Inhibitory effect of some medicinal plant extracts on some pathogenic fungi

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Abstract
Plants and their extracts preparations have been used as medicines against infectious diseases. In present work, *Cassia senna* (leaves), *Piper nigrum* (fruits) were extracted with different organic solvents to investigate their antifungal activities *in vitro.* However, the effective of plant extracts against some pathologic fungi (*Tricophyton rubrum, T. tonsurans, T. violaceum, Microsporum audouinii, M. canis* and *M. gypseum*) were evaluated at concentrations ranged between (0.005–5%) using agar diffusion methods and compared with standard antifungal drug (Clotrimazole). Results showed that methanol extract of *C. senna* and ethanol extract of *P. nigrum* displayed excellent inhibition on dermatophytes compared with standard antifungal drug, the MFC value for *C. senna* extract against dermatophyte isolates was (0.5%) except *T. rubrum* and *M. canis* (0.1%), the MIC was (0.05%). MFC value for *P. nigrum* extract against *T. violaceum, M. audouinii* and *M. gypseum* was (0.1%) while for *M. canis* was (0.05%), *T. rubrum* and *T. tonsurans* was (0.01%) and the MIC value was (0.005%). But MFC value for clotrimazole was (0.5%) and the MIC value was (0.1%). The yield of active compounds in *C. senna* and *P. nigrum* were (3, 6.7%) respectively. Also, the chemical compositions of these extracts were analyzed by FLC (Fast Liquid Chromatography) the result shows that the main components in *C. senna* were phenols, alkaloids and glycosides, while in *P. nigrum* were phenols, alkaloids and there was no terpens.

Key words: Plant extracts, Antifungal activities, dermatophytes, MIC, MFC, yield.

التاثير التثبيطي لبعض مستخلصات النباتات الطبية ضد بعض الفطريات الممرضة

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الخلاصه
استخدمت النباتات الطبيه ومستخلصاتها في العلاجات الطبيه وفي الوقايه من المسببات المرضه وفي هذا البحث تم استخلاص (اوراق السنمكي) و (ثمار الفلفل الاسود) المذيبات العضوية المختلفة للتحقيق في أنشطتها المضادة للفطريات في المختبر .ومع ذلك، تم تقييم فعالية المستخلصات النباتية ضد بعض الفطريات المرضيه (*Tricophyton rubrum, T. tonsurans, T. violaceum, Microsporum audouinii, M. canis*, *M. gypseum*) بتركيزات تراوحت بين (5 الى 0.005%) وباستخدام طريقة الانتشار في الاكار مقارنة مع المضادة القياسي للفطريات (Clotrimazole). وأظهرت النتائج أن المستخلص الميثانولي لاوراق السنمكي والايثانولي لثمار الفلفل الاسود فعاليه تثبيط ممتازة مقارنة مع المضاد القياسي للفطريات، بلغت قيمة MFC لمستخلص السنمكي ضد العزلات الفطور (0.5٪) باستثناء *T. rubrum* و *M. canis*

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(0.1٪) وكانت قيمه MIC (0.05٪). قيمة MFC لمستخلص الفلفل الاسود ضد *M.* T. *violaceum* *audouinii*, و *gypseum* (0.1%) في حين ل *M. canis* كانت (0.05%) و *T. rubrum*، و *T. tonsurans* كانت (0.01%) وقيمة MIC كانت (0.005%). ولكن قيمة MFC لكلوتريمازول كانت (0.5%) وبلغت قيمة MIC (0.1 ٪) لجميع الفطريات المعزوله كان العائد من المركبات النشطة في مستخلص السنمكي والفلفل الاسود هي (3.، 6.7) على التوالي . ايضا تم تحليل التراكيب الكيميائية لهذه المستخلصات بواسطة FLC اللوني السائل السريع (تظهر النتيجة أن المكونات الرئيسية في مستخلص السنمكي كانت المركبات الفينولات، والقلويديه والكلايكوسيديه بينما احتوى مستخلص الفلفل الاسود على الفينولات، وقلويدات ولاوجود للتربينات.

INTRODUCTION:

Plants have a long history of antibiotic usage for the cure of disease caused by antimicrobial, including antiviral, antibacterial and antifungal. The disease is widely distributed all over the world with various degrees and more common in men than in women. Skin, hair, nail, and subcutaneous tissues in human and animal are subjected to infection by several organisms, mainly fungi named dermatophytes and cause dermatophytoses [1,2]. Dermatophytoses are one of the most frequent skin diseases of human, pets and livestock [3].Therefore, in the last few decades, a variety of medicinal plants and plant extracts have been screened for their antimicrobial activity [4]. Plant chemical metabolic compounds were divided since nineteenth century to primary and secondary metabolic compounds [5]. Primary metabolic compounds were chemicals that produced by plants and they were essential in metabolic process of the cell [6].While secondary metabolic compounds were chemical that produced through primary metabolic process and do not have any role in plant growth and their production, but they have played a role in plant persist under special ecological conditions [5].

However, they play role in defense of plant against insects and pathogens causative [7].Secondary metabolite compounds were divided in to three main groups: Alkaloid, Phenols, and Terpenes [8]. Essential oils, derived from aromatic medicinal plants have been reported to be active against Gram-positive and Gram-negative bacteria as well as against yeasts, fungi, and viruses. They are mixtures of different lipophilic and volatile substances, such as monoterpenes, sesquiterpenes [9]. Due to the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effect of certain antimicrobial agents, there is a need to search for new agents. So the aim of present work is to evaluate the inhibitory effects of *Cassia senna* and *Piper nigrum* extracts against some dermatophytes.

Materials and Methods:

Fungal isolates

Tricophyton rubrum, *T. tonsurans*, *T.violaceum*, *Microsporum audouinii* ,*M. canis* and *M. gypseum* were obtained from the laboratories of Biotechnology department / College of Science / University of Baghdad. These fungi were identified according to [10; 11].

Collection and identification of the studied plants

Piper nigrum fruits and *Cassia senna* leaves were purchase from local herbal stores and the plant were classified and identified by Prof. Dr. Ali AL-Mosawi, the taxonomist at Dept. of Biology, College of Science, University of Baghdad.

Crude oils extraction

The oil extract of the *P. nigrum* fruits by ethanol 80% and *C.senna* leaves by methanol 95% were prepared by using soxhlet apparatus for continuous extraction of solids. This apparatus consists of condenser, extractor and flat bottom flask [12].

Evaluation of anti-fungal activity of the extracted oils

Various volumes of the crude and oil extracts were mixed apart with 100 ml of SDA (Sabouraud dextrose agar) to prepare the required concentrations (0.005, 0.01, 0.05, 0.1, 0.5, 1, 3, 5%).

1-The blend of both plant extract and SDA was shake well and poured in petri dishes and left to solidify in a sterile conditions.

2- Piece of 8 mm from the mycelial growth of mold culture of 15 days 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.

3- Diameters of fungal colonies were measured, and then the antifungal activity of each concentration of the studied extract was calculated by measuring the growth inhibition using the following formula [13].

$$\text{Growth inhibition\%} = \frac{[\text{Growth in control} - \text{Growth in treatment}]}{\text{Growth in control}} \times 100$$

Determination of extraction yield (% yield)

The yield (%) of all the dried extracts was calculated as follows:

Yield (%) = (W1 *100) / W2 where**:-**

W1 is the weight of the extract after lyophilization , and W2 is the weight of the plant powder.

Analysis of chemical compositions of the plants extracts

Analysis of the chemical compositions was made by injecting 20 µl of the extract of each sample in FLC (Fast Liquid Chromatographic). The concentration of each isolated compound was determined by following equation: [14].

$$\text{Conc. of sample (µg/ml)} = \frac{\text{Area of the sample}}{\text{Area of the standard}} \times \text{standard conc.} \times \text{Dilution factor}$$

Results and Discussions:

Evaluation of inhibitory effects of the extracted oils against dermatophytes

Inhibitory effects percentage (%) of extracted oils against fungi (*T. rubrum, T. tonsurans, T. violaceum, M. audouinii, M. canis* and *M. gypseum*) were tested using SDA medium, and clotrimazole was used as an antifungal agent due to its remarkable inhibitory effect on fungi under study. Results indicated that, (5%) concentration of each (*C. senna, P. nigrum* and Clotrimazole) appeared 100% inhibition against all dermatophytes under study.

Determination the MIC and MFC values for plant extracts against dermatophytes isolates.

- Cassia senna

The methanolic extract of *C. senna* shows a fungicidal activity against *T. rubrum* and *M. canis* at concentration (0.1%) Table 1, while *T. tonsurans, T. violaceum, M. audouinii* and *M. gypseum* were less sensitive, the inhibitory effects of this extract were (61.1, 50, 53.3 and 42.3%) respectively. There are significant differences ($P \leq 0.05$). And at concentration (0.05%), *T. rubrum* and *M. canis* were inhibited (15% and 40%) respectively. The MFC value for *C. senna* extract against dermatophytes isolate was (0.5%) except *T. rubrum* and *M. canis* (0.1%), the MIC was (0.05%), but the MFC for clotrimazole was (0.5%) and MIC (0.1%) against all dermatophytes under study.

Cassia alata leaves were extracted with petroleum ether followed by 85% hot ethanol under reflux and tested for its antifungal activity against *C. albicans, A. fumigatus, A. flavus, Mucor* spp., *Rhizopus* spp. and dermatophytes; *T. mentagrophytes, T. rubrum*and *M. gypseum*. They reported that 20% w/v crude extract did not show any significant activity against the contaminant fungi, whereas 2.5 and 3% crude extract completely inhibited the growth of dermatophytes [15].Whereas [16] found that the ethanolic extract of *C. alata* leaves showed antifungal activity at concentration 500mg/ml against *Trichophyton* sp, *Microsporum* sp, *Aspergillus* sp. and *Penicillium* sp, but not yeasts (*C. albicans* and *Cryptococcus neoformans*). The antifungal substances contained in the leaf extracts of *Cassia* species may have similar mechanism that could be related to fluid leaks in cells and the inhibitory effects percentage varied according to fungi species and the origin of the extracted oils [17; 18].

Table 1- Growth inhibition of *Cassia senna* methanolic extract against dermatophytes isolates on (SDA) medium at 30°C after 7 days .

Extract con%	*T. rubrum*	*T. tonsurans*	*T. violaceum*	*M. audouinii*	*M. canis*	*M. gypseum*
0.05	15	34	28.5	33	40	42
0.1	100	61.1	50	53.3	100	42.3
0.5	100	100	100	100	100	100
1	100	100	100	100	100	100
3	100	100	100	100	100	100
5	100	100	100	100	100	100

LSD ($P \leq 0.05$) between the means = 2.21

Clotrimazole used as control.

- Piper nigrum

The activity effects of *P. nigrum* ethanolic extract against (*T. rubrum, T. tonsurans, T. violaceum, M. audouinii, M. canis* and *M. gypseum*) were summarized in Table-2. At concentration (0.005%) *T. rubrum* and *T. tonsurans* were more sensitive than other fungal isolates; inhibitory effect was (83.3%). Only the growth of *M. canis* was inhibited (44.4%) at (0.01%) concentration. While *T. violaceum, M. audouinii*and *M. gypseum* were more resistant to this extract therefore (0.05%) concentration was used, inhibitory effects were (42, 34.3 and 50%) respectively. The statistical analysis showed significant differences at the level of probability ($P \leq 0.05$) between the concentrations. The MFC value for *P. nigrum* against *T. violaceum, M. audouinii*and *M. gypseum* were (0.1%) while *M. canis* (0.05%), *T. rubrum* and *T. tonsurans* was (0.01%) and the MIC value (0.005%).

Methanolic extract of *C. senna* and ethanolic extract of the *P. nigrum* displayed excellent inhibition on dermatophytes than clotrimazole Table-3.These extract might be exploited as natural drug for the treatment of several diseases caused by these organisms and could be useful in understanding the relations between traditional cures and current medications [19]. Generally, crude extracts are a mixture of active and non-active compounds (crude fusions) are expected [20].

The methanolic root extract of *P. longum*was evaluated for antifungal activity against *Chrysosporiu manum, C. keratirophilum, C. lobatum, C. tropicum, M. gypseum, M. nanum, T. ajelloi, T. mentagrophytes* and *T. terrestre,* the extract was effective in inhibiting species with zone of inhibition ranging between 3 mm and 11 mm but the extract showed no zone of inhibition for *C. keratirophilum*. The results indicate that the methanolic root extract of *P. longum* might be exploited as natural drug for the treatment of several infection caused by these organisms [21]. 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 [22]. Because of high volatility and lipophilicity of the essential oils, they are readily attached and penetrate into the cell membrane to exert their biological effect [23].

Table 2- Growth inhibition of *Piper nigrum* ethanolic extract against dermatophytes isolates on (SDA) medium at 30°C after 10 days.

Extract con.%	*T. rubrum*	*T. tonsurans*	*T. violaceum*	*M. audouinii*	*M. canis*	*M. gypseum*
0.005	83.3	83.3	N.D	N.D	N.D	N.D
0.01	100	100	N.D	N.D	44.4	N.D
0.05	100	100	42	34.3	100	50
0.1	100	100	100	100	100	100
0.5	100	100	100	100	100	100
1	100	100	100	100	100	100
3	100	100	100	100	100	100
5	100	100	100	100	100	100

LSD ($P \leq 0.05$) between the means = 1.04
Clotrimazole used as control .
N.D: not done.

Table 3- Growth inhibition of Clotrimazole against dermatophytes isolates on (SDA) mediumat 30°C after 7 days.

Extract con%	*T. rubrum*	*T. tonsurans*	*T. violaceum*	*M. audouinii*	*M. canis*	*M. gypseum*
0.1	10	66.6	2.8	6.6	6.6	5.8
0.5	100	100	100	100	100	100
1	100	100	100	100	100	100
3	100	100	100	100	100	100
5	100	100	100	100	100	100

LSD ($P \leq 0.05$) between the means = 2.21 .

Oil yields of the studied plants

The yields of oil extracts from *Cassia senna* leaves and *Piper nigrum* fruits were 3 and 6.7 % respectively Table-4. [24] found that, the yields of the methanolic extracts of *C. alata, C. fistula* and *C. tora* leaves were 6.4, 4.5 and 2.2%, respectively.

Table 4- Yields of the extracted oils from plants involved in this study.

Plant	Family	Type of oil	Collection place	Yield of the extracted oil%
Cassia senna	Leguminosae	Crude	Local market	3*
Piper nigrum	Piperaceae	Crude	Local market	6.7*

[25] have reported that the wide rang variation between the medical plant yields due to the extraction solvent and plant material used. In general, the yields obtained from these plants are quite adequate there by making further development of these herbal drugs economically feasible.

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

Results have been shown important and significant differences between the concentrations of each secondary metabolic compound among the extracted cruds, whether it were terpen, phenol, glycosids or alkaloid or even between their total concentration in the same cruds or between them and those in the other studied cruds plants.

- Phenolic compounds

Results of FLC (fast liquid chromatography) analysis indicated the presence of 5 phenolic compounds in *C.senna* and 10 in *P. nigrum* Table-5.All the isolated compounds appeared to have different retention time. Chrysophanol-1-O-B- glucopyranoside (55.08 µg/ml) and Trens –p-sinapyl-â-D-glucopyranoside (147.4 µg/ml) were the highest phenolic compounds in *C.senna* and *P. nigrum*, respectively while Anthraquionone (4.76 µg/ml) and Trans-p-feruloyl-â-D-glucopyranoside (3.68 µg/ml) were the lowest concentration in *C. senna* and *P. nigrum*, respectively.

The presence of phenolic compounds which can be held a good promise as a natural fungicide against common pathogens of crops [26).

Found that the phenolic compounds such as caffeic acid, α-thujone, cymene, ferulic acid, cimiracemoside, p-coumaric acid that used as antioxidants, anti-inflammatory, antitumor [27].

Table 5- Types and concentrations of phenols in plant extracts

Phenolic compounds(µg/ml)	Plant	
	C. senna	*P. nigrum*
Caffeic acid		-
Fistulic acid	**11.55**	-
Chrysophanol	**20.38**	-
Chrysophanol-1-O-B- glucopyranoside	**55.08**	-
Anthraquionone	**4.76**	-
Hydroxyl-anthraquionone	**35.74**	-
Chlorogenic acid	-	-
Gallic acid	-	**32.42**
Protocatcheic acid	-	-
Syringic acid	-	-
OH-benzoic acid	-	-
D-coumaric acid	-	-
Vanillic acid	-	-
Caetchol	-	-
Sinapic acid	-	-
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)	**127.51**	**274.5**

- Alkaloids

Alkaloids present in the extracted plants which identified by FLC were elaborated in Table-6. The total concentration of alkaloids in the extracted of *C. senna* and *P. nigrum* were (322.56 and 156.8 µg/ml) respectively. The highest concentration of alkaloids in *C. senna* was Senoside A (102.09 µg/ml) and in *P. nigrum* was Capsaicin (137.2 µg/ml), the lowest concentration of alkaloids in *C. senna* and *P. nigrum* were (Senoside B 70.47 and 2-dihdrocapsaicin 19.6 µg/ml).

Table 6- Types and concentration of alkaloids in plant extracts

alkaloids compounds(µg/ml)	Plants	
	C. senna	*P. nigrum*
Atropine	-	-
Hyocyamine	-	-
3-OH-tropane	-	-
Tropine	-	-
Scopolamine	-	-
7-OH-Hyocyamine	-	-
Senoside A	**102.09**	-
Senoside B	**70.47**	-
Hydroxymusicin	**75.00**	-
Muscicin	**75.00**	-
Capsaicin	-	**137.2**
2-dihdrocapsaicin	-	**19.6**
Total concentration (µg/ml)	**322.56**	**156.8**

- Terpens and glycosides:

The total concentration of glycosides in the extracted crude of *C. senna* was 319.29 µg/ml, Apigenin-6,8-di-c-glycosides (82.28 µg/ml) was the highest glycosides, while Tinnevellin (44.85 µg/ml) was the lowest concentration Table-7. But in *P. nigrum* extract there was neither terpens nor glycosides components. The phytoconstitutes of *P. nigrum* and *P. longum* fruits include minor alkaloids such as piplartin, piperlogumine, piperidine , starch, resin and pungent., alkaloid piperine was the main therapeutically active constituent [28, 29].

Table 7- Types and concentration of terpens and glycosides in plant extracts

Compounds (µg/ml)	Plants	
	C. senna	*P. nigrum*
α-pinene	-	-
Camphene	-	-
Myrcene	-	-
Thujone	-	-
Linalool	-	-
Cymene	-	-
β –phellandrene	-	-
*Isorhmnetin-3-OB-β-gentiosides	71.96	-
*Apigenin-6,8-di-c-glycosides	82.28	-
*Emodin-8O- β-glucopyanosides	49.71	-
*Kaempferol	70.49	-
*Tinnevellin glycosides	44.85	-
Total Concentration (µg/ml)	**319.29**	**Nile**

* glycosides compounds

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