RESEARCH ARTICLE

Isolation of Cardioactive Glycoside Peruvoside, and Phytoalexin Scopoletin along with Phytochemical Investigation of *Euphorbia Milii* Cultivated in Iraq

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ABSTRACT

Euphorbia milii is an ornamental and medicinal plant species used widely in folk medicine in the treatment of cancer and hepatitis in China, Nepal, Brazil, and other tropical areas. Various research reported many pharmacological properties for this species related to its phytoconstituents. This study will aim to provide a complete phytochemical profile for *E. milii* cultivated in Iraq. The methanolic crude extract was partitioned using different solvents with varying polarities for further analysis. The phytochemical analysis revealed cardiac glycosides, coumarins, flavonoids, phenols, sterols, terpenoids, and alkaloids. In the scope of this study, the compounds isolated from *E. milii* in this study are Peruvoside and Scopoletin. These compounds were detected and isolated by analytical thin-layer chromatography and preparative layer chromatography, respectively. The isolated compounds were identified by high-performance liquid chromatography (HPLC) with the corresponding standard, and the spectral method fourier transforms infrared spectra (FTIR) further confirmed the result. This study is the first to isolate the cardioactive glycoside Peruvoside from *E. milii*. In addition, this study is considered the first to detect and isolate the phytoalexin coumarin Scopoletin from Iraqi *E. milii*, highlighting the role of Iraqi flora in provoking secondary metabolites in the plant species. This suggests that *E. milii* is a good source of novel bioactive compound isolation. Furthermore, the compounds isolated were reported to have hepatoprotective, anti-cancer, and many pharmacological potentials that can justify its traditional use, which must be studied in future *in-vitro* studies as a part of drug discovery and development.

Keywords: *Euphorbia Milii*, Glycoside Peruvoside, Health, Therapy Treatment.

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INTRODUCTION

Drug discovery and development of new chemical entities having medical and pharmacological potential is a constant global process to treat diseases. Medicinal plants have always been a rich source for novel compounds or phytochemicals having medicinal importance; they have had historical contributions to new drug discovery and development. Medications derived from naturally based compounds are countless; one-third of approved drugs are of plant origin and natural sources.[1] *E. milii* from the spurge family (Euphorbiaceae) is a plant species from the widely distributed medicinal genus *Euphorbia*. It is commonly known as the "crown of thorns" due to its characteristic spiny stems. As a part of the spurge family, *E. milii* has the succulent property of producing a milky latex that has much biochemical and industrial significance.[2] *E. milii* is native to Madagascar and is widely distributed in tropical areas like western China, India, Nigeria, Brazil, Nepal, and Thailand,[3-5] and recently cultivated in Iraq. Its popularity rises as an ornamental plant and as folk medicine for curing diseases. It was anciently used in China to treat cancer and hepatitis,[6] in other tropical areas like Brazil and Nepal. It is widely used to treat warts, skin peeling and as an anti-inflammatory in eyesores and abscess.[7,8] Many studies performed the phytochemical analysis of the crude extract of *E. milii* reporting various phytochemical classes, and it was found that this species is rich in flavonoids, polyphenolics, and diterpenoids. Studies have also detected alkaloids, cardiac glycosides, anthocyanins, coumarins, saponins, and tannins as a part of the preliminary Phyto analysis of the crude extract of the plant.[4,9,10] Previous literature also reported that *E. milii* had Antinociceptive, analgesic,[5] anti-bacterial,[11,12] antioxidant[13-15] antiviral[16] and anti-cancer[17] properties based on analytical and *in-vitro* procedures. It was also found that *E. milii* was rich in Rosane type diterpenoids which gave it its osteoclast genesis inhibitor property that can aid in degenerative bone diseases and cancer therapy.[18] The previous research results mainly focused on the analytical

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and descriptive aspect of *E. milii* however, the pure isolation of specific compounds in chromatographic techniques are still limited and lacking. Therefore, the main objective of this study is to provide a clear phytochemical profile of *E. milii* in Iraq and to isolate compounds that are relevant to its medicinal folkloric uses.

Interestingly, Peruvoside has been detected in *E. milii* crude extract in previous literature,[13] and isolated from yellow oleander (*Thevetia peruviana*) is a cardiac glycoside that has been used in vivo as an alternative for digoxin in the treatment of congestive heart failure. Peruvoside showed an improved toxicity profile and GIT absorption than digoxin.[19] Furthermore, Peruvoside anti-cancer properties have also been evaluated and studies reported that it exhibited an apoptotic action by intrinsic mechanism, arresting the cycle on in the G2/M phase in an in-vitro study.[20] Many studies also revealed the antiproliferative potential of peruvoside in breast and lung cancer.[21] The latter indicates that Peruvoside requires more sufficient toxicological data to support its industrial production as a drug for CHF and further anti-cancer study due to the promising anti-cancer potentials.

Consequently, the isolation of this cardiac glycoside from *E. milii* is the first aim in this study. On the other hand, Scopoletin is a phytoalexin coumarin which is a secondary metabolite produced only as a result of mechanical stress and is one of the phytoconstituents found in vinegar well as other medicinal plants. Studies investigating the potential medicinal activities of Scopoletin reported hypoglycemic, anticonvulsant, and anti-asthmatic properties. In addition, its role in the modeling of hypo and hyper thyroiditis was evaluated and was indicated as a good antioxidant and hepatoprotective agent.[22,23] The latter studies indicate that Scopoletin is considered as a novel drug for future in vitro pharmacologic and toxicological research. Thus, this study will aim to explore the presence of this phytoalexin secondary metabolite in the Iraqi *E. milii*, to show the effect of Iraqi flora and environment in aggravating additional secondary metabolites.

MATERIALS AND METHODS

Plant Material

E. milii whole plant was collected in July 2020 as supplied from Baghdad greenhouses. The plant was identified and authenticated by assistant professor Dr. Sukaena Abbas from the Department of Biology, College of Science, University of Baghdad. All plant parts, including roots, stems, thorns, leaves, and flowers, were washed and air-dried for 14 days. The dried plant material was crushed and grinded with the aid of an electric grinder together to form a coarse powder.

Extraction

Four hundred grams (400 gm) of plant material was defatted using hexane for 24 hours. The plant material was filtered to be ready for extraction. The powdered plant material was extracted by Soxhlet apparatus using 7.3 L of 85% methanol for 12 hours. The extract was then filtered using Whatman's filter paper to remove boiling chips out of the extract.

Cardioactive Glycoside Extraction

Five grams of crude extract from cold and hot methods was placed in a conical flask with the addition of 85% aqueous methanol. A total of 10% Lead acetate solution was added to the methanolic extract, which was then centrifuged for 5 minutes to remove precipitated materials. The supernatant was taken in a conical flask with a 10% sodium phosphate solution; it was then centrifuged again for 5 minutes. The supernatant was then partitioned with chloroform: methanol (3:1) solution to extract the whole glycoside. The lower (organic) layer was collected for the identification and detection of cardiac glycoside presence. The organic layer is designated as (CG-1) to detect the suspected cardiac glycoside by further analysis.[24]

Fractionation and Solvent Extraction of *E. milii* Crude Extracts

Forty grams of crude extract from hot method was suspended in 200 mL distilled water and partitioned with 200 mL of chloroform, ethyl acetate for three times (200 mL × 3). Anhydrous sodium sulphate was added, filtered and each fraction was evaporated to dryness.

Preliminary Phytochemical Analysis on Crude Extracts and Fractions

Phytochemical screening was done on crude methanolic extract and on the chloroform fraction by performing the following tests to identify chemical compounds and different phytochemicals. This facilitates the identification step and allows selective guide to the following procedures since each chemical compound can vary and require more precise separation and detection methods.

Test for Alkaloids

From each extract, 2 mL was placed separately in a test tube and treated with few drops of Mayer's and Dragendorff reagents, and then each test tube was shaken. The formation of white and orange precipitate for Mayer's and Dragendorff's test respectively indicated a positive result for alkaloid.[25]

Test for Cardioactive Glycoside

Keller–Kiliani test was performed. 5 mL of each extract was mixed with 2 mL of glacial acetic acid containing one drop of 5% ferric chloride ($FeCl_3$) solution then 1 mL of concentrated sulfuric acid was added. Brown ring formed at the interface of 2 layers indicates the presence of deoxy sugar of cardenolides.[26]

Test for Flavonoids

From each, 1 mL was placed in a test tube, and few drops of dilute NaOH solution were added. An intense yellow color appears in the test tube, followed by the addition of a few drops of dilute HCl, where the yellow color becomes colorless. The latter is considered positive for the presence of flavonoids.[27]

Test for Phenolic Compounds

Lead acetate test: A 2-mL of each extract was placed in a test tube then few drops of lead acetate solution were added.

Formation of a white precipitate indicates the presence of phenolic compounds (tannins).[28]

Test for Anthraquinones

Borntrager's Test: A total of 3 mL of each extract placed in a test tube where diluted HCl solution was added then placed into a separatory funnel, and an equal volume of benzene is added. The solution was shaken well, and the organic layer (upper layer) was separated. An equal volume of dilute ammonia solution was added to the organic layer. Pink color in the ammonia layer indicated the presence of anthraquinone glycosides.[29]

Test for Coumarins

NaOH test: A total of 10% NaOH was added to each extract, then chloroform was added. Formation of yellow color shows the presence of coumarin.[28]

Test for Saponins

Foam Test: A total of 1-mL of each extract 20 mL distilled water was added, then shaken well in a measuring cylinder. The formation of froth that persists for 15 minutes indicates the presence of saponins.[29]

Test for Terpenoids

Salkowski test: A total of 5 mL of methanolic extracts were mixed in 2 mL of chloroform followed by the careful addition of 3 mL concentrated H_2SO_4. A layer of the reddish-brown coloration formed at the interface indicates a positive result for the presence of terpenoids.[28]

Test for Sterols

Liebermann-Burchard test: A total of 2 mL of acetic anhydride added to 0.5mL of plant methanolic crude extract with 2 mL H_2SO_4. The change in color from violet to blue or green in samples indicates the presence of steroids.[28]

Phytochemical Screening of Cardioactive Glycosides in CG-1

Specific chemical tests were carried out for CG-1 to identify the presence of cardioactive glycoside in these fractions. Keller–Kiliani test and Liebermann-Burchard test as mentioned above, were performed to identify deoxy sugar and sterol nucleus presence, respectively. In addition, a legal test was performed on CG-1 since it was used in previous studies to aid in identifying the type of cardiac glycoside present in *E. milii* as reported in the literature.[30,13] In the legal test, 2 mL of pyridine and sodium nitroprusside solution were added to each extract, followed by the addition of NaOH solution to make it alkaline. The appearance of red color indicates a positive result for this test.[31]

Examination and Separation

Thin-layer Chromatography (TLC)

The TLC was performed to analyze CG-1 and chloroform fraction to identify the presence of cardiac glycoside and coumarin Scopoletin, respectively, as part of the scope of this study. Readymade silica gel plates GF254 nm (20 x 20 cm) of 0.25 mm thickness (MERCK) were used as the stationary phase. The plates were activated at 110°C for 30 minutes prior to use. The solvent systems used in the aim to detect cardiac glycoside in CG-1 are Chloroform: methanol (80:20)[19] and Hexane: ethyl acetate (50:50).[32] for coumarin Scopoletin detection in chloroform fraction. The developing Solvent system was freshly prepared and then placed in a glass tank (22.5 cm × 22 cm × 7 cm) and covered tightly with a glass lid to ensure proper saturation in the tank before introducing the TLC plates. In addition, the glass tank was lined with filter paper (Whatman No. 2) to allow a better saturation process. Few milligrams of each sample were dissolved in few drops of absolute methanol, then applied on the baseline of TLC plates using a capillary tube and allowed to dry. Detection of the different separated components was done by examination under UV light utilizing 366 nm wavelength. The spots were marked with a pencil for each compound as evident from the florescent spots under UV or colored spots, then the R_f value (retention factor) for that compound as well as the standard used was calculated for further comparison:

$$Rf\ value = \frac{distance\ traveled\ by\ compound}{distance\ traveled\ by\ the\ solvent\ system}$$

Preparative Layer Chromatography

Readymade silica gel plates GF254 supplied by the Ministry of science and technology/Iraq having 0.75 mm thickness were used to isolate compounds that are suspected to be present when compared with the standard compounds on the analytical TLC plates for further identification and analysis. The plates were activated as they were placed in a heated oven at 110°C for one hour then left to cool to be ready for a sample application. The fraction was applied as concentrated solutions in streaks to form a plate using a capillary tube. The band was left to dry at room temperature and introduced into 20 × 20 glass tank saturated with the freshly prepared mobile phase used in the analytical TLC. After development, the separated bands were detected under UV light. The selected bands were each allocated using a needle and then scrapped out of the plate using a thin spatula. Each band was collected into a clean and dry beaker where enough volume of absolute methanol was added, stirred well, and shaken on a warm water bath. Each solution of separated bands was filtered using double filter paper. For further purification, each solution of the isolated bands was filtered by passing it through a disposable 2.5 µm filter.

Identification of Isolated Compounds

Identification by High-performance Liquid Chromatography (HPLC) for the Isolated Compounds

HPLC was used for the accurate and precise identification of the isolated compounds. HPLC was carried out at the Ministry of Science and Technology, Iraq; the identification was done by comparing the retention times (RT) obtained at specific chromatographic conditions of the analyzed samples and standards. For identification of the isolated compound from CG-1 by HPLC the solvent system used was A: 0.1% acetic acid in phosphate buffer 0.01 M, B: 0.1 % acetonitrile in acetic acid (Linear gradient program (A 100% >> 0% and B 0% >> 100%))

and C18 (3 µm 50 cm × 2.0 Id mm) HPLC column. While for the isolated coumarin was 1% formic acid: acetonitrile (10:90) and C18 (5 µm 25 cm × 4.6 mm) HPLC column. This HPLC analysis was carried out for the isolated compounds along with the proposed standard compounds Peruvoside and Scopoletin.

Identification by Fourier Transform Infrared Spectroscopy (FTIR)

Using FTIR, functional groups of the isolated compound were detected by assigning the chemical vibrations and bands in the spectrum to the characteristic functional group of the chemical compound structure. FTIR analyzed the isolated compounds at the Ministry of Water and Minerals, Iraq, where peaks are assigned to the functional groups accordingly.

RESULTS AND DISCUSSION

Preliminary Phytochemical Profile

The methanolic crude extract yielded 70 gm, while chloroform fraction from the 40gm crude partitioned with chloroform produced 3 gm. Phytochemical screening done on both crude and chloroform solvent extracts demonstrated the results illustrated in Table 1. At the same time, the specific screening on CG-1 gave the results in Table 1.

The chemical profiling of methanolic crude extract and chloroform solvent extract of the Iraqi Euphorbia milii whole plant was identical, as seen in Table 2, suggesting that chloroform could extract almost the same phytochemical classes the methanol. Phytochemical screening revealed that flavonoids, phenolic compounds (tannins), cardiac glycosides, alkaloids, coumarins, phytosterols, and terpenoids complemented the international phytochemical profile of *E. milii*. However, although alkaloids were present in crude and solvent extract of Iraqi grown *E. milii*, it was absent in some phytochemical assays carried out on the plant grown in Pakistan. On the other hand, CG-1 fraction from which the cardioactive glycoside is detected in gave positive results in the legal test, Liebermann Burchard's test, and a negative result with killer killiani test as seen in Table 1, indicating the presence of a cardioactive glycoside with a steroidal nucleus and a non-deoxy ribose sugar which matches the structural features of Peruvoside.

Table 1: Phytochemical test results for CG-1 to detect cardioactive glycosides.

Test	*CG-1*	*CG-2*
Legal	+	+
Keller-Kiliani	-	-
Liebermann-Burchard	+	+

(-): negative, (+): positive

Table 2: Phytochemical screening profile of methanolic crude extract and chloroform solvent extract of *E. milii* cultivated in Iraq.

Test	*Methanolic crude*	*Chloroform solvent extract*
Alkaloid (Dragendroff /Mayer)	+	+
Cardiac glycosides	+	+
Flavonoids	+	+
Phenols	+	+
Anthraquinone	-	-
Coumarins	+	+
Saponins	-	-
Terpenoids (Salkowski)	+	+
Steroids (Lieberman Burchard)	+	+

(–): Absent, (+): Present

Thin-layer Chromatography (TLC)

TLC for Cardiac Glycoside Detection in CG-1

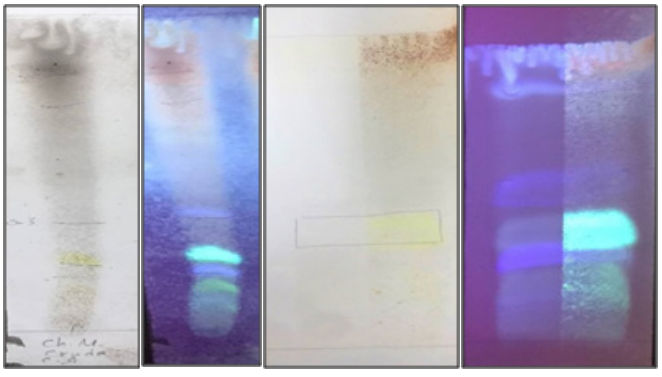
The solvent system chloroform: methanol (80:20) was used to examine and detect cardiac glycoside in CG-1. The TLC chromatogram of CG-1 development is shown in Figure 1. After spraying the plate with concentrated H_2SO_4, the cardiac glycoside was detected, a visible lemon-yellow spot appeared, and a florescent spot was detected under UV -366 nm. For better identification, CG-1 was applied as a band as seen in Figure 1. The latter is a characteristic of Peruvoside cardiac glycoside, as mentioned in the literature.[19,30]

TLC for Coumarin Detection in Chloroform Fractions

Using hexane: ethyl acetate (50:50) solvent system, the chloroform fraction was developed on TLC plate with Scopoletin standard. The fraction gave a spot with R_f value identical to that of Scopoletin standard (Rf 0.36 for standard Scopoletin and the corresponding spot) as in Figure 2. Scopoletin is a phytoalexin coumarin[22] that has never been detected in *E. milii* species; this is remarkably interesting as it suggests how Iraqi flora could influence the secondary metabolite content within the same species.

Preparative TLC of CG1 Fraction

The preparative TLC of CG-1 fraction using the same solvent system was used for its development by TLC and led to the isolation of cardiac glycoside (CG1-P). Which was detected by spraying a small portion of the plate with concentrated H_2SO_4,



Figures 1 A and B :TLC chromatogram of CG-1 developed in chloroform: methanol (80:20) solvent system detected visibly and under UV-366nm after spraying with H_2SO_4, respectively for CG-1. C and D : show CG-1 applied as a band and detected visibly and under UV-366nm after spraying with H_2SO_4, respectively.

which produced the lemon-yellow color band that was then visualized under UV-366nm.

Preparative TLC of Chloroform Fraction

The compound isolated from chloroform fraction is denoted as (S). This fraction developed in hexane: ethyl acetate (50:50) solvent system showed a uniform and clear spot detected under UV-366 nm when with Scopoletin standard. The creamy white powder is sent for further analysis.

Identification of Isolated Cardiac Glycoside CG1-P

The isolated CG1-P was identified primarily by HPLC with the related standard, spectral FTIR was carried out, and the subsequent vibrations and bands were compared to that in literature.

Identification of CG1-P by High-performance Liquid Chromatography (HPLC)

The isolated cardiac glycoside CG1-P was analyzed by HPLC with the suspected cardiac glycoside standard compound and was identified as the cardiac glycoside Peruvoside since the retention times (RT) obtained from HPLC data were very comparable (RT: 4.39 minutes for Peruvoside standard and 4.41 minutes for the isolated CG-1P), confirming that the CG1-P is Peruvoside. The HPLC chromatograms of CG1-P and Peruvoside standard are shown in Figure 3.

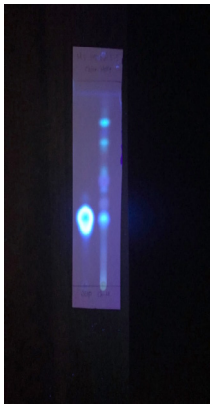


Figure 2: TLC chromatogram for chloroform fraction developed in hexane: ethyl acetate (50:50) solvent system with Scopoletin standard.

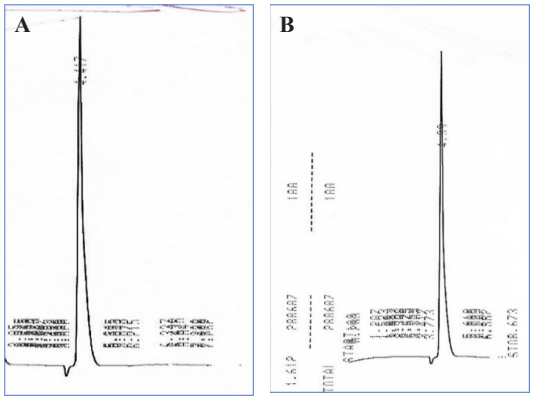


Figure 3: HPLC chromatogram of standard Peruvoside (A) and CG1-P (B).

Identification of CG1-P by FTIR

The FTIR spectrum was obtained for the isolated yellowish-white powder CG1-P cardiac glycoside from CG-1 which is identified as Peruvoside cardiac glycoside. Table 3 illustrates the bands and vibrations of the isolated Peruvoside and compares these bands with those reported for Peruvoside in previous studies.[33] Each band was assigned to its functional group as shown in Table 3.

From the latter results, we conclude that Peruvoside is the isolated cardiac glycoside from *E. milii* as detected in previous studies.[13] Peruvoside is a cardiac glycoside isolated from yellow oleander (*Thevetia peruviana*), and this study is considered the first to isolate Peruvoside from *E. milii*.

Identification of Isolated Compound S

The isolated compound S was identified using HPLC and FTIR methods and showed the following results.

Identification of isolated compound S by HPLC

The isolated compound S was predicted as the coumarin Scopoletin according to the results obtained by analytical TLC, where the calculated R_f values and the spot color under UV matched to the Scopoletin standard. However, precise confirmation that the isolated coumarin is Scopoletin was done by HPLC analysis of the isolated S with Scopoletin standard. The retention times were remarkably close and comparable (RT: 7.967 minutes for Scopoletin standard and 7.303 minutes for the isolated S), confirming that this coumarin S is Scopoletin. The HPLC chromatograms are shown in Figure 4.

Identification of isolated compound S by FTIR

FTIR further analyzed the isolated Scopoletin coumarin. The FTIR spectrum gave the bands illustrated in Table 4, the illustrated bands and vibrations of the isolated Scopoletin assigned according to the functional group range of Scopoletin.

The detection and isolation of Scopoletin from *E. milii* is considered the first in research. Scopoletin is a phytoalexin coumarin which is a secondary metabolite produced only as a result of mechanical stress and is one of the phytoconstituents found in vinegar and other medicinal plants.

Table 3: Illustrates the FTIR vibrations of CG-1P and Peruvoside as described in the literature with the assignment to their related functional group.

Functional Group	*FTIR bands for isolated CG1-P*	*FTIR bands for Peruvoside in previous studies*[33]
O-H stretching	3390.86	3448
C-H symmetrical stretching	2920.23	2940
C=O stretching of alpha -beta lactone ring	1732.08	1730
C=C double bond stretching	1647.21 1577.77	1636 1595
O-H bending	1465.90	Not recorded
C-O stretching	1045.42	Not recorded

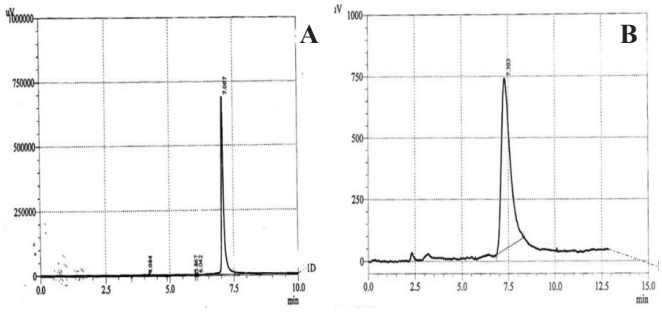


Figure 4: HPLC chromatogram of standard Scopletin (A) and isolated S (B).

Table 4: Illustrates the FTIR vibrations of isolated S with the assignment to their related functional group.

Functional Group	*Isolated Compound* *Scopoletin*	*Assignment*
O-H	3325.88	Broad stretching of phenolic OH
C=C	1627.07	Stretching of aromatic alkene
C=O	1697.98	Conjugated carbonyl stretching
C-H	2981.19 2946.39	Asymmetric and symmetric C-H stretching
C-O-C	1339.30 1287.56	Stretching of ether
O-H	1446.46	O-H bending
C-H	1458.61	C-H bending

CONCLUSION

E. milii cultivated in Iraq showed a comparable phytochemical profile as that provided in the literature. The cardiac glycoside Peruvoside and the phytoalexin Scopoletin coumarin bioactive compounds are first to be isolated from the species. Interestingly, Scopoletin detected and isolated from Iraqi *E. milii* is detected in this species for the first time, which is essential to prove the vital role of flora on the secondary metabolites produced by the plant. To conclude *E. milii* is considered a good source of novel bioactive compounds, which recommends further extensive analysis of this species.

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