Antifungal Activity of Alkaloids and Phenols Compounds extracted from black pepper *Piper nigrum* against some pathogenic fungi

فعالية مركبات القلويدات والفينولات المستخلصه من الفلفل الأسود ضد بعض الفطريات الممرضه

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Abstract

This study focused on the production of alkaloids and phenols from dry black pepper *Piper nigrum* fruit; and its effect on thirteen species of fungi isolated from dermal infection. The antifungal activity of different concentrations alkaloids and phenols were evaluated using agar dilution method. Statistics revealed different inhibition percentages; that are gradually increased with the increasing in the concentration of the used compound. Minimal inhibitory concentration MIC and maximum fungicidal concentration MFC were obtained 0.4, 0.002 mg/ml respectively. Confirming the presence of alkaloids and phenols were done by fast liquid chromatography; showed the presence of two types of alkaloids and ten types of phenols.

Key words: Black pepper, *Piper nigrum*, Antifungal activity, Alkaloid, Phenol compounds

الملخص

ركزت هذه الدراسه على انتاج القلويدات والفينولات من ثمار الفلفل الأسود ودراسة تأثيرها على 13 نوع من الفطريات المعزوله من اصابات جلديه، قيمت فعالية القلويدات والفينولات المحضرة بتراكيز مختلفه من خلال طريقة المزج بالأكار و أظهرت نسب تثبيط مختلفه تزداد مع زيادة تركيز المركب المستخدم بعد تحليل النتائج احصائيا.تم الحصول على قيمة التركيز المثبط الأدنى والتركيز الأعلى القاتل للممرضات المستخدمه في التجربه وكان 0.002, 0.4ملغم/ مل على التوالي تم التحليل والكشف عن القلويدات والفينولات باستخدام الكرموتوغرافيا السريعه السائلة وأظهر التحليل وجود نوعين من القلويدات و عشرة أنواع من الفينولات في المستخلص الذي تم تحضيره والكشف عنه.

الكلمات المفتاحية: الفلفل الاسود، مضادات الفطريات، مركبات القلويدا ت والفينولات

Introduction

Fungal infections are estimated to occur in over a billion people each year, and recent evidence suggests the rate is increasing, however fungi can infect almost any part of the body including skin, nails, respiratory tract, urogenital tract, alimentary tract, or can be systemic infection. Anyone can acquire a fungal infection, but the elderly, critically ill, and individuals with weakened immunity, due to diseases such as HIV/AIDS or use of immune suppressive medications, have a higher risk [1].

Fungal infections (dermatomycosis) caused by dermatophytes (a group of fungi cause fungal infection) represented by *Epidermophyton*, *Microsporum* and *Trichophyton*genera. They tend to invade keratinlayer and grow outwards on skin producing a ring like lesion called ringworm; these diseases are very common and affect different parts of the body. The most common of these organisms are *T.rubrum*, *T.tonsurans*, and/or *T. mentagrophytes*, *T. interdigitale*, *M.canis*, and *E. floccosum*[2].

The plant family Piperaceae is a source of many biologically active phytochemicals [3,4] with great potential for medicinal [5] and agricultural use [6]. Species in the genus *Piper* have a wide array of secondary compounds, principally alkaloids and amides [3]. The most widely recognized species is a black pepper, (*Piper nigrum* L) a spice traded around the world for hundreds, if not thousands, of years [7].

Alkaloids play a significant role in plant physiology, agriculture, host-plant resistance, entomology, as diet and medicine. Piperine alkaloid is the major chemical constitute responsible for the bitter taste of the black pepper. Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl groups and range from simple phenolic molecules to highly polymerized compounds [8]. Polyphenols may be associated with various carbohydrates and organic acids [9]. These compounds exhibit a wide range of physiological properties, such as anti-inflammatory, antimicrobial and antioxidant effects [8, 9].

The aims of this study were to evaluate the potential of alkaloids and phenols compounds extracted from *Piper nigrum* against some fungal dermal infections, and to determine of MIC and MFC of pathogenic fungi.

Material and Methods

Plant material and extraction

The Black pepper *Piper nigrum* fruits were purchased from local market. The fruits were air dried and powdered; and kept at 4°C until further investigations.

Preparation of plant extracts

a- Alkaloids

The extraction was prepared according to Harborne (1984) [10]. A quantity of 100g dried fruitswas homogenized in electrical shaker with 350 ml of (4:1) ethanol: D.W., then filtered Whateman filter paper No.1 with Bouknner funnel. The filtrate was concentrated to quarter of original volume, then acidified by drops of 2% H_2SO4 until the pH became between 1 and 2.The filtrate was extracted with (2:1) chlorophorm 3 times in the separating funnel. Alkaloids were precipitated by the addition drops of concentrated NH_4OH, at pH(9 –10). The precipitate was extracted with chloroform: methanol (1:3) Two and once with chloroform, two layers appeared; the lower layer which contained weak alkaloids was dried by a rotary evaporator. The aqueous upper layer was divided by Rotary, both residues were dissolved in methanol and kept in $-4c^0$ until use.

b- Phenols

The extraction was made according to flavbor and Ribbereau [11,10], A quantity of 200 g of dried fruits were divided into 2 equal quantities, one was mixed with 300 ml of D.W. and another one was mixed with 300 ml of 1% HCl. Then samples were homogenized by shaking for 5 min., after centrifugation the supernatants were mixed with equal volume of n-propanol and saturated with amount of NaCl through separation funnel, 2 layers were appeared: The lower one (aqueous layer) was extracted with amount of ethyl acetate and concentrated by rotary evaporator.

The upper layer which containing phenols were dried by rotary evaporator at 40°C.The dried material for both layers were dissolved with 5 ml of 96% ethanol, the 2 layers were dried using oven at 40°C, and kept in refrigerator until use.

Concentrations preparation:

Stock solutions were prepared by mixing 2 g of the dried extract with 20 ml Ethylene glycol, and then it was sterilized with Millipore membrane filter (0.22 µm). Then different concentrations of(0.002, 0.004, 0.02, 0.04, 0.2, and 0.4) mg ml^{-1}were prepared by mixing known volume from the stock solution with Ethylene glycol using the following equation:

C1V1=C2V2

C1= Concentration of stock solution.

V1= Volume that obtained from stock solution.

C2= Final concentration.

V2= Final volume.

Ethylene glycol was the solvent which used as diluents solution.

Fungal isolates collection

All fungal isolates were obtained from the laboratories of Biotechnology department/ College of Science/ University of Baghdad.

Evaluation of anti-fungal activity of the extracted compounds

The prepared compound evaluated against fungi in the study using Agar dilution technique according to Wang *et al*. (2005) [12] as follows:

1- Piece of 8 mm from the mycelia growth of 15 days mold culture was deposited in the center of each plate. The inoculated plates were incubated at 28 C˚ for 7-10 days. Replicates were made for each treatment.

2- Diameters of fungal colonies were measured, and then the anti-fungal activity of each concentration of the studied extract was calculated by measuring the growth inhibition using the following formula [12].

Growth inhibition% = [(Growth in control - Growth in treatment)/Growth in control] × 100

Analysis of chemical composition of the plant extracts by FLC:

The analysis of the chemical composition was made by fast liquid chromatography (FLC). FLC consists from a mobile phase which is polar and consists of a mixture of solvents such as water and acetonitrile, while the stationary phase comprises of a column which is usually stainless steel and packed with silica particles, a sample of 50µl was injected into the mobile phase and it passes along the stationary phase, the time taken for a sample to pass through the system is recorded as its retention time RT that is one of the characteristic used to identify

the compound, all the compounds were separated and identified using FLC with separation conditions C-18, 3 cm particle size, 50× 4.6 mm internal diameter of the column, detection U.V. set at 280 nm, flow rate 0.7 ml/min. and 30 C°. Mobile phase was (0.1 % acetic acid and acetonitrile with linear gradient from 0-100% B in 10 min). Phenolic compounds, and, (deionized water: methanol 40:60 V/V)or alkaloids and 0.1% acetic acid in deionized water: acetonitrile (20-80V/V). The area under a peak is used for calculating the concentration of a sample as the following formula:

Conc. of sample ($\mu g.ml^{-1}$) = Area of the sample × Standard conc. × Dilution factor

Area of the standard

Analysis of the chemical composition was made by injecting 20µl of the extract of each sample in FLC for identification. The conditions of separation were listed in Table (1,2). The peaks were detected by UV detector. The analysis was carried out in the laboratories of Ministry of Science and Technology [13].

Table (1): Conditions of Fast Performance Liquid Chromatographic used for analysis of alkaloid compounds of the plants extracts.

Parameter	Characteristic
Type of Column	C-18
Column dimensions	3µm particle size (50×4.6 mm ID)
Flow Rate	1.0 ml /min
Detector	UV spectrophotometer at 280 nm
Volume injection sample	20 µl
Mobile phase	Were 0.01 M phosphate buffer pH 6.2:acetonitrile (40:60 V/V)
Temperature	30 °C

Table (2): Conditions of Fast Liquid Chromatographic used for analysis of phenolic compounds of the plants extracts.

Parameter	Characteristic
Type of Column	C-18
Column dimensions	3µm particle size (50×4.6 mm ID)
Flow Rate	1.4 ml / min
Detector	UV spectrophotometer at 280 nm
Volume injection sample	20 µl
Mobile phase	Solvent A : 0.1% phosphoric acid in deionized water .Solvent B 20:80 V/V , 0.1% phosphoric acid in deionized water : acetonitrile HPLC grade , linear gradients 0% B-100% B.
Temperature	30°C

Statistical analysis

Complete Randomized Design (C.R.D.) was used as an experimental design. Data were analyzed using SAS [14] to study the effect of different factors on the diameters of inhibition zones. Least significant difference (LSD) was used to compare the significant difference between means at $P \leq 0.05$.

Results and Discussion

Inhibitory effects percentage (%) of extracted compounds against fungi was showed in Table (3,4). The inhibitory effects percentage varied according to fungi species, origin and type of the extracted compounds.

Table (3) indicated that, (0.4 mg/ml) concentration for alkaloids compounds showed completed inhibition against fungi under study, except *T.violaceum* which was more sensitive than other fungi that appears inhibition effect in concentration(0.2 mg/ml) of alkaloids. Besides the MIC concentration was 0.002 mg/ml for all fungi.

Table (4) showed that concentration (0.4 mg/ml) for phenols compounds appeared completed inhibition against fungi under study, while 0.2% concentration of phenols compounds showed less inhibition effect against *T. soudanense* and *M. cookie*. Also the MIC concentration was 0.002 mg/ml. Also Figure (1) and (2) showed significant differences at the level of probability ($P \leq 0.05$) between the concentrations.

Table (3): The Minimal inhibitory concentration MIC and maximum fungicidal concentration MFC of alkaloids compounds against some pathogenic fungi

		Concentrations of alkaloids (Growth inhibition %)						
	Fungus	**0.002 mg/mlMIC**	**0.004 mg/ml**	**0.02 mg/ml**	**0.04 mg/ml**	**0.2 mg/ml**	**0.4 mg/ml MFC**	**Mine A**
1	***Trichophytonrubrum***	**100.00**	**89.00**	**78.50**	**67.80**	**23.50**	**9.20**	**61.38**
2	***Trichophytonviolaceum***	**100.00**	**93.67**	**81.00**	**68.00**	**19.17**	**11.07**	**62.15**
3	***Trichophytonsoudanense***	**100.00**	**99.63**	**62.00**	**43.00**	**13.57**	**6.40**	**54.15**
4	***Trichophytonviolaceum***	**100.00**	**100.00**	**72.00**	**56.00**	**17.23**	**8.23**	**54.10**
5	***Trichophytonsoudanense***	**100.00**	**99.57**	**74.67**	**55.00**	**13.93**	**5.00**	**58.91**
6	***Trichophytonmentagrophytes***	**100.00**	**93.67**	**75.00**	**65.40**	**20.00**	**9.07**	**58.03**
7	***Trichophytonschoenleinii***	**100.00**	**93.67**	**81.20**	**50.00**	**10.30**	**3.90**	**60.52**
8	***Trichophytonverrucosum***	**100.00**	**91.00**	**79.00**	**69.00**	**24.43**	**10.43**	**56.51**
9	***Trichophytontonsurans***	**100.00**	**95.00**	**90.00**	**85.00**	**33.03**	**15.83**	**62.31**
10	***Microsporumcanis***	**100.00**	**99.80**	**99.80**	**72.93**	**23.33**	**8.17**	**69.92**
11	***Microsporum cookie***	**100.00**	**89.00**	**89.00**	**63.00**	**14.00**	**5.40**	**65.54**
12	***Microsporumaudouinii***	**100.00**	**91.50**	**91.50**	**67.50**	**18.03**	**3.93**	**58.23**
13	***Epidermophytonfloccosum***	**100.00**	**89.20**	**89.20**	**67.80**	**16.40**	**4.47**	**60.15**
	Mine B	**100.00**	**94.18**	**94.18**	**70.00**	**23.59**	**9.00**	**59.39**
	L.S.D.	**A= 0.295**		**B=0.166**		**A X B=0.723**		

Table (4):Minimal inhibitory concentration MIC and maximum fungicidal concentration MFC of phenolic compounds against some pathogenic fungi.

		Concentrations of alkaloids (Growth inhibition %)						
	Fungus	**0.002 mg/mlMIC**	**0.004 mg/ml**	**0.02 mg/ml**	**0.04 mg/ml**	**0.2 mg/ml**	**0.4 mg/ml MFC**	**Mine A**
1	***Trichophytonrubrum***	**9.03**	**24.36**	**71.40**	**64.83**	**98.50**	**100.00**	**64.83**
2	***Trichophytonviolaceum***	**14.30**	**33.26**	**92.83**	**72.06**	**96.00**	**100.00**	**72.06**
3	***Trichophytonsoudanense***	**8.00**	**21.66**	**70.00**	**63.17**	**95.10**	**100.00**	**63.17**
4	***Trichophytonviolaceum***	**9.60**	**25.76**	**75.00**	**63.09**	**89.70**	**100.00**	**63.09**
5	***Trichophytonsoudanense***	**12.80**	**28.86**	**89.00**	**70.43**	**100.00**	**100.00**	**70.43**
6	***Trichophytonmentagrophytes***	**7.20**	**19.06**	**75.00**	**61.54**	**87.00**	**100.00**	**61.54**
7	***Trichophytonschoenleinii***	**8.33**	**22.30**	**62.00**	**62.15**	**93.10**	**100.00**	**62.15**
8	***Trichophytonverrucosum***	**12.60**	**35.33**	**85.06**	**69.96**	**95.80**	**100.00**	**69.96**
9	***Trichophytontonsurans***	**3.63**	**17.16**	**75.00**	**62.11**	**93.56**	**100.00**	**62.11**
10	***Microsporumcanis***	**4.03**	**12.60**	**75.00**	**63.88**	**99.00**	**100.00**	**63.88**
11	***Microsporum cookie***	**6.33**	**24.33**	**89.00**	**69.11**	**100.00**	**100.00**	**69.11**
12	***Microsporumaudouinii***	**1.83**	**12.06**	**77.06**	**62.08**	**97.20**	**100.00**	**62.08**
13	***Epidermophytonfloccosum***	**5.43**	**23.30**	**81.40**	**67.03**	**100.00**	**100.00**	**67.03**
	Mine B	**7.93**	**23.08**	**78.28**	**64.83**	**95.76**	**100.00**	
	L.S.D.	**A=0.29**		**B=0.16**		**A x B=0.72**		

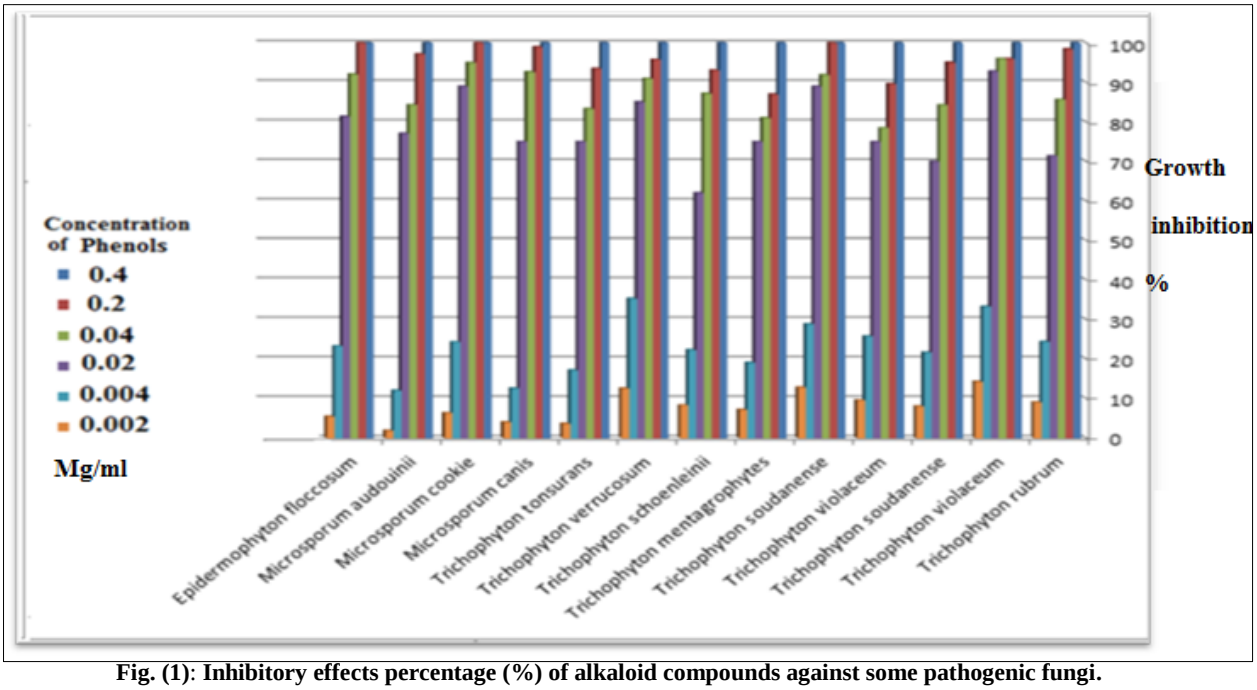

Fig. (1): Inhibitory effects percentage (%) of alkaloid compounds against some pathogenic fungi.

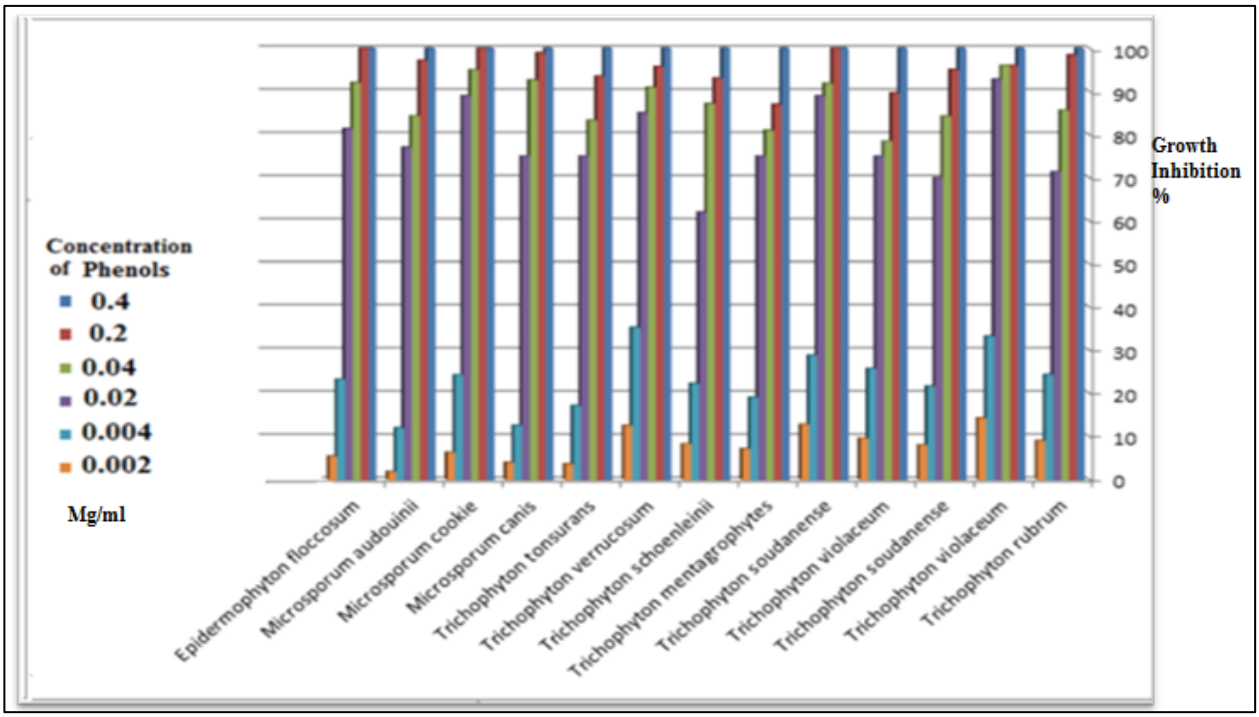

Fig. (2):Inhibitory effects percentage (%) of phenolic compounds against some pathogenic fungi.

Fast Liquid Chromatography (FLC) analysis for active compounds inplants extracts.

Alkaloids compound

The alkaloids extracted from plants identified by FLC, were elaborated in Table (5) The peaks of each compound showed in Figures (3 and 4). The total concentration of alkaloids in the extracted *P. nigrum* was156.8 μgml^{-1}.Capsaicin 137.2μgml^{-1} was the major alkaloid, while 2-dihdrocapsaicin (19.6μgml^{-1}) was the minors in the *P. nigrum.*

Table (5) Types and concentration of alkaloids in plant extracts

alkaloids compounds	(µg/ml)
Capsaicin	137.2
2-dihdrocapsaicin	19.6
Total concentration (µg/ml)	156.8

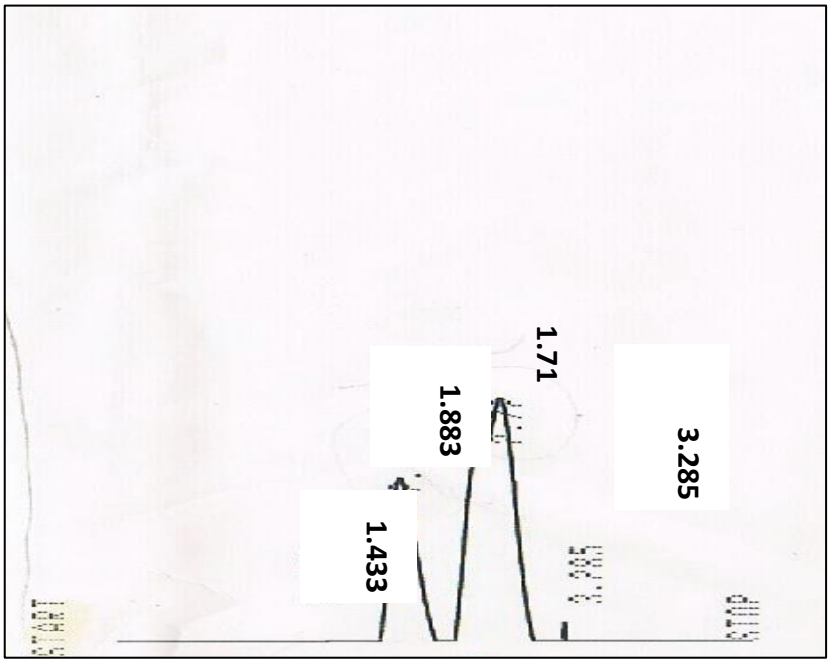

Fig. (3): FLC profile of alkaloids standards of *Pipernigrum* (1) Capsaicin,(2) 2-dihdrocapsaicin.

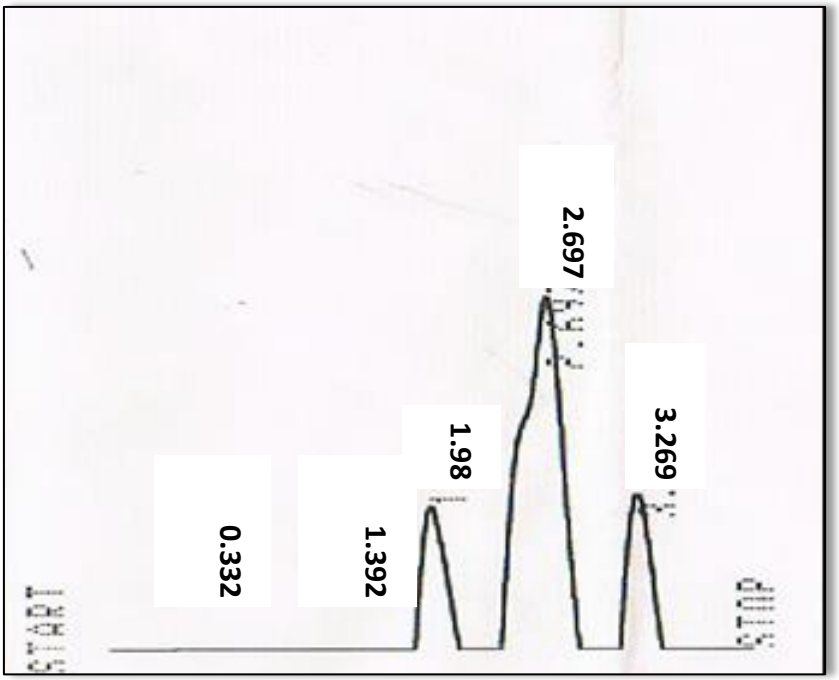

Fig. (4): FLC profile of *P. nigrum* alkaloids (1) Capsaicin, (2) 2-dihdrocapsaicin

The total concentration of alkaloids in the extracted *P. nigrum* was 156.8 µg/ml. The phytoconstitutes of *P. nigrum*minor alkaloids such as piplartin, piperlogumine, piperidine, starch, resin and pungent alkaloid. Piperine were the main therapeutically active constituent [14, 15].

Phenolic compounds

Results of FLC (fast liquid chromatography) analysis indicated the presence of ten phenolic compounds in *P. nigrum* Table (6) and figure (5 and 6). All the isolated compounds appeared to have different retention time. Chrysophanol-1-O-B- glucopyranoside (55.08 µg ml^{-1}) and Trens –p-sinapyl-â-D-glucopyranoside (147.4 μgml^{-1}) were the highest phenolic compounds in *P. nigrum*, while Anthraquionone (4.76 μgml^{-1}) and Trans-p-feruloyl-â-D-glucopyranoside (3.68 µg/ml) were the lowest concentration in *P. nigrum*.

Table (6) concentration of phenols in *p.nigrum*extract

Phenolic compoundsin *P. nigrum*	(µg/ml)
Gallic acid	32.42
Trans-p-feruloyl-â-D-glucopyranoside	3.68
Trens –p-sinapyl-â-D-glucopyranoside	147.4
Quercetin3-O-R-L-rhamnopyranoside-7-O- â-D- glucopyranosyl	62.6
Quercetin3-O-R-L-rhamnopyranoside	4.492
Luteolin 6-C- â-D-glucopyranoside-8-C-R-L-arabinopyranoside	8.02
Luteolin 7-O- [2-(â-D-apiofuranosyl)- â-D-glucopyranoside-8-C-R-L-arabinopyranoside	5.048
Luteolin 7-O- [2-(â-D-apiofuranosyl)- 4-(â-D-glucopyranosyl)	10.84
Kaempferol	11.46
Coumarins	12.92
Total concentration (µg/ml)	274.5

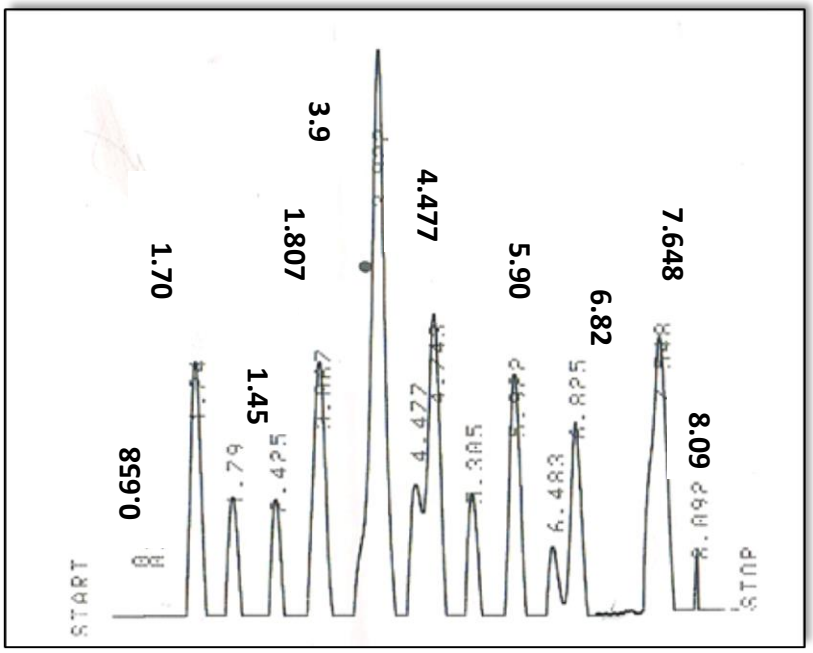

Fig. (5) FLC profile of phenols standards

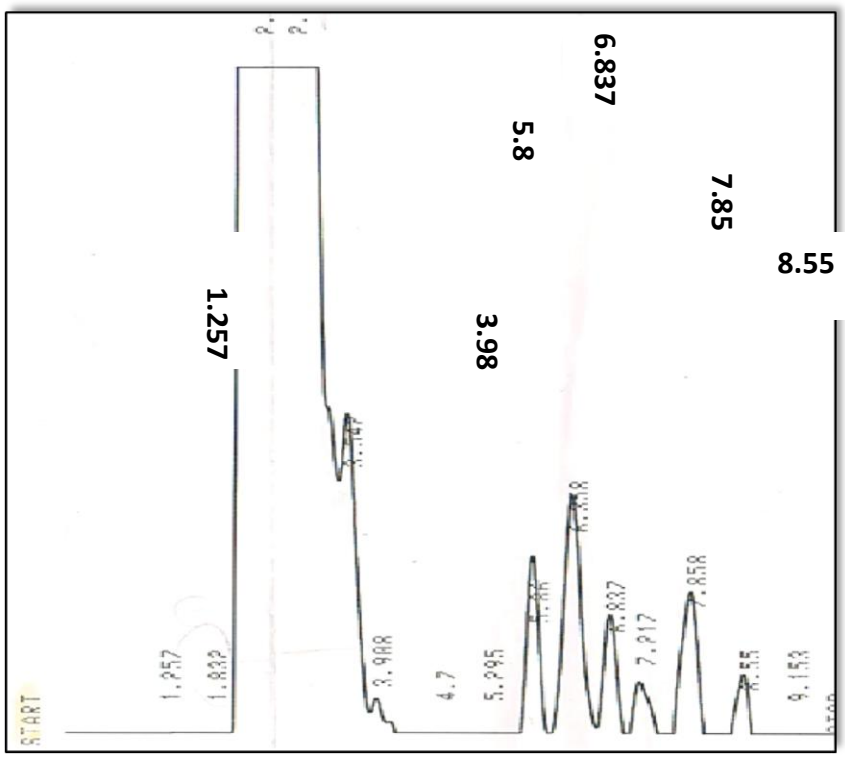

Fig. (6): FLC profile of phenols in *P. nigrum*

The present of phenolic compounds which can be hold a good promise as a natural fungicide against common pathogens of crops[17] .

Aly and Bafiel (2008) [18] found that phenolic compounds such as caffeic acid, α-thujone, cymene, ferulic acid, cimiracemoside, p-coumaric acid that used as antioxidants, anti-inflammatory, antitumor.

This variations in inhibitor effect due to presence of some active components (Two alkaloids with ten phenols compounds) that present in *P. nigrum*as showed in Table (5, 6).

Their mechanism of action appears to be predominantly on the fungal cell membrane, disrupting its structure causing leakage and cell death; blocking the membrane synthesis; inhibition of the spore germination, fungal proliferation and cellular respiration [19]. Because of high volatility and lipophilicity of the extracts, they are readily attached to penetrate into the cell membrane to exert their biological effect [20]. Also, the extracts inhibit the synthesis of DNA, RNA, proteins and polysaccharides in fungal and cells [21]. In fungi, they evoke changes similar to the effects of antibiotic action [22,23].

Fungicidal effect of phenols and alkaloids may be due to its activity in lyses of fungal cell wall and cytoplasmic membrane due the liberation of antimicrobial products and it was also plants used in reported that plant lytic enzymes act on the fungal cell traditional medicine, wall causing breakage of B-1,3 glycan, B-1,6, glycan and chitin polymer[19, 22]

Identification of medicinal plant and Antimicrobial effectiveness of species which possess antimicrobial activity against plant spices: an approach for use in food conservation [17].

Conclusion

Development of more effective and less toxic antifungal compounds is required for the treatment of dermatophytosis. Plants and their extraction preparations have been used as medicines against fungal diseases. Therefore, recommend performing further studies to separation, identification and purification of active compounds of *Piper nigrum.* Furthermore, to extract the fungicidal active ingredient from *Piper nigrum* using it as therapeutic remedy by implementing experimental trials through induced infection and treatment in laboratory animals.

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