid The sample (100 μg/mL) solution was prepared using high performance liquid chromatography (HPLC) analytical grade solvent of MeOH, filtered using a membrane disc filter (0.2 μm) then subjected to LC-ESI-MS analysis. Samples injection volumes (10 μL) were injected into the UPLC instrument, Sample mobile phase was prepared by filtering using 0.2 μm filter membrane disc and degassed by sonication before injection. The parameters for
analysis were carried out using negative ion mode as follows: source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in the ESI between m/z 100–1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

**Results and Discussion**

**Analysis of fractions by high-performance thin-layer chromatography (HPTLC)**

HPTLC is one of the most advanced forms of TLC, efficient for qualitative and quantitative analysis. Table 3-1 shows HPTLC results of standard flavonoids, alkaloids, phenolic glycoside, and the analyzed fractions.

| Fractions | Mobile phase | Isolated compounds | Reference standard | Rf of compound | Rf in fraction |
|-----------|--------------|-------------------|--------------------|----------------|---------------|
| chA       | M5           | C1                | protopine          | 0.73           | 0.73          |
| B.B       | M4           | S1                | salicin            | 0.29           | 0.28          |
|           | M4           |                   | NeohesperidinN1    | 0.17           | 0.17          |
|           | M5           | S1                | Salicin            | 0.56           | 0.56          |
|           | M5           |                   | Neohesperidin. N1  | 0.60           | 0.60          |

**Figure 1**. HPTLC plate analyzed fraction with reference standard detection under UV light at 256nm (b.b=butanol before hydrolysis fraction, Sst=salicin standard, Nst=neohesperidin standard, S1=isolated compound from b.b fraction) developed in [chloroform : methanol : acetic acid (70:20:10)] solvent system symbolized M4.

**Figure 2**. HPTLC of analyzed fraction and reference standard at 256nm (Pst=protopine standard, Sst=salicin standard, Nst=neohesperidin standard, P1=C1=isolated compound from chA fraction, aq.f=aqueous fraction after butanol hydrolysis, S1=isolated compound from B.B, chA=acid-base purification of chloroform fraction. Developed in [ethyl acetate: acetic acid: formic acid: water (70:10:10:10)] solvent system symbolized M5.
UPLC-ESI-MS/MS for tentative identification of compounds in fractions

Twenty phytochemicals have been putatively characterized by UPLC-ESI-MS/MS in Populus euphratica leaves based on comparison of mass fragmentation pattern, LC retention time and molecular weight with previous study and the literature in phytochemicals mass library such as Respect (http://spectra.psc.riken.jp/menta.cgi/respect/search/keyword?page=1), MONA (http://mona.fiehnlab.ucdavis.edu/), GNPS (https://gnps.ucsd.edu/ProteoSAFe/gnpslibrary.jsp?library=GNPS-NIST14-MATCHES), Melton (https://metlin.scripps.edu/landing_page.php?pagecontent=batch_search), comparing the results of mass fragmentation with the previous study also helpful in confirming the result. Sometime, the precursor ion is not as the molecular weight this is due to adduct formation which is common in ESI–mode especially with phenolic glucoside (11). All the analyses have done, using XEVO TQD triple quadruple instrument (Milford, MA01757 USA, mass spectrometer).

Figure 3. UPLC chromatogram of isolated C1

Figure 4. UPLC chromatogram of isolated S1.
Figure 5. UPLC chromatogram of isolated aq1 compound.

Mass 1 of isolated compounds.

Figure 6. Mass 1 of isolated C1 compound in positive ion mode showing M and M+H
Figure 7. Mass 1 of isolated S1 compound in negative ion mode showing M and M-H.

Figure 8. Mass 1 of predicted S1 in B.B fraction in negative ion mode showing M and M-H without adduct formation.
Figure 9. Mass 1 of isolated aq1 compound in negative ion mode showing M and M-H with adduct formation.

Table 3. UPLC-ESI-MS/MS of the isolated compounds.

| Peak no. | Rt     | Compound sample | M.W | M+H, [M+FA-H], M-H | Ms/Ms fragments               | Ref. |
|----------|--------|-----------------|-----|--------------------|-------------------------------|------|
| 2        | 23.34  | C1              | 353.1| 354                | 148,163,159,188,190,275,247,354 | (12,13) |
| 2        | 2.33   | S1              | 286  | 331²               | 121,123,124,207,93,144        | (14,11) |
| 6        | 7.59   | Aq1             | 424  | 469⁶              | 423,317,316,299,285,155,123,111,121 | (11,16) |
| 2        | 8.72   | N1              | 610  | 609                | 301,300,609,489,283,34,325,7 | (17,18) |

A, b these precursor ions are due to adduct formation presumably arising from combination with the formic acid present in the LC mobile phase which is common. In negative ESI mode especially with phenolic glycoside, the unmodified molecular ion is also present but with small intensity⁴⁴¹. 

Figure 10. UPLC chromatogram of chloroform fraction
Table 4. Identification result by UPLC-ESI-MS/MS fragmentation of chloroform fraction (number in Bold line represent base peak)

| Peak no | Compound name | Compound class | MS/MS fragments | M+H | M.W | Rt |
|---------|---------------|----------------|-----------------|-----|-----|----|
| 20      | Protopine     | Benzylisoquinoline alkaloid | 148,163,159,188,190,273,247,317,354 | 354 | 353.1 | 22.67 |
| 3       | Cheilanthifoline | Benzylisoquinoline alkaloid | 148,324,233,112,178,91,89,165 | 326 | 325 | 8.84 |
| 4       | Solasodine    | Glycoalkaloid    | 119,295,107,109,272,219,344,491,81 | 414 | 413 | 10.78 |
| 2       | B-sitosterol  | Steroids        | 119,295,107,109,272,219,344,491,81 | 415 | 414 | 8.05 |

Reference MS/MS fragments are reported in (310)
Figure 11. UPLC chromatogram of B.B fraction
Continue to Figure 11. UPLC chromatogram of B.B fraction.

Table 5. UPLC-ESI-Ms/MS of B.B fraction.

| Reference | Structure | MS/MS fragments | M-H & [M+FA-H] | MW | Compound class | Compound name | Rt | Peak no. |
|-----------|-----------|-----------------|----------------|----|---------------|---------------|----|---------|
| (30)      |           | 264, 240, 260, 266, 242, 216, 216 | 247 | 448.38 | Flavonoid glycosides | Luteolin 7-O-beta-D-glucoside | 8.42 | 12 |
| (11)      |           | 233, 216, 235 | 45 | 470[M+FA-H] | Phenolic glycosides | Salicortin | 7.75 | 11 |
Phytochemicals in *Populus euphratica* Oliv. leaves extract

| Flavonoid glycosides | Neohesperidin | 8.72 |
|----------------------|--------------|------|
| Phenolic glycosides  | Salicin       | 2.66 |
| Flavonoid O-glycosides | Phloretin-2' O-glucoside | 10.49 |
| Coumarin glycosides  | Scopolin      | 5.44 |

| 301, 300, 609, 89, 283, 33, 114, 286, 280, 273, 314, 274, 167, 353, 354, 191, 191, 176, 179, 133, 37, 325, 148, 255 |
| 267, 121, 123, 124, 207, 93, 331, 332, 286 |

| 477, 315, 477, 478, 286, 285, 243 |
| 267, 121, 123, 124, 207, 93, 331, 332, 286 |

| 37, 325, 148, 255 |
| 267, 121, 123, 124, 207, 93, 331, 332, 286 |

| 301, 300, 609, 89, 283, 33, 114, 286, 280, 273, 314, 274, 167, 353, 354, 191, 191, 176, 179, 133, 37, 325, 148, 255 |
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A,b these precursor ions are due to adduct formation presumably arising from combination with the formic acid present in the LC mobile phase which is common in negative ESI mode especially with phenolic glycoside, the unmodified molecular ion is also present but with small intensity (11).

Figure12. UPLC chromatogram of B.A fraction
| reference | Structure | MS/MS fragments | M-H | MW | Compound class | Compound name | Rt | Peak no. |
|-----------|-----------|-----------------|-----|----|----------------|---------------|----|---------|
| (30)      | ![Structure](image1) | 190,9,178,126,8,84,9 | 353 | 354 | Phenolic acid | Chlorogenic Acid | 2.23 | 3       |
| (38)      | ![Structure](image2) | 137,299,241,138,123,937,85 | 405 | 406 | Phenolic glycoside | Salicyloylsalicin | 13.07 | 33      |
| (39)      | ![Structure](image3) | 191,62,4,173,127,171,85 | 337 | 338 | Phenolic acid | (S)-p-coumaryloyquinic acid | 6.58 | 10      |
| (40)      | ![Structure](image4) | 406,155,527,137,121 | 575 | 528 | Phenolic glycosides | Tremulacin | 12.90 | 30      |
| (41)      | ![Structure](image5) | | | | | | |

Table 6. UPLC-ESI-MS/MS of B.A fraction.
Identification of isolated compounds

**Compound C1:** is isolated from fraction ChA and give positive results with alkaloidal identification results such as Dragendorff’s, Mayer’s, and Hager’s. So it is alkaloid. the UPLC-ESI-MS/MS method is powerful method for identification of phytochemicals it revealed that the molecular weight of this compound is 354 da and fragmentation pattern as in figure 13 during search in various mass libraries such as RESPECT, MONA, GNPS. the only alkaloid that matches both the molecular weight and fragmentation pattern is protopine alkaloid and to get 100% identification conformity protopine standard is compared with the isolated compound in HPTLC so both gave the same rf 0.75 value as in figure 2(12,13).

| Compound | Molecular Weight | Fragmentation Pattern | Identification Results |
|----------|------------------|-----------------------|------------------------|
| NCG00347678 | 311.461,205,189,109,143,163 | Phenypropanoids and polyketides | Dragendorff’s, Mayer’s, and Hager’s |
| 133,122,137,176,148 | 191 | Scopoletin | |

Figure 13. Mass 2 fragmentation of protopine.
The protopine alkaloids can fragment by the RDA reaction, and, it also can undergo another characteristic fragmentation pathway. Selecting protopine as an example, fragment ion at m/z 206.0823 and 149.0603 in the MS/MS spectrum is generated by RDA C ring-opening (Figure 14) but given the presence of hydroxyl groups, the product ion at m/z 336.1209, m/z 188.0721 are probably formed by loss of H2O from the molecular ion and from the m/z 206.0823 ion (27).

Figure 14. MS/MS fragmentation pathway of protopine. (27).

**Compound S1:** is isolated from butanol before hydrolysis fraction this compound gave positive results with KOH so it is glycoside and also gave gray violet color with Vanillin –glacial acetic acid reagent (VGA) this reagent gave gray violet color with salicin and its derivatives (46) the isolated S1 gave the same rf value 0.56 with standard in HPTLC analysis as in figure 2, in UPLC-ESI-MS/MS it gave molecular ion 331 instead of 285 of salicin, this precursor ion (331) is due to adduct formation presumably arising from combination with the formic acid present in the LC mobile phase which is common in negative ESI mode especially with phenolic glycoside [M+FA-H] (11). Compound S1 has displayed the fragment ions at m/z 267, 123 (base peak), 121 and 93 in accordance with the loss of [M–H–18], [M–H–162], and [M–H–164], [M–H–192]. A comparison of these MS/MS ions with the literature, Moreover, this compound has previously been reported in this plant, according to these data S1 is salicin (14) (11), as in fragmentation pattern (Figures 15 and 16).

Figure 15. Mass fragmentation of salicin
Compound **aq1**: it is isolated from aqueous fraction (aqf) it gives positive result with KOH, it is also gave positive result to VGA reagent which confirm that it is from salicin derivatives, HPLC-ESI-MS/MS gave precursor ion 469 and mass fragmentation as that of salicortin and again due to adduct formation with formic acid salicortin (M.W 424 ) had result this molecular ion [M+FA-H] m/z 469 and mass fragment as in figure 17\(^{(11,15)}\).

**Figure 17.** Mass fragmentation pattern of salicortin.

Compound **aq1** has displayed fragmentation ion m/z 423 in the mass1 as in figure 9 due to loss of formate adduct [M-H-HCOOH] [469-46=m/z 423] the fragment ions at m/z 285,155,137,123 and 120 in accord with the loss of [M–H(m/z423) -138]–, [M–H–268]–, [M–H–300]–, [M–H–303]–, A comparison of these MS/MS ions with the literature, Moreover, this compound has previously been reported in this plant, according to these data compound S2 has been identified as salicortin\(^{(47)}\).

**Figure 18.** Fragmentation pathway of salicortin
Phytochemicals identified in hexane fraction of *Populus euphratica* leaves. 
The n-hexane fraction was analyzed using GC-MAS spectroscopy and revealed the following compound as shown below.

Figure 19. GC-MS of n-hexane fraction of *Populus euphratica* leaves

Figure 20. (A1) Mass spec. of peak 1

As shown in the figure 20 complete match of mass spec. between A1 and B1 it indicates the peak 1 is palmitic acid trimethylsilyl ester.
From mass spec. of the figure 21 complete match of mass data between A2 and B2 indicate that peak 3 is Diisooctyl phthalate.

Peak 5 is beta-sitosterol due to the exact mass pattern between A3 and B3 mass charts.
Conclusion

Based on the result the following point may be concluded

1. UPLC-ESI-MS/MS is a powerful method for identification of compounds in mixture base on their molecular weight, retention time and MS/MS fragmentation

2. Twenty compounds have been tentatively identified with this method as in tables 3.8, 3.9 and 3.10

3. protopine alkaloid is identified for the first time in Populus euphratica and genus populus.

4. salicinoids are major compounds in Populus species 4 of them have been identified, salicin, salicortin, salisaoylsalicin, and trumulacin.

5. salicin, neohesperidin, protopine, and salicortin have been isolated from different fractions and identified by various methods.

6. Hexane fraction of leaves of Populus euphratica is rich in beta-sitosterol with 37.14% of total hexane fraction, this may be the cause that extracts of this plant is used traditionally by farmers for various skin conditions like dermatitis and eczema, so the leaves of this tree may be exploited as a rich source for beta-sitosterol

7. spermidine derivatives, zeatin-9-glucoside, and solasodine have been identified for the first time in this plant.

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