



Isolation, Characterization and Identification of Active Antifungal Compound from the Ethanolic Leaf Extract of *Pluchea dioscoridis*

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SECONDARY metabolites from medicinal plants have antimicrobial properties and thus are used to cure chronic and acute diseases worldwide. This study aimed to isolate and identify the antifungal compound in the ethanolic extract of *Pluchea dioscoridis* leaves. Qualitative and quantitative phytochemical analysis for the secondary metabolites of *P. dioscoridis* was assessed. Thin layer chromatography (TLC) was used. Preliminary screening for antifungal activity against otomycotic *Aspergillus niger* was carried out for all isolated fractions at different concentrations. Results showed that phenolic compound RF₁ exhibited excellent antifungal activity against *A. niger* (MIC= 2mg/mL). UV spectrum, IR, HNMR, mass spectrum and high performance liquid chromatography (HPLC) analysis identified this compound as gallic acid, with a molecular formula of C₇H₆O₅. Therefore, gallic acid is the potential antifungal compound of *P. dioscoridis* extract.

Keywords: Antifungal activity, Gallic acid, *Pluchea dioscoridis*.

Introduction

Plants are a valuable source of natural products for human health maintenance, and natural therapies have been intensively studied. The pharmaceutical use of plant compounds has gradually increased (Romero et al., 2005). According to the World Health Organization, medicinal plants can be a source of various drugs. The pharmacological industries have produced a number of new antibiotics due to the resistance to many drugs by microorganisms (Janicsak et al., 2006). Isolating and identifying active constituents from traditional medicinal plants ensure their usage as proper medication that is safe and effective.

Otomycosis is a chronic fungal infection of the external auditory canal caused by molds. *Aspergillus niger* and *Penicillium* species frequently cause otomycosis (Loh, et al., 1998) and have genetic resistance to synthetic antifungal drugs (Nagl et al., 2003). Therefore, research

focuses on minimizing the use of antifungals, developing new drugs, and controlling pathogenic antifungal-resistant microorganisms (Gabor et al., 2006).

In this study, the chemical constituents of *Pluchea dioscoridis* were analyzed to determine their activity against otomycotic fungal infections. This investigation provides information on valuable compounds and new drug candidates for otomycosis treatment.

Materials and Methods

Collection of plant material

Fresh leaves of *P. dioscoridis* (Conyza) were collected from three habitats in Nile Delta, namely, highways, railways, and waste lands (Ahmed, 2003). Plants were classified based on authentic materials in Tanta Herbarium, Tanta University (TANE), and all collected sheets are kept in it. The leaves were washed thoroughly 2–3

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times with running water and once with sterile distilled water, then air-dried under shade (Satish et al., 2007).

Preparation of P. dioscoridis extract

P. dioscoridis extract was prepared in ethanol at room temperature through simple extraction (Deshpande et al., 2004). A grind mixer was used to grind the dried leaves to a fine powder (150mg), which was then mixed with 10mL of 70% ethanol in 250mL conical flask. The flasks were plugged, placed on a shaker for 24h, and allowed to stand for 5h to settle the plant materials. The extract was filtered and centrifuged at 5000rpm for 15min. The supernatant was collected, and the solvent was evaporated at 45°C to obtain the final volume (1/5th of the original volume) and stored at 4°C in airtight bottles for further studies.

Microorganism and inoculum preparation

A. niger strain was isolated from a patient with otomycosis who visited the outpatient clinic of Otolaryngology Department at Tanta University Hospital. The specimen was maintained on agar slants. Fungal spores were inoculated by adding 10 ml of sterile distilled water to Sabouraud Dextrose Agar (SDA) slants and then dislodged using a sterile inoculation loop under aseptic conditions. One ml of spore suspension (5x10⁶ spores/mL) was used as the inoculum.

Determination of antifungal activity

Antifungal activity was determined for *P. dioscoridis* ethanol extract and fluconazole as a positive control by employing the hole-plate method (Igbiosa et al., 2009; Abd El-Zaher et al., 2019). Freshly prepared spore suspension of isolated *A. niger* (1mL with 5x10⁶ spores/mL) was mixed with 14mL of sterile SDA medium at 45°C, poured in sterile Petri dishes, and left to solidify at room temperature. Regular wells were formed in the inoculated agar plates by a sterile cork borer with 0.8 cm diameter. Each well was aseptically filled up with 0.2mL of plant extract or fluconazole with different concentrations of (0, 5, 10, 15, 20, and 25mg/mL). Three replica plates for each concentration were incubated at 25°C for 3–4 days and then observed for inhibition zones.

Phytochemical evaluation of P. dioscoridis leaf extract

Qualitative analysis of secondary metabolites

P. dioscoridis leaf extract was preliminary screened for the presence of secondary

metabolites (alkaloids, saponins, terpenoids, flavonoids, steroids, and phenolics) using standard procedures (Trease & Evans, 1989) modified by Sazada et al. (2009), Bishnu et al. (2011) and Helal et al. (2019).

Detection of alkaloids: In brief, 5 ml of the extract was dissolved in 1 ml of diluted HCL and then filtered. The filtrate was treated with Mayer's reagent (potassium mercuric iodide). Formation of yellow cream precipitate indicates the presence of alkaloids.

Detection of saponins: a) Froth test: In brief, 5mL of the extract was diluted with 20mL of distilled water and shaken for 15min. Formation of 1cm foam layer indicates the presence of saponins.

(b) Foam test: A small amount of the extract was mixed with water and shaken. If the produced foam persists for 10min, then saponins are present.

Detection of terpenoids: In brief, 5mL of the extract was mixed with 2mL of chloroform. The mixture was then added with 3mL of concentrated H₂SO₄ to form a layer. A reddish brown hue indicates the presence of terpenoids.

Detection of flavonoids: In brief, 1mL of the extract was treated with 1mL of dilute NaOH. The presence of a cloudy precipitate confirms the presence of flavonoids.

Detection of steroids: In brief, 2mL of acetic anhydride was added to 0.5mL of the ethanolic extract and 2mL of H₂SO₄. Color change from violet to blue or green in some samples indicates the presence of steroids.

Detection of phenolics: In brief, 1mL of the extract was mixed with 1mL of 10% FeCl₂. Formation of blue precipitate confirms the presence of phenolics.

Quantitative analysis of secondary metabolites

Estimation of total phenolic compounds: Phenolic compounds were extracted from *P. dioscoridis* leaf extract using the method of Jindal & Singh (1975). In brief, 1gm of powdered extract was extracted with 80% aqueous ethanol and centrifuged at 3000rpm for

20min. Afterward, 1mL of sample was mixed with 1mL of Folin–Ciocalteu phenol reagent and 1mL of 20% anhydrous sodium carbonate to a final volume of 5mL with the addition of distilled water. The absorbance of blue color was measured after 30min at wavelength 650nm against blank. Phenolic content was obtained from a standard curve of pyrogallol and then calculated as mg per gm dry weight.

Estimation of total flavonoids: Aluminum chloride colorimetric technique was used for flavonoid estimation (Chang et al., 2002). *P. dioscoridis* leaf extract (0.5mL of 1:10g/mL) in methanol was separately mixed with 1.5mL of methanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1M potassium acetate, and 2.8mL of distilled water. The mixture was left at room temperature for 30min, and the absorbance of the reaction was measured at 416nm with a double beam UV-visible spectrophotometer. Total flavonoid content was obtained from calibration curve plotted by preparing the quercetin solutions at concentrations of 12.5–100g/mL in 90% methanol.

Estimation of total alkaloids: Alkaloids were extracted according to the method of Harborne (1973): 5g of the extract was weighed into a 250 ml beaker and 200mL of 10% acetic acid in ethanol was added, covered, and allowed to stand for 4h. This solution was filtered, and the extract was concentrated on a water bath to 1/4 of the original volume. Concentrated ammonium hydroxide was dropwise added to the extract until the precipitation was complete. The whole solution was allowed to settle, and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed as mg alkaloid per 5 gram dry weight.

Estimation of terpenoids: *P. dioscoridis* leaf extract was filtered using Whatman filter paper no.1. The filtrate was then evaporated to 1/10 volume at 40°C and acidified with 1mL of concentrated sulfuric acid (pH= 0.89), followed by chloroform extraction (three times the volume). The sample was stirred and allowed to stand in a separation funnel. The non-aqueous layer was isolated and evaporated until dry. The residue is terpenoid, which was weighed as mg per gm dry weight. (Majaw & Moirangthem, 2009).

Separation of active compound by thin layer chromatography (TLC)

P. dioscoridis extract was also characterized by TLC on silica gel plates (Merck) using the following developing systems (butanol:acetic acid: water (14:1:5 v:v:v), (2:1:1), (3:1:1), chloroform: methanol (9:1) and water: methanol(4:1) (Khan & Nasreen, 2010). The plates were sprayed (Aly, 1997) and modified (Esmaeili et al., 2011) with silver nitrate to create dark spots for phenols, lead acetate to generate yellow to orange spots with flavone compounds, Ehrlich reagent to produce reddish brown spots only with alkaloids, and sulfuric acid that create yellowish brown spots only with terpenoids. These compounds were then scratched from TLC using a clean and dry spatula, collected in beaker containing 70% methanol, and left overnight (Bishnu et al., 2011).

Effect of all isolated active components on A. niger growth

A concentration of 0.5 mg/ml in ethanol was prepared from all isolated active components (phenolics, flavonoids, terpenoids, and alkaloids). Antifungal activity was assayed by the hole-plate method (Igbinsosa et al., 2009). Given that the most efficient active compounds for antifungal activity were phenolics, a concentration of 0.5mg/mL in ethanol was prepared from the isolated three bands of phenolics (R_f -1, R_f -2, R_f -3). The antifungal activity was also assayed by the hole-plate method (Igbinsosa et al., 2009).

Minimal inhibitory concentrations of phenolics R_{f1}

Different concentrations (0, 0.5, 1, 1.5, 2, and 2.5mg/mL) were prepared from phenolics R_f -1 (highly antifungal active component). Colony forming units were counted and graphically represented, and MIC was recorded using the methods of Shadomy et al. (1985) and Radhika et al. (2008). Three replica were prepared for each concentration and compared by all plates.

Identification of active component R_f -1 phenolics

Active components were identified by spectroscopic analyses (UV, IR, ¹H-NMR, mass spectrum, and high performance liquid chromatography (HPLC) at the National Research Center, El Dokky, Giza Governorate.

Results

Table 1 showed the inhibitory effect of *P.*

dioscoridis leaf ethanol extract on the radial growth of *A. niger* and fluconazole. The results revealed that *A. niger* growth decreased with the increasing concentration of *P. dioscoridis* extract.

TABLE 1. Effect of *P. dioscoridis* leaf extract on radial growth of *A. niger*, in comparison to commercially available Fluconazole

Concentration (mg/ml)	Diameter of inhibition zone (cm)	
	<i>P. dioscoridis</i> extract	Fluconazole
0	0.0	0.0
5	2.0±0.11	0.0
10	3.9±0.12	0.0
15	6.0±0.11	0.5
20	8.0±0.13	1.0±0.12
25	9.0±0.12	1.3±0.14
ANOVA	F	4392.6
	P value	<0.001*
		196.0
		<0.001*

* P value is statistically highly significant at the 0.001 level.

Qualitative analysis on *P. dioscoridis* leaf extract (Table 2) showed the presence of alkaloids, phenols, flavonoids, and terpenoids and the absence of saponins and steroids. Table 2 showed the quantitative analysis of *P. dioscoridis* leaf extract. The contents of total phenolics and terpenoids were 200 and 18mg/g gallic acid equivalent, respectively, as measured by Folin–Ciocalteu reagent. In addition, the contents of total flavonoids were 9.57mg/g quercetin equivalent. Alkaloids showed the lowest content of 4mg/g.

TABLE 2. Qualitative and quantitative phytochemical screening for secondary metabolite production in *P. dioscoridis* leaf extract

Component	Qualitative detection	Quantitative (mg/g)
Saponins	-ve	-
Terpenoids	+ve	18
Phenolics	+ve	200
Flavonoids	+ve	9.57
Alkaloids	+ve	4
Steroids	-ve	-

The leaf extract was loaded onto preparative silica gel thin layer plates, which were then

developed in different systems. The best solvent systems were as follows: butanol: acetic acid: water (14: 1: 5, V : V : V), followed by chloroform: methanol (9: 1, V: V) and water : methanol (4: 1, V: V). Other solvents systems showed a rapid tailing of the active substances in *P. dioscoridis*. With the first solvent system, four compounds were detected under the UV lamp with R_f values of 0.9, 0.8, 0.7, and 0.6. With the second solvent system, one compound was detected with $R_f = 0.3$. In the third solvent, two compounds were detected with R_f of 0.75 and 0.88 as shown in Table 3.

The spots were developed in the TLC plate and sprayed with different color reagents to roughly determine their nature. As shown in Fig. 1, the extract contains one flavonoid compound, which produced a yellow color with lead acetate, two terpenoid spots, which generated a yellowish brown color with sulfuric acid, and one alkaloid compound, which created a reddish brown color with Ehrlich reagent. In addition, the reaction with silver nitrate produced three brown spots of phenolic compounds. Spots on the preparative silica gel plate were scratched with dry spatula, collected in beaker containing 70% ethanol, left overnight, and then filtered and used to determine the antimicrobial effect. Table 4 shows that only the fraction of phenolic R_f-1 exhibited an inhibitory effect on *A. niger* growth.

Figure 2 reveals the effect of different R_f-1 concentrations on the radial growth of *A. niger*. The growth rate decreased and eventually stopped completely. The MIC of phenolic R_f-1 was determined as 2mg/mL (Table 5). The structures of the isolated compounds were established by UV analysis, IR, NMR, and mass spectroscopy (Figs. 3–6). Phenolic compound (R_f-1) was obtained as a pale buff powder from TLC and identified with two peaks at 220 and 270nm, which were called the B-band of benzenoid peak by UV analysis. IR (cm^{-1}) also showed broad bands at 3446.7cm^{-1} (OH), 3064cm^{-1} (C-H), 1700.86cm^{-1} (C=O), 1618.6 (C=C), and 1400.86 (C-C). $^1\text{H NMR}$: (DMSO) $\delta 4.724\text{ppm}$ (3H, OH), $\delta 6.96$ ppm (2H, Ar-H), and $\delta 9.5\text{ppm}$ (1H, COOH). Mass spectra of phenolic compound (R_f-1) produced a base peak at 170m/z.

The HPLC fingerprint in Fig. 7 of the phenolic compound R_f-1 showed major peak at retention time of 2.415min and a wavelength of 240nm. This phenolic compound R_f-1 was identified as gallic acid.

TABLE 3. Visualization of phytochemical components of *P. dioscoridis* leaf extract

Coloring reagent	Reaction	No. of spots	Recognized component	R _f	Solvent
Silver nitrate 5%	+	3	Phenolics	0.8-0.7-0.6	Butanol:aceticacid:H ₂ O (14:1:5)
Lead acetate	+	1	Flavonoids	0.9	Butanol:aceticacid:H ₂ O (14:1:5)
Ehrlich reagent 1%	+	1	Alkaloids	0.3	Chloroform:methanol (9:1)
Sulphuric acid 5%	+	2	Terpenoids	0.75-0.88	Water:methanol (4:1)

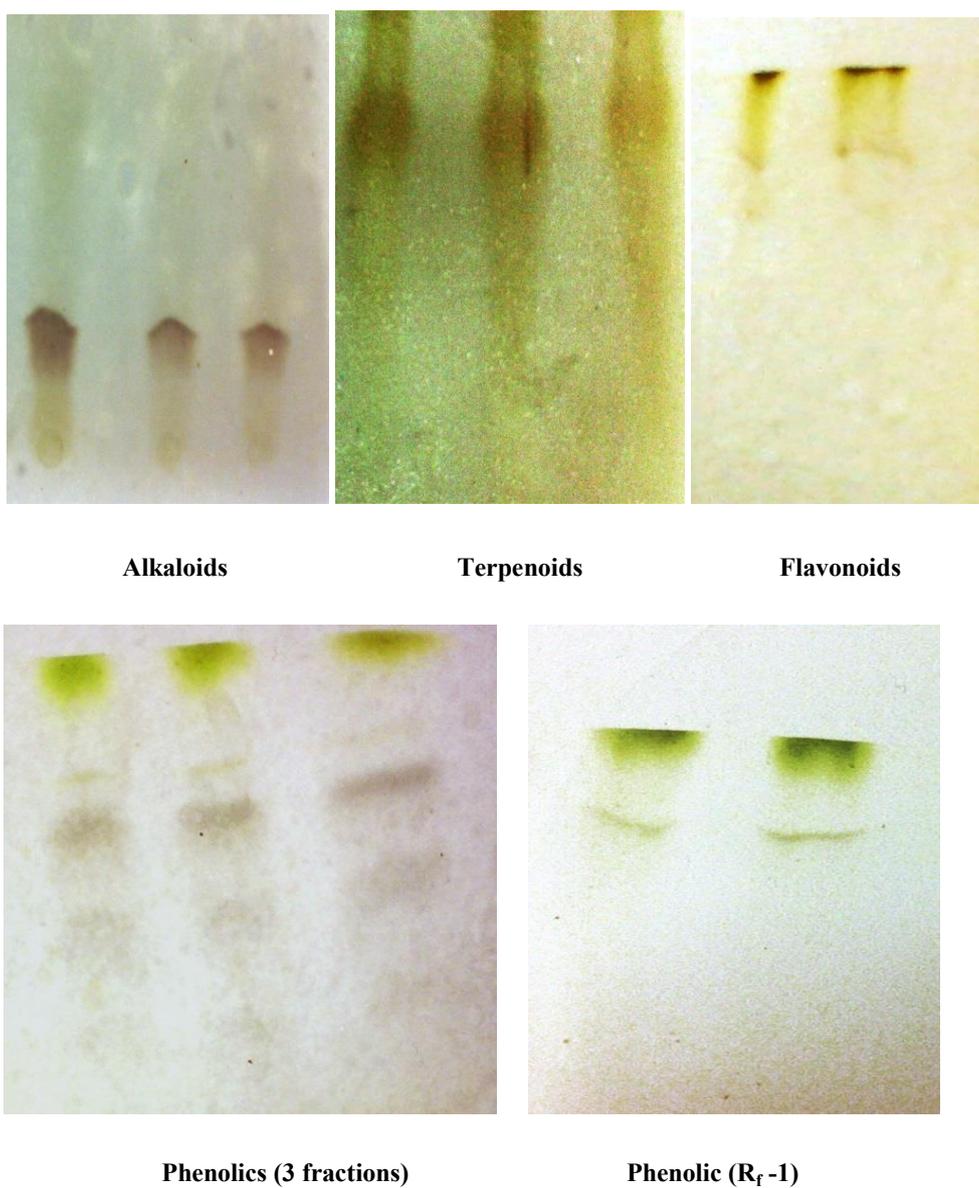
**Fig. 1. Thin layer chromatography for separation, and characterization of different phytochemical components of *P. dioscoridis* leaf extract**

TABLE 4. Minimal inhibitory concentration (MIC) of the most efficient phenolic fraction (R_f -1) of *P. dioscoridis* leaf extract against *A. niger*

Concentration (mg/mL)	Number of surviving cells (C.F.U)
0.0	35±1.0
0.5	12±1.0
1.0	8±1.0
1.5	2±0.6
2.0	1
2.5	0
ANOVA	F
	P value
	942.1
	< 0.001*

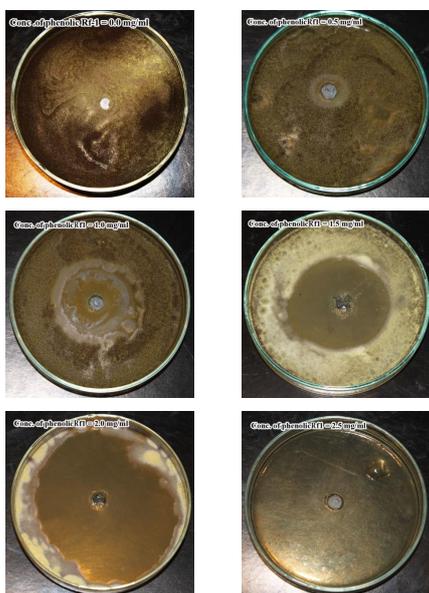


Fig. 2. Inhibitory effect of the most efficient phenolic fraction (R_f -1) of *P. dioscoridis* leaf extract against *A. niger*

TABLE 5. Minimal inhibitory concentration (MIC) of the most efficient phenolic fraction (R_f -1) of *P. dioscoridis* leaf extract against *A. niger*

Concentration (mg/mL)	Number of surviving cells (C.F.U)
0.0	35±1.0
0.5	12±1.0
1.0	8±1.0
1.5	2±0.6
2.0	1
2.5	0
ANOVA	F
	P-value
	942.1
	< 0.001*

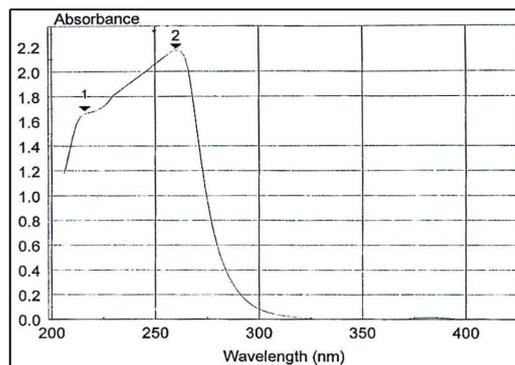


Fig. 3. UV-analysis for phytochemical composition of *P. dioscoridis* leaf extract (R_f -1 phenolic fraction)

Discussion

The beneficial medicinal effects of plant materials are typically attributed to their secondary products, whether a single compound or a combination of metabolites (Paerkh & Chanda, 2008).

The antifungal activity of *P. dioscoridis* leaf extract is attributed to its active constituents. Phytochemical screening revealed the presence of phenolics, flavonoids, terpenoids, and alkaloids. This finding is in agreement with El-Hamouly & Ibraheim (2003), who reported that the aerial parts of *P. dioscoridis* contain 3%–5% volatile oil and consist mainly of phenolics and terpenoids. Phytochemical analysis on *Conyza sumatrensis* leaf extract revealed the presence of active components such as tannins, flavonoids, and steroids (Jack & Orubite, 2008). Among the four groups of secondary metabolites from *P. dioscoridis* leaf extract, phenolics (200mg/g) were the most abundant, followed by terpenoids (18mg/g), flavonoids (9.57mg/g), and alkaloids (4mg/g). This result is in agreement with Shahwar et al. (2012), who revealed that *Conyza bonariensis* contains 108.6–395.6 mg of gallic acid/g of extract. Shahid et al. (2003) also reported the presence of four phenolic compounds in *Conyza canadensis* leaf extract. To the best of the authors' knowledge, the active components on *P. dioscoridis* leaf extract have not been identified.

This work is the first to detect the active compounds of *P. dioscoridis* leaf extract. The results were in agreement with Si et al. (2011) who detected phenolics and flavonoids from *Paulowniato mentosa* extract by TLC. The active

TLC fractions in the present study were subjected to antifungal activity assessment, and phenolic (R_f -1) exhibited the highest antifungal activity against *A. niger*. UV, IR, and NMR successfully established the structure of this compound, and mass spectroscopy identified it as gallic acid. This result was in accordance with Nayeem & Karvekar (2010), who isolated gallic acid from *Tectona grandis* methanolic extract by using the same techniques. HPLC fingerprinting is the optimal method for chemical characterization (Springfield et al., 2005) and accurately identifies secondary

metabolites within a short period (15min). In the current work, the HPLC fingerprint for the active phenolic acid (R_f -1) showed the same peak at a retention time of 2.415min and a wavelength of 240nm compared with gallic acid standard. This finding was in agreement with Samee & Vorarat (2007) who detected gallic acid in the flower extracts of *Michelia alba* by HPLC. High antifungal activity against *A. niger* was detected for gallic acid, one of the active compounds of *P. dioscoridis* leaf extract. This result is similar to the report of Aziz et al. (1998).

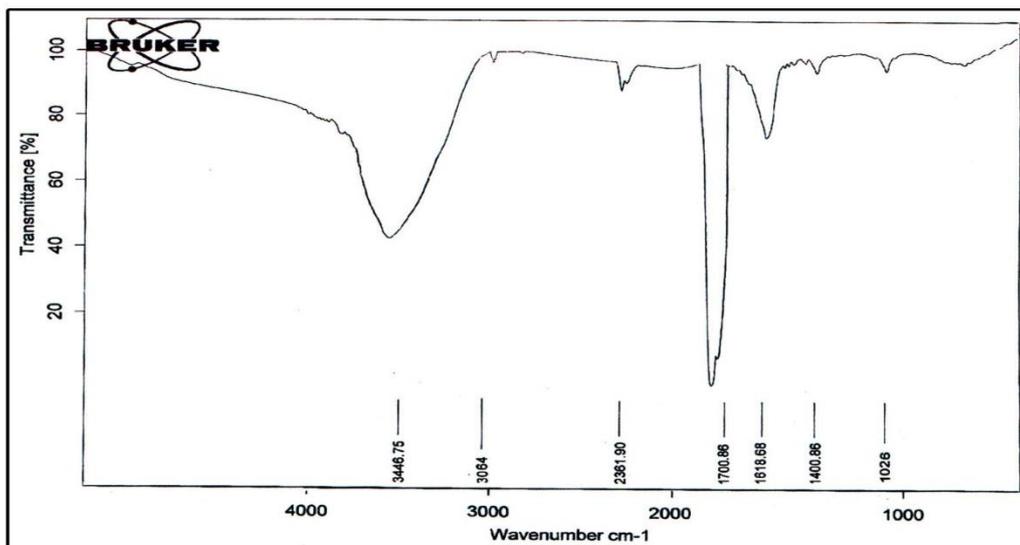


Fig. 4. IR-analysis for phytochemical composition of *P. dioscoridis* leaf extract (R_f -1 phenolic fraction)

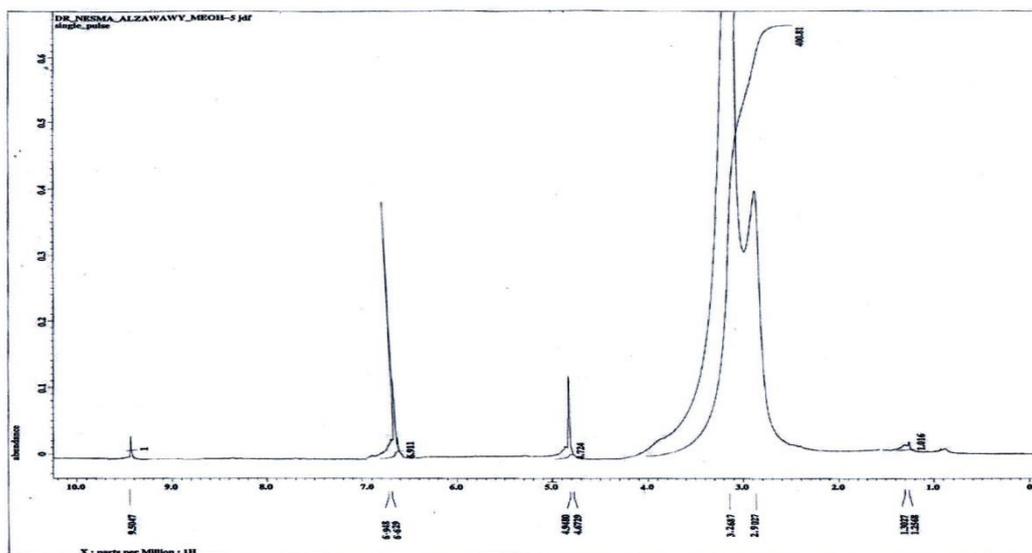


Fig. 5. NMR-analysis for phytochemical composition of *P. dioscoridis* leaf extract (R_f -1 phenolic fraction)

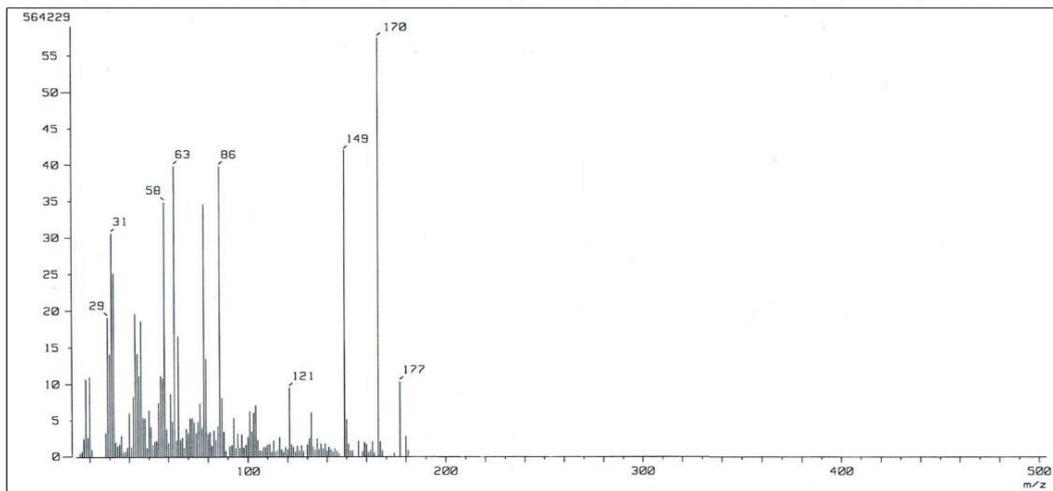


Fig. 6. Mass spectrum analysis for phytochemical composition of *P. dioscoridis* leaf extract (R_f -1 phenolic fraction)

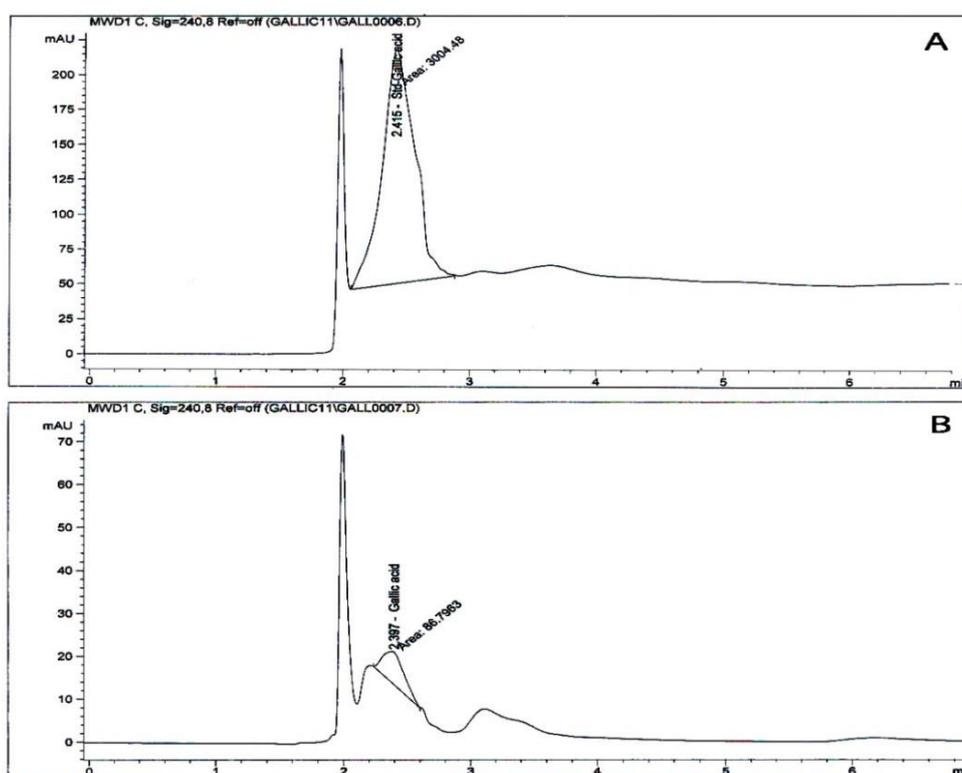


Fig. 7. HPLC- analysis for phytochemical composition of *P. dioscoridis* leaf extract (R_f - phenolic fraction), A: Standard Gallic acid, and B: Unknown sample

Conclusion

The gallic acid component of *P. dioscoridis* extract exhibits potential *in vitro* antifungal activity against *A. niger* isolated from otomycosis.

Conflicts of interest: No conflicts of interest have been declared.

Author contribution: M.A.M: Experimental design, Reviewing and Editing; H.M.M: Data curation, Reviewing and Editing; A.M.G: Methodology and Reviewing; N.A.E: Methodology, Investigation and Writing original draft.

Ethical approval: Not applicable

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عزل وتوصيف مركب مضاد للفطريات من المستخلص الإيثانولي لأوراق نبات البلوشيا ديسوكوريديس

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تستخدم نواتج الأيض الثانوية من النباتات الطبية كمضادات ميكروبيه لعلاج بعض الأمراض المزمنة والحادة في جميع أنحاء العالم. ولذلك أجريت هذه الدراسة في محاولة لعزل وتعريف مركب مضاد للفطريات من المستخلص الإيثانولي لأوراق نبات البلوشيا ديسوسوريديس. وتم أيضاً إجراء تحليلات كميّة وكيفية للمكونات الثانوية لنبات البلوشيا ديسوسوريديس. وقد تم إجراء الفصل الكروماتوجرافي للطبقات الرقيقة لتحليل المكونات الثانوية الموجودة في المستخلص. وتم إجراء فحص أولي لنشاط مضاد الفطريات لكافة المركبات المعزولة بتركيزات مختلفة ضد فطره أسبرجيليس نيجر المسببه للإلتهاب الفطري لقناة الأذن الخارجية. وقد اظهرت النتائج، أن الجزء النشط ضد فطره أسبرجيليس نيجر هو الفينول المركب R₁ (عند تركيز = 2 مجم/مل). وتم تعريفه على أنه حمض الجالك (C₇H₆O₃) عن طريق الطيف فوق البنفسجي، والأشعة تحت الحمراء والرنين النووي المغناطيسي و كتله الأطياف و تحليل استشراب السائل عالي الأداء. وقد تم إستنتاج أن حمض الجالك في مستخلص نبات البلوشيا ديسوسوريديس هو مركب مضاد للفطريات.