Phytochemical investigation And high performance thin layer chromatography (HPTLC) identification of flavonoids and phenolic acids in *Euphorbia cyathophora* (Family: Euphorbiaceae) cultivated in Iraq

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Abstract

The objective of the current work was to investigate the phytochemicals, and to determine the types of phenolic acids and flavonoids in aerial parts of *Euphorbia cyathophora* since no previous work has been done in Iraq. The aerial parts of *Euphorbiacyathophora* were defatted by petroleum ether 40-60°C, then extracted by 80% ethanol using soxhelt apparatus. The hydroalcoholic extract was subjected to preliminary phytochemical analysis. Then Fractionation using chloroform, ethyl acetate, and n. butanol. The ethyl acetate and n. butanol fractions were separately reflexed with 5% HCl for several hours. Fractions (ethyl acetate and n. butanol) before and after hydrolysis were examined by HPTLC (high performance thin-layer chromatography) for their flavonoids and phenolic acids contents. Anthraquinon, cardiac glycosides, alkaloid and steroids were not detected in crude extract. Flavonoids and phenolic acids were detected in analyzed fractions. Quercetrin and rutin respectively were the main identified flavonoids in ethyl acetate and n. butanol fractions before hydrolysis; quercetin was the main detected aglycone in acidic hydrolyzed fractions. Chlorogenic acid and caffeic acid only detected in n. butanol fractions.

Key words: *Euphorbia cyathophora*, phytochemical investigation, quercetin, rutin


Introduction

Natural phenolics are the most abundant secondary metabolites containing benzene rings, with one or more hydroxyl substituents, these phytochemicals ranging from simple phenolic compounds (phenolic acids) to complex high molecular weight polymers (tannins). Biosynthetically phenolics originated from shikimate/ phenylpropanoid pathway, or from 'polyketide’ acetate/malonate pathway. The merging of both pathways leads to flavonoids

These phytochemicals are divided into several groups based on the number of phenol rings and to the structural components that bind these rings to one another, the main groups of phenolics are flavonoids and non-flavonoids which include (phenolic acids, tannins, stilbenes and lignans)⁵. Stilbenes and lignans are less common than the other groups ⁶. Natural phenolics from dietary plants and medicinal herbs exert various activities including anticancer ⁷, antioxidant ⁸, anti-Inflammatory, antimicrobial, anti-aging¹⁰,¹¹, immuno-modulatory¹², antidiabetic activity¹³, phytotoxic, antifungal, nematicidal, insecticidal, antihypertensive ¹⁴,¹⁵, hypolipidemic and other activities ¹⁶.

Fire on the mountain is the common name of *Euphorbia cyathophora* = *Euphorbia cotinifolia* L. (Family: Euphorbiaceae) ¹⁷, owing to the yellow and red batches at the base of leaves and bracts that look like fire when observed from distance ¹⁸. It is annual, stem standing usually branched up to 1 meter height ¹⁹, and grown as ornamental especially in US, in Brazil the red blotched leaves are used as dye²⁰. Juice from leaves used for lactation in women ²¹,²², and it is applied externally to relieve pain of scorpion bite²³. In folk medicine the latex is used for management of irritation caused by other species of euphorbia²⁴. Pharmacologically *E. cyathophora* reported to have antioxidant and hepatoprotective²⁵, antibacterial, antifungal, antidiarrheal, antidiabetic effects ²⁶-²⁸.

The objective of this study was to investigate the phytochemicals and to determine the types of phenolic acids and flavonoids in the aerial parts of *Euphorbia cyathophora* cultivated in Iraq since no previous work has been accomplished in Iraq.

**Figure 1: Euphorbia cyathophora**

**Materials and methods**

**Collection and authentication**

The aerial parts of *Euphorbia cyathophora* were collected from the garden of medicinal plants in College of Pharmacy /University of Baghdad, authenticated by Dr Sokaena Abassin College of Science/ University of Baghdad. The desired parts of the plant were shade dried at room temperature for about two weeks, grind to a fine powder with the aid of electrical grinder.
**Equipment and chemical**

The equipment used was rotary evaporator (BUCHI Rotavapor R-205, Swiss) and high-performance thin-layer chromatography (HPTLC) (Eike Reich/CAMAG-Laboratory, Switzerland). The used solvents and chemicals were of analytical grade and acquired from Riedel-de Haen, Germany. The standards (rutin, quercetin, chlorogenic acid, caffeic acid, p-coumaric acid, quercetin, kaempferol, luteolin, apigenin, isorhamnetin) were purchased from Chengdu Biopurify Phytochemicals, China (purity >97). Silica gel GF$_{254}$ TLC plates used were obtained from E. Merck Ltd., India.

**Extraction of flavonoids and phenolic acids**

The powdered plant (75 g) was defatted in (750 ml of petroleum ether 40-60˚C) in soxhelt apparatus (successive hot continuous extraction) for 12 hours until complete extraction. The defatted plant material was dried to ensure complete evaporation of defatting solvent then extracted using (750 ml) 80% ethanol for 12 hours using soxhelt$_{29}$. The extract was filtered and the filtrate was evaporated under reduced pressure to obtain dry residue. The hydroalcoholic residue was suspended in water (60 ml), successively extracted with organic solvents of different polarities such as chloroform, ethyl acetate and n. butanol (60 ml for each three times). The first two extracts (chloroform and ethyl acetate) were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under vacuum. 0.5 g of both ethyl acetate and n. butanol fractions were hydrolyzed separately by refluxing with 25 ml of 5% hydrochloric acid for 8 hours, cooled and extracted with 25 ml x 2 ethyl acetate. The organic layers were collected together, dried over anhydrous sodium sulfate, filtered, and evaporated till dryness$_{30}$. The crude hydroalcoholic extract was analyzed for its phytochemical constituents. The fractions ethyl acetate and n. butanol before and after hydrolysis were subjected to HPTLC for their phenolics (flavonoids and phenolic acids) profile.

**Preliminary phytochemical investigation**

The hydroalcoholic extract was subjected to preliminary qualitative investigations to identify the various phytoconstituents in aerial parts of plant.

1. Test for flavonoids: few drops of dilute NaOH solution was added to a test tube contain 1 ml of crude extract. An intense yellow color was appeared, which became colorless upon the addition of few drops of dilute acid that indicated the presence of flavonoids.

2. Test for saponins: (1 ml) of stock solution was taken in a test tube and diluted with 20 ml of distilled water, shaken for 15 min by hand. Foam was formed on the top of the test tube. This layer indicates the presence of saponin$_{31}$.

3. Test for phenolic acids: about 10 ml of distilled water was added to a test tube contains about 0.5 g of crude extract then stirred and filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate development of a blue-black, green or blue-green indicates the presence of phenols.

4. Test for steroids: To 0.2 g of crude extract, 2 ml of acetic acid was added, the solution was cooled well in ice, then conc. H$_2$SO$_4$ was added carefully. The steroidal ring is indicated by color change from violet to blue or bluish-green$_{32}$.
5. Test for alkaloids: To a test tube contains 2 mg of extract few drops of the Mayer’s reagent, were added. The presence of alkaloids is indicated by formation of white or pale yellow precipitate.

6. Test for condensed tannins (phlobatannins test): to a test tube, contains around 3 ml of extract 3 ml of 1% HCl was added and the mixture was boiled. The presence of phlobatannins is conformed through the deposition of a red precipitate.

7. Test for anthraquinone glycosides: to a test tube containing dried extract (few milligrams), 2 ml of 5% HCl solution was added and heated for about 5-10 minutes, and filtered while hot, to the filtrate add 2 ml of chloroform, and 1 ml ammonia solution, shake well, after few minutes the aqueous layer acquired rose pink color which indicate the presence of anthraquinone glycosides.

8. Test for cardioactive glycosides: Keller-Kiliani Test: to a test tube, about 2 ml plant extract, 2 ml glacial acetic acid, one drop of 5% FeCl₃ were added, then conc. H₂SO₄ added on the wall of the test tube. Reddish brown ring appears at the interphase and upper layer appears bluish green which indicate the presence of glycosides.

9. Test for cardioactive glycosides Baljet test: to a test tube containing 2 ml of extract, 2 ml of sodium picrate was added. The presence of cardineolides was conformed through the transformation of sodium picrate yellow color to orange.

10. Test for reducing sugar (Benedict’s test): 2ml of extract was mixed with 2 ml of Benedict’s reagent and heated gently. Birke red precipitate shows the existence of reducing sugars. This test was done for extract before and after hydrolysis with dilute hydrochloric acid.

Preparation of the fractions and standard material for analysis

The assigned fractions (ethyl acetate and n. butanol fractions before and after hydrolysis) were prepared by dissolving 2 mg of each fraction in 2 ml of absolute methanol. Standard materials (rutin, quercetrin, chlorogenic acid, caffeic acid, p-coumaric acid, quercetin, kaempferol, luteolin, apigenin, isorahmnetin) were prepared by solubilizing 0.5 mg of each standard in 0.5 ml of absolute methanol.

HPTLC analysis

The phenolic profile for the mentioned fractions was analyzed by HPTLC using Eike Reich/CAMAG–Laborator. 10× 20 cm Silica gel TLC plates, which was loaded with standards and samples (2 µl of each) via a Hamilton syringe and CamagLinomat 5 instruments. The TLC plate was developed in automatic developing chamber (ADC, CAMAG), followed by pre-conditioning of the plate with 10 ml of a mobile phase composed from ethyl acetate: formic acid: acetic acid: water (50:3:3:4). The plate was developed in the mobile phase up to 75 mm, hot air was used to evaporate solvent from the developed plate. The plate was kept in Photo-documentation chamber and the images at white light and under UV light (254 nm and 366 nm) were captured.

Result and discussion

The protection against diverse metabolic conditions and diseases had been attributed to phytochemicals. Preliminary examination confirm the presence of flavonoids, saponins, deoxy sugar and phenolic acids, while
alkaloids\textsuperscript{27}, steroids, anthraquinone and condensed tannins had not been identified. The basic structures of cardioactive glycosides have steroidal nucleus, sugar moieties and lactone 2-furanone or lactone 2-pyrone\textsuperscript{41}. Steroids were not identified in extract, so cardioactive glycosides are absent in \textit{Euphorbia cyathophora}\textsuperscript{extract.} The reducing sugar identified in the extract only after hydrolysis. The results for preliminary phytochemical screening of crude aerial part extract are shown in table 1.

Table 1-Phytochemical screening of \textit{Euphorbia cyathophora}\textsuperscript{crude extract}

<table>
<thead>
<tr>
<th>phytochemical</th>
<th>Accomplished test</th>
<th>Observation</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>alkaline reagent test</td>
<td>yellow color observed</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>foam test</td>
<td>foam is formed</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>ferric chloride test</td>
<td>blue-black color observed</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>Lieberman’s test</td>
<td>color not change</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>Mayer’s test</td>
<td>white precipitate not formed</td>
<td>-</td>
</tr>
<tr>
<td>Condensed tannins</td>
<td>phlobatannins test</td>
<td>red precipitate not formed</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinone glycosides</td>
<td>Borntrager's test</td>
<td>pink color not formed</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Keller-Kiliani test</td>
<td>brown ring at the interphase</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Baljet test</td>
<td>orange color</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugar before hydrolysis</td>
<td>Benedict’s test</td>
<td>brick red precipitate not formed</td>
<td>-</td>
</tr>
<tr>
<td>Reducing sugar after hydrolysis</td>
<td>Benedict’s test</td>
<td>brick red precipitate formed</td>
<td>+</td>
</tr>
</tbody>
</table>

Regarding HPTLC analysis, scanning under the two designated wavelengths manifest the presence of several peaks of discrete compounds. Ethyl acetate and n. butanol fractions before hydrolysis had the highest number of separated components, 14 and 13 separated spots with \( R_f \) values ranging from 0.03-0.92, and 0.02-0.87 respectively, while in fractions after hydrolysis 5 and 4 discrete spots were detected with \( R_f \) values ranging from 0.02-0.79 for both ethyl acetate and n. butanol fractions successively. Based on \( R_f \) values of standard materials only rutin, quercitrin, caffeic acid, chlorogenic acid and quercetin were recognized. Results for \( R_f \) values of standards, identified flavonoids and phenolic acids in ethyl acetate and n. butanol fractions of are shown in table 2 and 3.

Table 2-\( R_f \) values of standard materials. Flavonoids detected in ethyl acetate fractions.

<table>
<thead>
<tr>
<th>Standard compounds</th>
<th>( R_f ) value</th>
<th>EtOAc fraction before hydrolysis</th>
<th>EtOAc fraction after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>0.14</td>
<td>Rutin</td>
<td>quercetin</td>
</tr>
<tr>
<td>Quercitrin</td>
<td>0.50</td>
<td>Quercetin</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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In TLC or HPTLC flavonoids detection is commonly accomplished under the UV light at 254 nm (short wave length), were all phenolics as flavonoids give rise to fluorescence quenching or at 366 nm (long wave length), were the flavonoids based on their structural type, demonstrate dark yellow, green, or blue fluorescence. Quercetin, apigenin and p-coumaric acid have the same $R_f$ value in the solvent system used for separation, similar fluorescent quenching at 254 nm, but different quenching at 366 nm. P-coumaric acid standard observed as blue fluorescent spot at 336 nm, apigenin as dark opaque spot, queretin appear as yellow spot, chlorogenic acid, and caffeic acid appear as blue spots.

| Kaempferol | 0.82 |
| Luteolin | 0.77 |
| Apigenin | 0.79 |
| Isorhamnetin | 0.81 |

EtOAc: Ethyl acetate

HPTLC chromatograms showing max retardation factor values (Max $R_f$) of standard flavonoids, phenolic acids and analyzed fractions are shown in figure 2. The HPTLC plate of tested fractions and standard materials are shown in figure 3.
Standard apigenin Standard isorhamnetin``

Ethyl acetate fraction before hydrolysis

Ethyl acetate fraction after hydrolysis
n. butanol fraction before hydrolysis

n. butanol fraction after hydrolysis

Figure 2-HPTLC chromatograms showing max retardation factor values (Max Rf) of standard flavonoids, phenolic acids and analyzed fractions.
Figure 3- (a, b and c) HPTLC plates of analyzed fractions with reference standards, detection under UV light at 254 nm, 366 nm and at white light (1: rutin, 2: quercetrin, 3: chlorogenic acid, 4: caffeic acid, 5: p-coumaric acid, 6: quercetin, 7: kaempferol, 8: luteolin, 9: apigenin, 10: isorhamnetin, 11: ethyl acetate fraction before hydrolysis, 12: n. butanol fraction before hydrolysis, 13: ethyl acetate fraction after hydrolysis, 14: n. butanol fraction after hydrolysis.

In HPTLC in unhydrolyzed ethyl acetate fraction before, the % area were 5.99 and 9.91 for identified rutin and quercitrin respectively. In n. butanol fraction before hydrolysis, the % area were 13.67 and 3.17 for identified rutin and quercetin respectively. So quercetrin and rutin were the major identified constituents in ethyl acetate an n. butanol fractions before hydrolysis. This could be attributed to the effect of solvent polarity on which will affect the type and the amount of the extracted compounds. In hydrolyzed fraction the % area for identified quercetin were 88.63 and 58.5 respectively, consequently quercetin (flavonolaglycone) was the main separated constituent in these fractions. This could be attributed to the hydrolysis of rutin (quercetin-3-rutinoside) and other unidentified quercetin glycosides as hyperoside and the biflavones as amentoflavone (bis-apigenin) which might present in extract. Quercetin and its glycosides structures are demonstrated in figure 4.
Figure 4- Chemical structures of rutin, quercitrin and quercetin.

Caffeic acid detected in n. butanol fraction before and after hydrolysis, which reflect its presence in free and conjugated form as chlorogenic acid (an ester of caffeic acid with quinic acid) and other esters. The chemical structures of these acids are demonstrated in figure 5.

In n. butanol fraction before hydrolysis, the % area of chlorogenic acid was 16.52 while for caffeic acid % area was 4.97, since free form of phenolic acids exist in minor fraction and the vast majority exist in conjugate forms. Caffeic acid result from hydrolysis of chlorogenic acid and other derivatives which might present in n. butanol fraction before hydrolysis.

Figure 5- Chemical structures of quinic, chlorogenic acid and caffeic acid.

**Conclusion**

Quercetin and rutin were the main identified flavonoid glycosides in ethyl acetate and n. butanole fractions before hydrolysis, while quercetin was the main detected aglycone in acid hydrolyzed fractions. Chlorogenic acid detected only in n. butanole fraction before hydrolysis, caffeic acid detected only in n. butanol fractions. Additional identification techniques are required for conforming the detected phytochemicals plus quantitative estimation since this plant might be considered as a valuable source of quercetin.
Conflict of interest

The authors declare no conflict of interest.

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