RESEARCH ARTICLE

Isolation of Two Phenolic Compounds from the Ethyl Acetate Fraction of Iraqi *Eucalyptus Camaldulensis Dehnh.*

Zahraa S. Al-Tameemi[1]*, Maha N. Hamad[2]

[1]*Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Baghdad, Baghdad, Iraq.*
[2]*Department of Pharmaceutical Chemistry and Pharmacognosy, Baghdad College of Medical Sciences, Baghdad, Iraq.*

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ABSTRACT

Background: *Eucalyptus camaldulensis Dehnh*. (Family: Myrtaceae) is one of the most widely distributed species of eucalyptus trees and the original land of this variety in Australia. In Iraq, *E. camaldulensis Dehnh*. is the main species that distributed in various regions of the country. *E. camaldulensis* has active constituents rich in pharmacologically significant secondary metabolites with proven activities including anti-bacterial, anti-fungal, anti-viral, anti-oxidant, and others.

Objectives: Phytochemical screening of Iraqi *E. camaldulensis* was the main aim of this study with the isolation and some secondary metabolites.

Methods: Defatting step of leaves and fruits separately with n-hexane was preceding the extraction process by using soxhlet apparatus with 80% ethanol followed by subsequent fractionation process with and the isolation step from the ethyl acetate fraction that achieved by thin-layer chromatography (TLC).

Results: The resulted ethanolic extract undergoes preliminary chemical tests suggesting the presence of flavonoids, coumarins, anthraquinone, and cardiac glycosides, while the saponins and alkaloids were absent. The isolation process gives rise to the separation of two phytoconstituents identified by high-performance liquid chromatography (HPLC), high-performance thin layer chromatography (HPTLC), and Fourier-transform Infrared spectroscopy (FT-IR) and results in the confirmation of C1 compound as the coumarin scopoletin and C2 compound as the phenolic compound chlorogenic acid.

Conclusion: The Iraqi *E. camaldulensis* plant is rich in various secondary metabolites present. The first isolation might be around the world from this species is the phytoconstituent scopoletin. The phenolic acid chlorogenic acid was isolated for the first time from the Iraqi species.

Keywords: *Eucalyptus camaldulensis,* Isolation, Phytoconstituents

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INTRODUCTION

Since a long time ago and progressed to the present days, medicinal herbs remain one of the major sources consumed to treat different diseases.[1] A few decades ago, the utilization of natural products expanded widely, confirmed by the annual consumption of medicinal plants in different countries. This direction toward using natural products extracted from the plant due to their ease of availability and the prevailing belief in the lack of side effects compared to synthetic medicines.[2] In Iraq, several kinds of research and statistical studies in different provinces[3-6] showed a wide variety of medicinal plants, which are still used in herbalism, but lack studies that investigate their effectiveness, pharmacological significance, and its use properly as therapeutic agents along with synthetic drugs. This widespread consumption of herbs needs awareness by both prescribers (physician and pharmacists) and consumers to take the proper medicinal action from the natural plants.

Eucalyptus camaldulensis Dehnh. (Common names: eucalyptus kamali, kaffour, Murray River gum), one of the most widespread types of eucalyptus trees worldwide belongs to the Myrtaceae family. The major origin of this species in Australia with large distribution within diverse regions of the country grows more on the banks of rivers, especially the Murray River.[7] *E. camaldulensis Dehnh*. plant cultivated in Iraq within a broad area and considered the major *Eucalyptus* species distributed.[8] Historically, this species was introduced with different folkloric applications especially the oil as a traditional remedy for respiratory infections such as bronchitis, tuberculosis, gastritis, skin ulcers, and kidney diseases. Currently, different pharmacological activities have been

*Author for Correspondence: zahraa.salam.kadhim@gmail.com

proved for the *E. camaldulensis Dehnh.* such as anti-bacterial, anti-viral, anti-oxidant, and anti-inflammatory, and others.[9,10] This distinct and medical significance might attribute to the diverse phytoconstituents detected in the plant-like essential oils, terpenes, flavonoids, phenolic acids, and tannins.[11] This article's objectives are mainly to isolate some secondary metabolites that have not been detected yet in the Iraqi species and/or around the world.

EXPERIMENTAL WORK

Collection and Authentication of Plant Materials

The *E. camaldulensis* plant was gathered during October month 2020 from a local garden in Baghdad-Iraq. The leaves were detached from the stem linked to and dried alone in the shade for about 14 days. The plant was authenticated at the Iraqi Research Center & Natural History Museum as *E. camaldulensis Dehnh.* (Family: Myrtaceae).

Defatting, Extraction & Fractionation of Plant Materials

Dried leaves of 140 gm were pulverized by a grinder, defatted with 450 mL n-hexane for 24 hours, and filtered by a Whatman filter paper. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator (BÜCHI Rotavapor R-205 / Merck-German) and the obtained residue was kept for the extraction step.

The extraction for leaves had been achieved using Soxhlet apparatus. Powdered defatted leaves (weight:70gm) were extracted with 800 mL of 80% ethanol (Hayman Kimia Ltd., UK) on 5 days with a total time of running approximately 20 hours. The total extract was filtered and evaporated to dryness to get the residue.[12] The fractionation step was carried out using organic solvents to increase polarity. About 12 grams of residue were dissolved in 70 mL distilled water and then fractionated with an equal volume of petroleum ether (40–60°C - Sinopharm Chemical Reagent/China), chloroform (Alpha Chemika, India), ethyl acetate (98.4%-HIMEDIA, India) & n-butanol (99.9% - BDH limited, England). The repetition for each solvent was three times.[12,13] Anhydrous sodium sulfate was added to each fraction (except n-butanol fraction) to remove any water residue and then allowed to be evaporated to dryness and weighed to be utilized for the next analysis step. The ethyl acetate fraction was of interest to be analyzed and identified.

Qualitative Tests for Phytochemical Screening

Preliminary chemical tests were carried out to determine qualitatively the presence or absence of some phytochemicals in Iraqi *E. camaldulensis* that were achieved for the general extract.

1-Detection of Alkaloids

Mayer's test: About 2-3 drops of Mayer's reagent were added to 2 mL of ethanolic extract.[14]

2-Detection of Flavonoids

The sodium hydroxide test for flavonoids was the sodium hydroxide test (NaOH test). 2 mL of 10% aqueous NaOH was added to the ethanolic extract followed by a few drops of 10% aqueous hydrochloric acid.[15]

3-Detection of Cardiac Glycosides

Keller-Killiani test including 1-mL of ethanolic extract mixed with glacial acetic acid (1.5 mL) followed by the addition of 5% ferric chloride ($FeCl_3$) and 1-mL concentrated sulfuric acid and observe the acetic acid layer.[15]

4-Detection of Saponins

The crude grinded plant was dissolved in about 15 mL distilled water and shaken for 15 minutes.[16]

5-Detection of Coumarins

About 0.1-mL of 10% NaOH mixed with 1ml of chloroform added to 2 mL of alcoholic extract.[17]

6-Detection of Anthraquinone

Bontrager's test is done for the detection of anthraquinones. 3 mL of chloroform was added to 2 mL of ethanolic extract to form two layers, then about 0.5 mL of diluted ammonia solution added to the mixture.[18]

Analysis of the Ethyl Acetate Fraction

The analysis procedure is achieved mainly through the chromatographic technique, thin-layer chromatography (TLC). The major determinant of this chromatographic analysis are the stationary phase and the mobile phase. The stationary phase is the silica already available as an aluminum sheet (20 × 20 cm) coated with silica gel 60 GF_{254} with a thickness of 0.25 mm (Sigma-Aldrich, Germany). Plates activation is achieved through heating in an oven at 110°C for 10–15 minutes to eliminate any moisture absorbed from the atmosphere. The mobile phases consumed for the analysis of the ethyl acetate fraction are:

- *S1*: Toluene: acetone: chloroform (55:45:5)[19]
- *S2*: Ethyl acetate: formic acid: acetic acid: water (50:3:3:4)[20]

Sample and standards (Scopoletin & Chlorogenic acid-Chengdu Biopurify/China) prepared by dissolving in a few milliliters methanol and applied in the diluted form on the base line of the plate that developed in a glass TLC jar (22.5 cm x 22 cm x 7 cm) covered with a glass lid. The separated spots on the TLC plate have been visualized (if not colored) by non-destructive way utilizing ultra-violet (UV) light (Desaga /Germany) with wavelengths of 254 nanometer (nm) and 365 nm. The detected spots circled with a pencil and the retardation factor (R_f) value specific for each isolated spot is calculated and compared to the R_f value of the standard (if available) which is equal to the distance moved by the sample to the distance moved by the solvent. It should be noted that the distance marked from the center of the spot.

Rf value =(Distance moved by the sample)/(Distance moved by the solvent)

Isolation of C1 and C2 Compounds

Isolation of some natural products from Iraqi *E. camaldulensis Dehnh*. denoted by C1& C2 that isolated both from the ethyl acetate fraction had been achieved mainly by preparative

liquid chromatography (PLC). Plates of preparative liquid chromatography are ready-made glass plates covered with silica gel GF_{254} (20*20 cm) and (20*10 cm). The thickness of silica utilized for the separation is 0.25 mm (Tklst/ China) applied for C2, 0.5 mm (BBC chemical for lab / China) for C1, which is activated in the oven at 110°C for 10 minutes. The mobile phases used to isolate compounds C1, C2 isolated from the ethyl acetate fraction of the leaves were S1 and S2, respectively. About 0.1 mg of the ethyl acetate fraction dissolved in 1.5 mL methanol and set on the plate as a band with a capillary of the boiling point that developed to the front line of the plate and get out after entire dryness to be detected under UV (wavelength: 365 and 254 nm) and enclitic by pencil and crushed by a spatula.

Identification of Compounds C1 and C2

Confirmation of the separated compounds had been achieved through high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and fourier-transform infrared spectroscopy (FT-IR).

- Analytical HPLC for the isolated C1 and C2 compounds and standards were injected (1-µL) separately and compared both the sample and the standard by the retention time (RT). Chromatographic conditions utilized for this analysis are:[21]
  - Instrument model: LC-2010 Shimadzu, autosampler
  - Stationary Phase: Column C18- ODS (25 cm* 4.6 mm)
  - Mobile Phase: Water: acetonitrile (70:30 % v/v)
  - Mode of Operation: Isocratic elution
  - Detector: UV-visible detector with wavelength 335 nm
  - Flow Rate: 1-mL/min
  - Injection Volume: 2 microliters
- HPTLC is another way to further identify the isolated secondary metabolites. The instrument utilizes TLC plate (20.0 x 10.0 cm, 0.25 mm) with silica gel GF_{254} (Tklst/ China). The mobile phase applied for this analysis is S2. The sample and standard dissolved in methanol and spotted by autosampler on the plate, detected by UV with a wavelength of 366 nm.
- FT-IR with attenuated total reflection (ATR) technique has also identified the isolated C1 and C2 compounds as a spectrum of absorptive peaks each representing the functional group of the compound analyzed.

RESULTS

Preliminary Phytochemical Screening

The primary analysis for the Iraqi *E. camladulensis Dehnh.* made with the chemical tests screening the existence or absence of the secondary metabolites found in the plants. Table 1 describes the main chemical tests achieved to the general ethanolic extract of the leaves.

The phytochemical analysis for the leaves part extracted by the hot method suggests the presence of flavonoids, anthraquinone glycosides, cardiac glycosides, and coumarins, and the absence of saponins and alkaloids.

Examination of the Ethyl Acetate Fraction

The ethyl acetate fraction analysis has been achieved to detect some phenolic compounds using two different solvent systems to be compared with the standards. The solvent system S1 composed of the toluene: acetone: chloroform (45:55:5) was the best solvent system achieved for the ethyl acetate fraction compared with the scopoletin standard and as shown in Figure 1.

The previous capture to the TLC plate clarifies under the UV light with the wavelength 365 nm where the standard spot (on the right) fluoresces and observed well with an R_f value of 0.65. Fluorescent spots appeared in the ethyl acetate fraction (on the left) with an R_f value of 0.66.

Another mobile phase (S2) mixture of ethyl acetate: formic acid: acetic acid: water (50:03:03:04) also tried for the ethyl acetate fraction and compared to chlorogenic acid standard, the outcome of development is shown in the Figure 2.

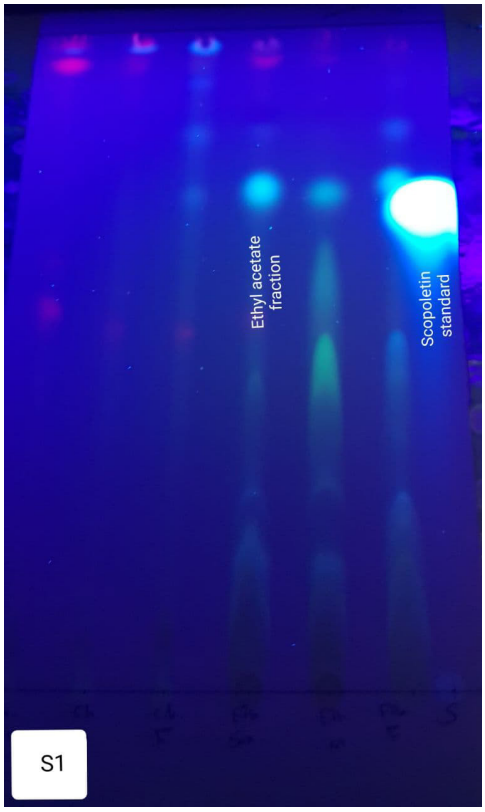

Figure 1: Analysis of the ethyl acetate fraction compared to scopoletin standard / detected under UV-365nm

Table 1: Phytochemical screening of Iraqi *E. camaldulensis Dehnh.* plant

Test achieved	*Observation*	*Outcome*
Mayer's test	White precipitate (not formed)	-
Alkaline reagent test	Yellow color (disappear with the addition of the acid)	+
Keller-Killiani test	Brown ring at the interphase	+
Foam test	Formation of foam (not formed)	-
Coumarin's test	Yellow color	+
Bontrager's test	Pink color	+

As seen in the previous figure of the plate at wavelength 365 nm, a blue fluorescent spot in the ethyl acetate fraction and the chlorogenic acid standard spot have been detected with no specific finding under the short wavelength. The R_f value for the detected spots was 0.4 for both the chlorogenic acid standard and the spot from the fraction, suggesting a close match between the retardation factor of the standard and the detected spot, observed under the long wavelength but not detected as an isolated spot in the short wavelength.

Isolation of Compounds C1 and C2

The isolation process achieved for compounds C1 and C2 from the ethyl acetate fraction of leaves after the TLC analysis process suggests too close R_f value of scopoletin and chlorogenic acid standards spots compared to the spots that appeared in the ethyl acetate fraction and occur as isolated spot. These phytoconstituents were of interest to be isolated consuming the solvent systems S1 for suspected scopoletin (C1) and S2 for suspected chlorogenic acid (C2) and as shown in Figures 3 and 4, respectively and the arrow referred to the isolated bands.

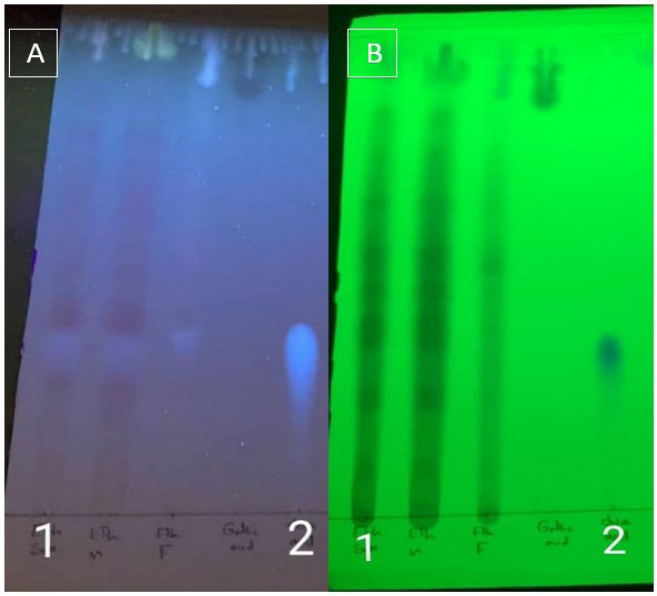

Figure 2: TLC analysis of the ethyl acetate fraction using the solvent system S2. 1. Fraction analyzed. 2. Chlorogenic acid standard / Detected under A-UV-365nm. B-254nm

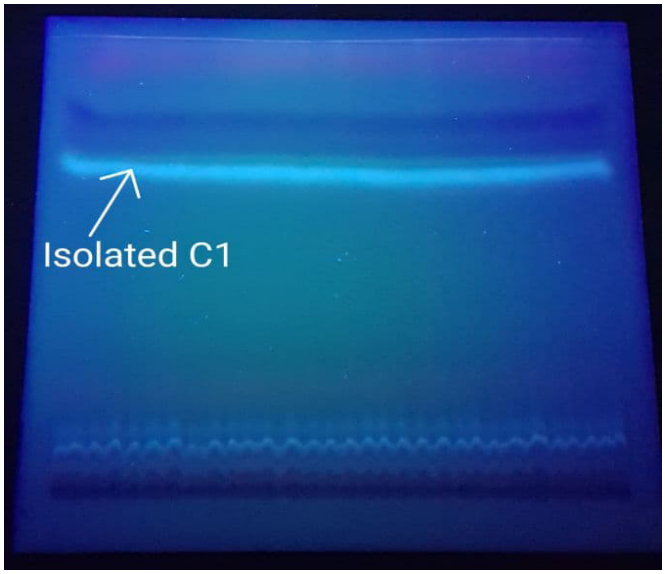

Figure 3: Isolation of C1 compound using the mobile phase S1 that developed on silica gel GF_{254} / Detected under UV-365nm

Identification of the Isolated Compound C1 and C2

The isolation of compound C1 and C2 had been attained after close matching with the standards scopoletin and chlorogenic acid done by TLC. The HPLC fingerprint first confirmed the identification to determine the purity of isolation, as shown in Figure 5.

HPLC analysis achieved through the chromatographic conditions mentioned previously and suggest a close retention time of the isolated C1 peak in 3.037 minutes compared to that of scopoletin standard recognized in 3.038 minutes.

The isolation seems to be almost pure and also compared to the standard (Figure 6), there is close matching in the time of retention of the isolated C1 compound at 2.055 minute and the chlorogenic acid standard observed at 1.920 minute.

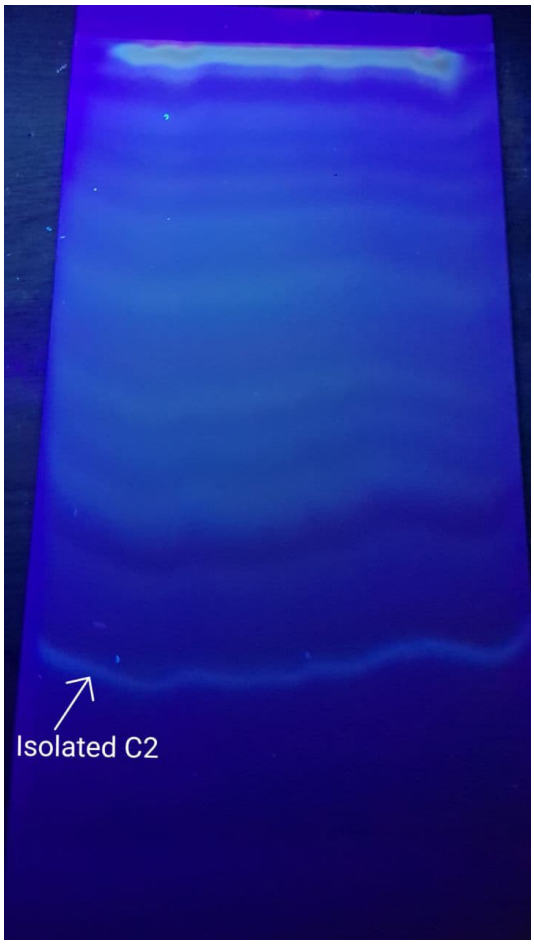

Figure 4: Separation of C2 compound from the ethyl acetate fraction using the mobile phase S2 that developed on silica gel GF_{254} / Detected under UV-365nm

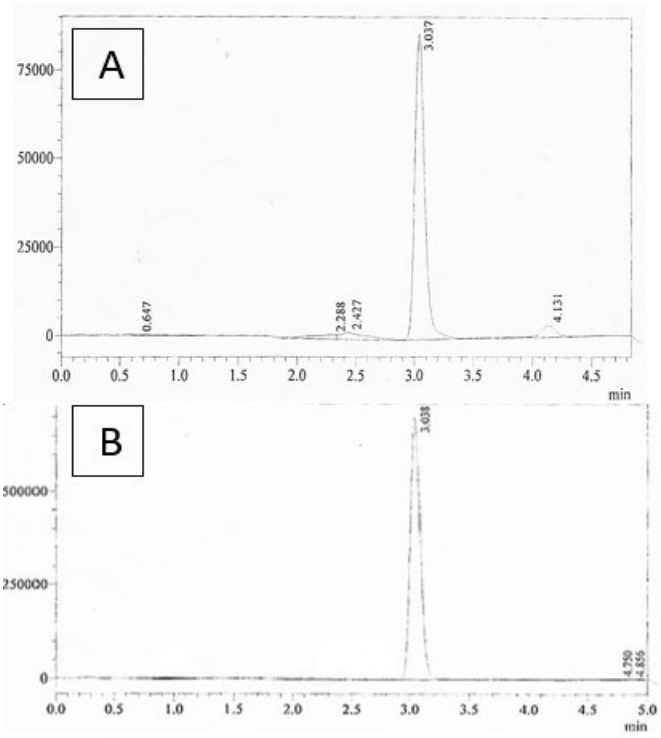

Figure 5: A. HPLC chromatogram of the isolated C1 compound, B. HPLC chromatogram of the scopoletin standard

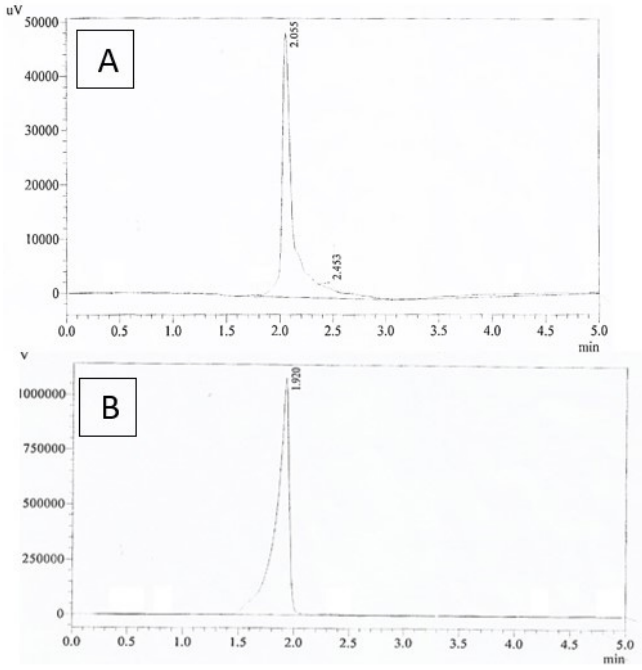

Figure 6: A. HPLC chromatogram of isolated C2 compound, B. HPLC chromatogram of chlorogenic acid standard

Another technique consumed for the identification is the HPTLC by comparing the maximum retardation factor of the standard and the isolated compounds that detected

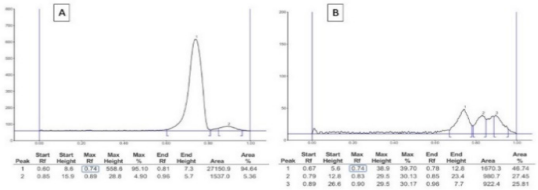

Figure 7: HPTLC analysis detected under UV-366nm. A. Scopoletin standard, B. Isolated C1 compound

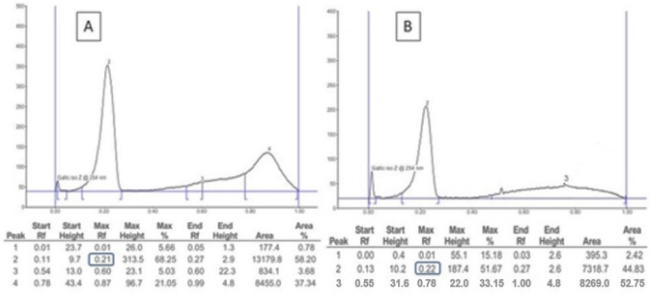

Figure 8: HPTLC analysis detected under the UV-254nm. A. Chlorogenic acid standard, B. Isolated C2 compound

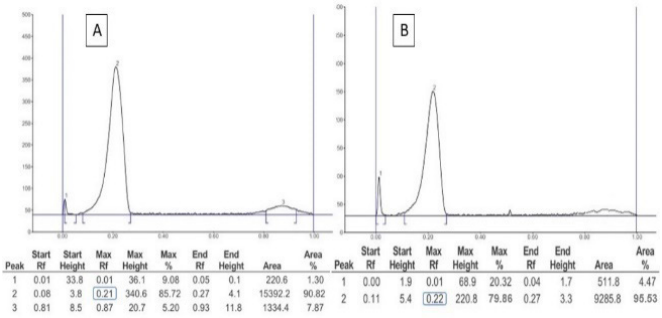

Figure 9: HPTLC analysis detected under the UV-366nm. A. Chlorogenic acid standard, B. Isolated C2 compound

under the UV-366 nm for C1 compound (Figure 7) and UV-254 nm (Figure 8) and 366 nm (Figure 9) for isolated C2 compound.

As displayed in the previous figure, the max. The Rf value (encircled) of the standard and the sample was the same (0.74), observing the same fluorescent blue spots of both the sample and the standard.

As shown in the HPTLC chromatogram of the UV-254 nm and 366 nm, there is a clear correlation between the maximum retardation factor (encircled) of the standard (0.21) and the isolated C2 (0.22). The visualization of the spots shows the same fluorescent color of the standard spot and the isolated C2 under the long-wavelength and approximate quenching at the short wavelength.

Finally, the FT-IR also achieved for the isolated C1 and C2 compounds. The main absorptive peaks and the chemical structures of possible isolated compounds observed from the

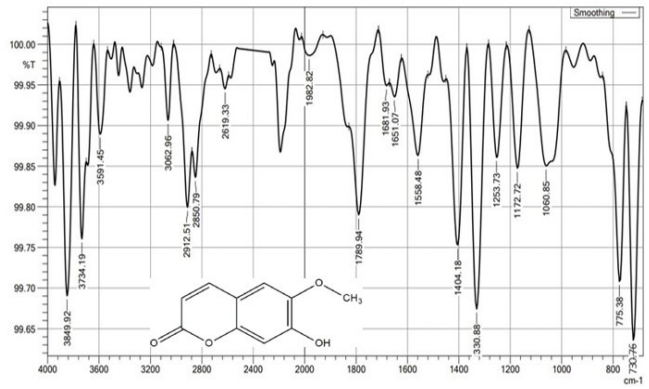

Figure 10: FT-IR spectrum of the isolated C1 compound

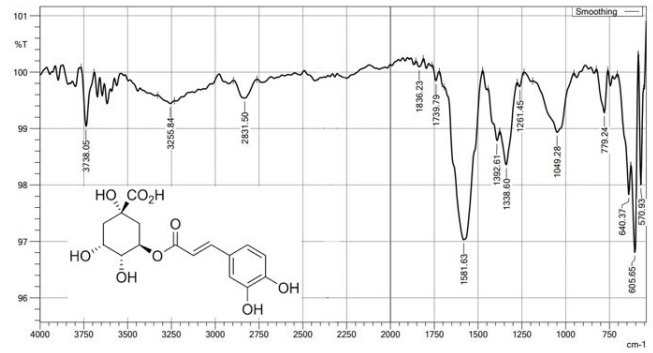

Figure 11: FT-IR spectrum of the isolated C2 compound

IR spectrum are shown below in Figures 10 and 11 for C1 and C2, respectively.

DISCUSSION

The main step that determines the type of secondary metabolites available in the extract is the extraction method and the solvent used to perform this process. The methods achieved in this study is the hot method done by using soxhlet apparatus with relatively high temperature that reaches more than 70°C. The solvent consumed for leaves is 80% ethanol (ethanol: water, 80:20 v/v). The selection of ethanol is based on its high efficiency in extracting wide ranges of phytochemicals including both polar and non-polar compounds in addition to its safety. Water in low percent with an organic solvent as an aqueous-ethanolic extract helps to improve the extraction, especially of phytoconstituents dissolved in water and in organic solvents.[22] The resulting residue from the extraction method now ready for the fractionation step which had been done using solvents with different polarities starting with the low solvent polarity petroleum ether followed by chloroform, ethyl acetate, and n-butanol. Each solvent pulls from the ethanolic extract dissolved in water. The phytoconstituents resolve in that solvent (like dissolve like) to facilitate the next steps of analysis and isolation since each fraction can be expected by its active constituents according to the polarity. The analysis step achieved for the ethyl acetate fraction through thin layer chromatography using the standards scopoletin and the chlorogenic acid compared to the fraction suggests a high correlation in the R_f value between the spots identified in the ethyl acetate fraction and the standards detected in the long wavelength as a blue fluorescent. The next step following the analysis and observation of spots was their isolation made by preparative liquid chromatography (PLC) as a simple and available way for separation. Their analysis seems that it could be isolated possibly pure without separating other compounds. The last and major step was the identification and confirmation achieved by the HPLC, one of the most sensitive and accurate chromatographic techniques utilized for the analysis based on the compound's retention time that might compare to the retention time of the standard. The close matching in the retention time observed between the isolated C1 and C2 compounds with their standards gives a primary confirmation for the isolated compounds identified by another chromatographic technique, HPTLC, which results in the same maximum retardation factor for the samples and the standards. Final identification was with the FT-IR, which is interpreted according to Sigma-Aldrich company's official website and the article.[23]

The confirmation of isolated compounds was achieved in the mid IR region between 400–4000 cm^{-1} to identify possible functional groups that might be distinguish and prove to the isolation phytoconstituents. The main distinguished peaks observed with the isolated C1 compound are 1789.94, 3591.45, 2850.79, 1404.18, and 1681.93 cm^{-1} that linked respectively to carbonyl stretching, hydroxyl group stretching, asymmetric C-H stretching, asymmetric C-H bending, and carbon double bond carbon stretching of alkene, as well as the peaks of 775.38 cm^{-1} and 730.76 cm^{-1} that may relate to the benzene ring di-substitution at meta and para positions. Analysis of isolated C2 compound by FT-IR also identifies the number of absorptive peaks interpreted as the following: 3255.84 cm^{-1}/1392.61 cm^{-1} for hydroxyl group stretching and bending, respectively, 2831.50 cm^{-1} related to the asymmetric C-H stretching of methyl group, 1739.79 cm^{-1} linked to the carbonyl group. Other peaks related to the benzene ring are 1836.23 cm^{-1}, 1581.63 cm^{-1}, and 779.24 cm^{-1} linked consecutively to C-H bending of benzene, C=C aromatic ring stretching, and benzene ring substitution. All the previous identifications starting from the positive chemical tests for flavonoids and coumarins along to the analysis by TLC and comparison with the standards, to the isolation and final identification by HPLC, HPTLC, and FT-IR all confirms that the isolated C1 compound is the coumarin scopoletin and the isolated C2 compound is the phenolic acid chlorogenic acid.

CONCLUSION

Based on the results of this study, The isolation of the coumarin scopoletin from Iraqi *E. camaldulensis Dehnh.* plant is considered the first isolation worldwide, while the isolation of the phenolic acid chlorogenic acid is considered the first one from the Iraqi species.

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