

In vitro* effects of laser beam on antifungal activity of crude chloroform extract produce from leaves of *concarups spp

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Abstract

The current study aimed to evaluate the effects of laser irradiation on the antifungal activity of the crude chloroform extract which was produced from leaves of *Conocarpus* species by which DMSO concentration (50 mg / ml) of this extract was exposed to laser radiation and other concentration was not exposed. Both concentrations were tested against the growth of *Cryptococcus neoformans*, *Microsporium canis*, *Trichophyton mentagrophytes*, and *Rhodotorula mucilaginosa*. Results showed that *C. neoformans* was sensitive only laser- exposed chloroform extract. While, the growth of other fungal species was unaffected either from non-laser - exposed chloroform extract or laser-exposed one. GC-MS analysis resulted in a content of the laser exposed crude chloroform extract form [pyrazolo [1,5-a]pyrimidine-3-carbonitrile,2-methylthio-7-(2-pyridyl), [benzaldehyde,2,4-bis(trimethylsil oxy),[3-hydroxymandelic acid, ethyl ester, di-TMS, and [pyrimidine-4,6(3H,5H)-dione, 2-butylthio-] while non-laser exposed extract had [carbamic acid, methyl-, phenyl ester], [acetic acid, phenyl ester], [phenol], [pentadecanoic acid, methyl ester], [hexadecanoic acid, methyl ester], [methyl 8-methyl-nonanoate], [phytol, acetate], [3,7,11,15-tetramethyl-2-hexadecen-1-ol], [citronellyl isobutyrate], [5H-2a,4a,7a-triaza-7b-phosphacyclopent[cd]indene-7b-thione, 1,2,3,4,6,7-hexahydro-], [olean-12-en-28-oic acid, 3-oxo-, methyl ester], [4-amino-5,7-dichlorobenzofurazan], [phenol, 2,4-dichloro-6-nitro], [], [cyclobarbital], [benzo[h]quinoline, 2,4-dimethyl-], [pyrido[2,3-d]pyrimidine, 4-phenyl-], [1H-indole, 5-methyl-2-phenyl-], and [carbonic acid, butyl octadecyl ester].

Keywords: Antifungal activity. *Conocarpus* species, Laser radiation.

Introduction

Medicinal plants represent a rich source for producing drugs, traditional medicines, and agents for drug synthesis (1,2). Traditional medicine is currently as a system for the health of the peoples. Developing countries still use traditional medicine. Also, it has been increasingly used in developed countries (3).

Many drugs belong to plants as natural products for their synthesis; therefore, studies carry out to get new drugs for treating the diseases. For these reasons, plants are sources of benefit compounds used as commercially consumed drugs (4). *Conocarpus lancifolius* and *C. erectus* are two species of *Conocarpus* in the family Combretaceae. Somalia, Djibouti, and Yemen are the origin of this genus which grows in the coastal and riverine areas (5) *C. lancifolius* can grow in the extreme and stressful environmental conditions. They can produce antimicrobial, antioxidant, and anticancer agents (6,7). The present study was designed to evaluate a promotion of the laser radiation on the antifungal activity of the crude chloroform extract produced from leaves of the *Conocarpus* species.

Material and methods

Preparation of plant extract

Extraction was done as described previously (9). A tree of *Conocarpus* species (Fig.1), growing in the Province of Thi-Qar, south of Iraq, was selected to collect some of its healthy leaves. The leaves were washed with

tap water distilled water, and stand at room temperature for three days to dry. The dried leaves were pulverized using a small mill for getting them a powder. The powder (200 g) were placed in a glass beaker (sized-1 liter). Absolute petroleum ether (700 ml) was then poured on this powder.

Beaker was tightly covered with two parafilm layers, and stand at room temperature for 4 days. After that, the soaked powder was filtrated using a filter paper containing a film layer (2 g) of the activated charcoal. The filtrate and soaked powder were separately left to evaporate the petroleum ether. Then, dried powder was treated with 700 ml of aqueous methanol (80%) for 7 days. The separation method of the filtrate was similar to the petroleum ether. Finally, the methanol free powder was also treated again, but the absolute chloroform was used as the same method for getting filtrate, which was evaporated at room temperature to obtain a crude chloroform extract (solid-state).

Antifungal activity test

An amount (50 mg) of the laser-exposed crude chloroform extract was dissolved in DMSO to obtain a concentration was 50 mg / ml and the same concentration of the non-laser exposed extract was also prepared. Both concentrations and discs of ketoconazole were tested against *Microsporium canis*,, *Trichophyton mentagrophytes*, *Rhodotorula mucilaginosa* and *Cryptococcus neoformans* obtained from isolate bank of Veterinary Microbiology Laboratory/ Hospital in Wasit, in which collected from different clinical

infected animals. This test was done using preparing Petri dishes of the Sabourad dextrose agar (SDA), and each dish was inoculated with an inoculum of one fungal species. After 5 min, two wells (6 mm in diameter of each well) were aseptically done and 100 µm of each concentration was loaded into a well. In addition, ketoconazole, positive control, was also placed on the dishes. All dishes were incubated at 25 °C, but the incubation periods were different at three days for *R. mucilaginoso* and *C. neoformans*, *M. canis* and *T. mentagrophytes* were left for 10 days. Finally, inhibitory zones (I.Z.s) were measured by mm (9).

Colored chemical tests of the non-laser exposed chloroform extract

Detection of sterol /terpenoids

1-Salkowski's test: was done by adding 2 to 3 milliliters of chloroform to a solution of chloroform extract that had not been subjected to laser light before filtering. While handling the filtrate, surface applying a few drops of strong sulphuric acid to the test tube's surface was customary without shaking. However, the advent of golden yellow hue indicates the presence of triterpenes, if reddish - brown coloring of the interface forms, it indicates to the existence of terpenoids.

2-Copper acetate test: was done by adding a few drops of copper acetate solution to (5%) 2-3 ml obtained from the non-laser exposed chloroform extract solution. Emerald green coloration is a sign of the diterpenes presence.

Detection of alkaloids

1-Wagner's - test: was applied by combining 2 ml of Wagner's reagent (Iodine in a content of the potassium iodide) with 2-3 ml obtained from the non-laser exposed chloroform extract. Alkaloids were presented when a brown or reddish-brown precipitate forms.

2-Dragendroff's test: was applied using 0.5 ml of Dragendroff's reagent (solution of potassium bismuth iodide) after 2-3 ml of the plant extract had been treated with 1 drop of sulfuric acid. The alkaloids were presented when a red precipitate forms.

Detection of Glycosides

General test

It was done using non-laser exposed chloroform extract solution (5 ml), treated with a few drops of aqueous NaOH solution (10%). If a yellow color forms, it indicates the glycoside presence.

Detection of phenolic compounds/ Flavonoids

1- Alkali test: was done by adding a few drops of aqueous sodium hydroxide solution into (40%) 2 ml of the non-laser exposed chloroform extract. A yellow-orange color which leads to get the colorless due to adding a few drops of dilute acetic acid indicates the flavonoids presence.

2-Lead acetate test: was done by adding a few drops of lead acetate solution (10%) into 2 ml of plant solution. The appearance of a white precipitate indicates the presence of phenolic compounds.

Detection of tannins

1- Ferric chloride test: was done by adding non-laser exposed chloroform extract (1ml) into 2 ml of water. Then 2-3 drops of diluted ferric chloride solution (5%) was added. If a green to blue-green color forms, it indicates catecholic tannins presence and a blue-black indicates the gallic tannins presence.

2-Gelatin test: was performed by adding sodium chloride gelatin solution (1%) into 2-3 ml obtained from the plant extract solution. When a white precipitate forms, it indicates the tannins presence.

Detection of saponin glycosides

1-Foam test: was applied by mixing 2-3 ml of distilled water with the non-laser exposed chloroform extract (1 ml). Once, the mixture was shaken vigorously. If the foam which lasts for ten minutes, it is an indicator for the saponin presence.

2-Froth test: was applied using plant extract diluted (1 ml) with distilled water (20 ml) and agitated for 15 min in a graduated cylinder. If a one - centimeter foam layer lasts was formed for 15 min, it is an indicator for the saponins presence.

GC-MS Analysis

Using Agelint 7820A, USA GC Mass Spectrometer, laser –exposed crude chloroform extracts and non-laser - exposed one was submitted to the GC-MS device and conditions of analysis were illustrated in (Table 1).

Antifungal activity

Non-laser - exposed crude chloroform extract exhibited no inhibitory effect against tested fungal species, (*C. neoformans*, *R. mucilaginosa*, *M. canis*, and *T. mentagrophytes*). Regarding laser - exposed extract appeared, an inhibitory zone (30 mm) against *C. neoformans* was only detected (Table,2) (Fig. 3).

Colored chemical tests of the non-laser exposed chloroform extract

The tests appeared the non-laser crude chloroform extract contained sterol /terpenoid and no alkaloids in its content. Other tests did not reveal any result because the plant extract could not be dissolved in the test solutions (Table, 3).

GC-MS Analysis

GC-MS analysis appeared compounds in contents of the non-laser crude chloroform extract through 20 peaks during 7.190 - 27.462 min. and the sum of corrected areas: 297062543. While, solvent (DMSO) of the sample was detected in all peaks except peak 3. Only peak 3 of the non-laser exposed extract did not appear in the solvent (Table 4). Related to the laser-exposed extract exhibited compounds by 20 peaks during 6.780 - 31.659 min. and sum of corrected areas: 193728824.



Fig.1: *Conocarpus* spp. tree



Fig. 2: Exposure of the crude chloroform extract to the laser radiation.

Colored chemical tests of the non-laser exposed chloroform extract

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Detection of saponin glycosides

1-Foam test: was applied by mixing 2-3 ml of distilled water with the non-laser exposed chloroform extract (1 ml). Once, the mixture was shaken vigorously. If the foam which lasts for ten minutes, it is an indicator for the saponin presence.

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Table 1: Parameters and conditions of CG-MS analysis

Parameters of analysis	
Injection volume	1µl
G.C. inlet line temperature	250 °C
Pressure	11.933 psi
Injector temperature	250 °C
Scan range	m/z 25-1000
Analytical column	Agilent HP-5ms ultra lneit: 30 m length x 250 µm internal diameter x 0.25 µm film thickness
Aux heaters temperature	300 °C
Service gas	Helium 99.99 %
Injection type	Split less
Application temperature	Ramp 1: 60 °C for 3 min. Ramp 2: 60 -180 °C for 7 min. Ramp 3: 180°C - 280 for 8 min. Ramp 4: 280°C for 3 min.

Results

Antifungal activity

Non laser - exposed crude chloroform extract exhibited no inhibitory effect against tested fungal species, (*C. neoformans*, *R. mucilaginosa*, *M. canis*, and *T. mentagrophytes*). Regarding laser - exposed extract appeared, an inhibitory zone (30 mm) against *C. neoformans* was only detected (Table,2) (Fig. 3).

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Table 2: Producing antifungal activity from laser - exposed crude chloroform extract and non-laser - exposed crude chloroform extract

Tested fungi	Inhibitory zones (I.Z.s) measured by mm		
	LEC	NLEC	Ketoconazole
<i>Cryptococcus neoformans</i>	30	No effect	No effect
<i>Rhodotorula mucilaginosa</i>	No effect	No effect	No effect
<i>Microsporium canis</i>	No effect	No effect	No effect
<i>Trichophyton mentagrophytes</i>	No effect	No effect	No effect

LEC: Laser 450 wavelength and 50 mw power - exposed crude chloroform extract. NLEC: Non-laser exposed crude chloroform extract.

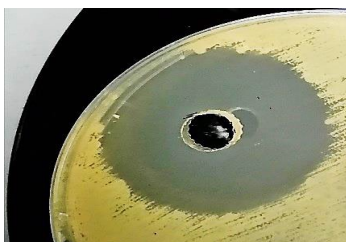


Fig. 3: Antifungal activity of laser - exposed crude chloroform extract against *C. neoformans*.

Table 3: Compounds in the non-laser exposed chloroform extract using colored chemical tests

Compounds	Results
Sterol /Terpenoids	Present
Alkaloids	Absence
Glycosides	-----
Phenolic compounds	-----
Tannin	-----
Saponone glycosides	-----

--The test solution could not dissolve the plant extract

Table 4: GC-MS analysis of crude chloroform extract.

Content of laser - exposed crude chloroform extract			
Compounds	Peaks	R.T.s	Areas
Pyrazolo[1,5-a]pyrimidine-3-carbonitrile,2-methylthio-7-(2-pyridyl)			
Benzaldehyde, 2,4-bis(trimethylsil oxy)-	3	7.892	2.33
3-Hydroxymandelic acid, ethyl ester, di-TMS			
Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	11	19.209	0.32
Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	12	19.446	0.34
Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	14	21.032	0.74
Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	15	21.464	1.90
Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	16	21.841	0.23
Content of non laser - exposed crude chloroform extract			
Carbamic acid, methyl-, phenyl ester			
Acetic acid, phenyl ester	3	7.104	2.54
Phenol			
Pentadecanoic acid, methyl ester			
Hexadecanoic acid, methyl ester	10	18.594	1.45
Methyl 8-methyl-nonanoate			
Phytol, acetate			
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	14	22.014	2.41
Citronellyl isobutyrate			
5H-2a,4a,7a-Triaza-7b-phosphacyclopent[cd]indene-7b-thione, 1,2,3,4,6,7-hexahydro-	18	27.419	2.19

 Olean-12-en-28-oic acid, 3-oxo-, methyl ester

4-Amino-5,7-dichlorobenzofurazan

Phenol, 2,4-dichloro-6-nitro

Cyclobarbital

19 30.170 0.90

Benzo[h]quinoline, 2,4-dimethyl-

Pyrido[2,3-d]pyrimidine, 4-phenyl-

1H-Indole, 5-methyl-2-phenyl-

20 31.659 0.64

Carbonic acid, butyl octadecyl ester

Discussion

Plants contain compounds, alkaloids that possess antimicrobial activity. Example of the plants is *Conocarpus lancifolius* and *C. erectus* (7,10,11,12). Methanol and chloroform were used to extract antimicrobial compounds produced from *Conocarpus* that inhibited the tested microorganisms (11,12). Selection a solvent has been considered a great step to obtain antimicrobial products from a plant extract. A consideration of the solvent selection has interested because extraction of the plant contents depends on the affinity of the contents and solvents. Therefore, plant essential oils are extracted by absolute petroleum ether (13). Petroleum ether extract the fixed and essential oils. While, chloroform used to obtain the alkaloids. Petroleum ether and chloroform represent non-polar ones that can extract non-polar compounds in substance (14,15). Other researchers demonstrated the extracted essential oil of two plants, *Cinnamomum zeylanicum* and *Citrus limonum* had effects on some microbial pathogens (16,17). In addition researchers showed that essential

oil produced from the plant of *Lippia alba* resulted in antimicrobial activity (18).

Relating to solvent selection, the aim of using water and alcohol is to extract the glycosides (19,13). Researchers showed that the plant extracts of three different solvents varied in their antimicrobial activity against tested microorganisms that the aqueous methanol extract had more bioactivity than both extracts of petroleum ether and chloroform (20). Leaf extract of *Conocarpus lancifolius* [Engl.] tannins are natural products from several plants with a large content of phenolic compounds. Tannins are classified into hydrolyzable and condensed. The structure of hydrolyzable tannins is the mixing of simple phenols with ester linkage. While, the condensed tannins having flavonoids as units possess several degrees of condensation. Tannins have antimicrobial activity as microbial inhibitors (21,15). Scientists found that using methanol in plant extraction produced a large amount of phenolic yielding (22, 23,24). Moreover, *C. erectus* could inhibit the soil-born pathogenic fungi and human pathogenic

bacteria besides *C. albicans* and GC-MS analysis detected phenolic compounds within the extract (25)

This study showed the laser - exposed crude chloroform extract exhibited antifungal activity against *C. neoformans* only where no effects were on the growth of other tested fungi. Non-laser exposed extract had no inhibitory effects. Based on a concept of the solvent and laser effect, the results may be attributed to some reasons. First: The extraction process of the crude extract produced from leaves of *Conocarpus* species was carried out sequentially by which petroleum ether was first used to extract essential oils and other products. In water and methanol extracted most of the antimicrobial compounds was in the *Conocarpus* leaves powder. Finally, chloroform (Table, 3) was used in the extraction. This may lead to chloroform extract having a very small amount of antifungal compounds that were insufficient to reveal effects on the tested fungi. The second reason may be due to the effect of the laser radiation on the extract of chloroform that was fabricated (Table, 4) leading it to produce remarkable inhibitory effect on the growth of *C. neoformans*. This explanation was supported by a previous study (26) which showed that the plant extracts contain alkaloids, polyphenols, flavonoids, polysaccharides, proteins, enzymes, steroids, and reducing sugar may help to cause the fabrication in their structures when they are exposed to the effective agent. As well as, the composition of the fungal cell walls especially in the filamentous fungi where two species of them, *M. canis* and *T. mentagrophytes*, were

used in the present study . The composition may prevent permeability of the little compounds in the content of the crude chloroform extract into these walls making the dermatophytic fungi unaffected. However, the inhibitory result was noticed against *C. neoformans* (Fig.3) due to laser - exposed extract.

Conclusion

This study concluded that the laser radiation fabricated structure of the crude chloroform extract produced from leaves of *Conocarpus* species. The fabrication produces an inhibitory effect on one species of the tested fungi compared with non-laser exposed crude extract which did not give any effect against the growth of these fungi. However, this study needs further works to test other powers of the laser radiation besides other fungal species.

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Ethical statement: There is original research work not yet published elsewhere, and the authors declare no conflict of interest.

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التأثير المختبري لشعاع الليزر على النشاط المضاد للفطريات لمستخلص الكلوروفورم الخام المنتج من أوراق *Conocarpus Spp*

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الخلاصة

هدفت هذه الدراسة إلى تقييم تأثير تشعيع الليزر على النشاط المضاد للفطريات لمستخلص الكلوروفورم الخام الذي تم إنتاجه من أوراق أنواع كونوكاربوس حيث تعرض تركيز 50 DMSO (مجم / مل) من هذا المستخلص لأشعة الليزر. تم اختبار كلا التراكييز ضد نمو *Trichophyton*، *Microsporium canis*، *C. neoformans*، و *Rhodotorula mucilaginosa*، أظهرت النتائج أن *C. neoformans* كانت حساسة فقط لمستخلص الكلوروفورم المعرض بالليزر بينما لم يتأثر نمو الأنواع الفطرية الأخرى سواء من مستخلص الكلوروفورم غير المعرض لليزر أو المعرض لليزر. نتج عن تحليل GC-MS محتوى من مستخلص الكلوروفورم الخام المعرض لليزر المحتوي على [1-pyrazolo-5-a] بيريميدين-3-كاربونيتريل، 2-ميثيلثيو-7 (2-بيريديل) [di-TMS]، بنزالديهيد، 2،4 - مكرر (ثلاثي ميثيل سيل أوكسي)-، حمض 3-هيدروكسي مانديليك، إيثيل إستر، [di-TMS]، و [بيريميدين-4، 6 (3 ساعات، 5 ساعات) -ديون، 2-بوتيلثيو-] بينما يحتوي المستخلص غير المعرض لليزر حمض كارباميك، ميثيل، فينيل إستر [، حمض أسيتيك، إستر فينيل [، فينول [، حمض خماسي ديكانويك، ميثيل إستر [، حمض هيكساديكانويك، ميثيل إستر [، ميثيل 8 ميثيل نونوات [، ytol]، أسيتات [، [15، 11، 7، 3-رباعي ميثيل-2-هيكساديسين-1-أول [، [سيترونيل أيزوبوتيريت [، [5، 2a، 4، a-7-triaza-olean-12-en-28-]، [7b-phosphacyclopent [cd] indene-7b-ثيون، 7، 6، 4، 3، 2، 1-هيكساهيدرو-]، [28-olean-12-en-28-]، [7-dichlorobenzofurazan-4-amino-5-methyl ester]، [3-oxo-، oic acid]، [6-نيترو [، [، [سيكلوباربيتال [، [بنزو [ح] كينولين، 4، 2-ثنائي ميثيل-]، [بيريدو [3، 2-د] بيريميدين، 4-فينيل [، [H-indole 1، 5-ميثيل-2-فينيل-]، و [حمض الكربونيك، بوتيل أوكتابيسيل إستر].

الكلمات المفتاحية: النشاط المضاد للفطريات، جنس *Conocarpus*، شعاع الليزر.