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In Vitro Effects of Laser Radiation on the Antifungal Activity of *Eucalyptus spp.* Leaves Extract by chloroform

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SOME plants have been interested as important sources in which the drugs including antimicrobial agents can be prepared. This study aimed to evaluate effects of the laser radiation on the Chloroform crude extract that was produced from *Eucalyptus* species leaves. Both extracts either laser-exposed crude chloroform extract and non-laser exposed one were *in vitro* tested against the growth of some pathogenic fungal species were *Candida albicans, Candida parapsilosis, Aspergillus fumigatus, Microsporum canis,* and *Trichophyton canis* isolated from animal obtained from isolate bank of Veterinary Microbiology laboratory/ Collage of the Veterinary Medicine/University of Mosul. The results of this study showed the laser-exposed Chloroform crude extract produced inhibitory zones against C. *parapsilosis* and *M. cains* that the observed inhibitory zones were 15 and 10 mm, respectively but the non-laser exposed extract resulted in 10 mm against *C. parapsilosis* only. In this context, ketoconazole was also used as positive control and inhibited growth of the *C. albicans* and *A. fumigatus* only to give 20 and 18 mm against these fungi, respectively. Both extracts appeared compounds in their contents using GC-MS which showed three similar compounds in them were methanesulfonylacetic acid, pyrimidine-4,6(3H,5H)-dione, 2-butylthio- , and hexanedioic acid, bis(2-ethylhexyl) ester. Increasing antifungal activity of these compounds after exposure to laser radiation must be taken in attention. Although, there were no remarkable inhibitory results on the other fungi, the exact mechanism as antifungal is unclear and needs to be clarified in the future.

Keywords: *Eucalyptus* species, Laser application, Fungi, GC-MS.

Introduction

The plants are still used as one of important sources to produce drugs including antimicrobial agents are prepared [1,2]. Traditionally, peoples use plants for protect of their bodies against diseases [3, 4] the previous studies referred that two thirds of tested plant extracts have been used for treating diseases [5, 6]. Historically, peoples in the Mesopotamia (Iraq) where the ancient written scripts referred that the peoples used plants for their treating from different infections [7-9]. *Eucalyptus* species is a one of these plants that indicate great antimicrobial properties and contain volatile oils which occupy in many parts of the plants, depending on the species, but in the leaves the oils were most plentiful. *E. camaldulensis* vital oil has a sensitivity range of 0.125 to 1.0% for the majority of fungal model organisms. The extracts (0.2-200 mg/mL leaf extracts and 0.5 mg/mL bark extracts) are effective against *C. albicans* and in opposition to a number of dermatophytes [10]. The imperative 1,8-cineole used to be a component of the dangerous oil made

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from the oily, clean leaves of Eucalyptus species. The reported 1,8-cineole content material cloth ranges from 54 to 95%. Limonene, -terpineol, monoterpenes, sesquiterpenes, globulol and, as well as -eudesmol, and aromatic components were the most common co-occurring compounds with 1,8-cineole. As according pharmacological research, eucalyptus has numerous medicinal properties, including those that are gastrointestinal, analgesic, antidiabetic, antioxidant, anticancer, antibacterial, antiparasitic, insecticidal, repellant, oral and dental, dermatological, and nasal. one-of-a-kind outcomes [11-13].

All fungal species have been observed to develop drug-resistant strains, and fungi have become resistant to poliens, azoles, and echinocandins [14,15].Researchers showed that leaves of *Eucalyptus* species possess activities against microbial pathogens (16-18].

Laser has been applied in the different aspects including medical and pharmaceutical applications such as stimulation of the antimicrobial natural compounds [19- 21]. According to recent studies, irradiation could change the cell growth and metabolites of different fungal species, and experimentally it may have an effect on some compounds which act as antifungal upon pathogenic fungi [21]. Our study was designed to evaluate effects of the laser radiation on the antimicrobial activity of the chloroform plant extract produced from leaves of the *Eucalyptus* species.

Material and Methods

Preparation of Plant Extract

The procedure was done according to Husein et al. [22]. Collecting *Eucalyptus* species healthy leaves (Fig. 1) were done and they are washed twice for removing derbies and dust. The first wash was performed with tab water and while distilled water was used to perform the second wash. Leaves were left at room temperature to dry and pulverized the Using to get powder of a small mill till the leaves were obtained to become powder. One hundred fifty g of powder were flooded by 700 ml of petroleum ether for 4 days. After completing period, the powder used to be separated from the petroleum ether using filter paper had a layer (0.5 g) of the activated charcoal, to remove plant chlorophyll, for 7 days. The powder used to be left at room temperature until the petroleum evaporated from the powder totally and the later used to be additionally placed in a clean glass beaker (1000 ml potential), soaked with 700 ml of aqueous methanol 80 % (80 % of an absolute methanol with 20 % of distilled water). Other steps were done similarly to the petroleum procedure. The powder, which had totally evaporated methanol, was soaked by absolute chloroform and remaining steps were sequentially done as in petroleum ether and methanol, which were above mentioned that the chloroform filtrate was separated from the powder. Finally, the filtrate was left at 37 °C for 3 days until getting solid extract.

Phytochemical Colored Tests

Detection of sterol and terpenoids

-**Salkowski's test**: 2.3 ml of the extract solution were mixed with 2 ml of the chloroform and filtered. The filtrate was placed in a tube and few drops of the concentrated sulphuric acid were added on the tube side carefully without shaking. If reddish brown appears, it indicates to terpenoids but a presence of triterpenes when golden yellow forms.

Fig. 1. Tree of *Eucalyptus* species.

-**Libermann Burchard's test:** 2 ml of the extract solution were mixed with chloroform and filtered. Treating the filtrate with few drops of the acetic anhydride (1-2 ml), boiled and cooled. Then, adding concentrated sulphuric acid on the sides of the test tube carefully. If brown ring forms at the junction gives sterol presence.

-**Copper acetate test:** 2-3 ml of the extract solution were treated with few drops of 5% copper acetate solution. Diterpenes present if emerald green forms

Detection of alkaloids

-**Wagner's test:** 2-3 ml of the extract solution were treated with 2 ml of Wagner's reagent (Iodine in potassium iodide). Alkaloids present if reddish brown precipitate forms.

- **Dragendroff's test:** Acidifying 2-3 ml of the extract solution with 1 drop of sulfuric acid then treated with 0.5 ml of Dragendroff's reagent (solution of potassium bismuth iodide). If red color (precipitate) forms, it indicates to alkaloid presence.

Detection of carbohydrates

-**Molisch's test:** 2-3 ml of the extract solution were treated with few drops of 10% alcoholic – α – naphthol solution (2-4 drops). Then 2 ml of concentrated sulphuric acid was added carefully to the test tube side. A presence of the carbohydrate if purple color occurs.

Detection of glycosides

- **General test:** 5 ml of the extract solution were treated with few drops of 10% aqueous NaOH solution. If yellow color forms, it indicates to glycoside presence.

- **Keller Kiliani test** (for deoxysugar glucosides): 2 ml of glacial acetic acid containing a few drops (3-4) of 5% FeCl3 solution were added to 2-3 ml of the extract solution. Then adding 1 ml of concentrated H_2SO_4 to along the test tube side carefully. Deoxysugar of cardenolides present if reddish brown ring forms at the interface

- **Legal test:** Acidifying 2 ml of the extract solution with 1 drop of concentrated HCl, then treating with 1 ml sodium nitroprusside in 1 ml pyridine and methanolic alkali. cardiac glycosides present if pink-red color developes.

Detection of coumarin glycosides, phenolic compounds, flavonoids, and tannins

- **Alkaline Reagent test:** 2-3 ml of the extract solution were treated with few drops of 20% sodium hydroxide solution. Flavonoids present if intense yellow color forms and disappear when adding few drops of dilute acetic acid to the solution..

-**Lead acetate test:** 2 ml of the extract solution were treated with few drops of 10% lead acetate solution. If white color (precipitate) develops, it indicate to a presence of the phenolic compounds

- **Ferric chloride test:** Adding 1 ml of the plant extract solution to 2 ml of water and adding 2-3 drops of the diluted 5% ferric chloride solution.If green to blue-green forms, it indicates to catechol tannins but the blue-black indicates to a presence of the gallic tanninscoloration.

- **Gelatin test:** Adding 2-3 ml of the extract solution to a 1% gelatin solution containing sodium chloride (1%). Tannins present if white color is (precipitate) forms.

Detection of proteins and amino acids

-**Xanthoproteic test:** 3 ml of the extract solution were treated with few drops of concentrated nitric acid. Proteins present if yellow color forms.

-**Ninhydrin test:** 3 ml of the extract solution were treated with few drops of 2% (w/v) ethanolic ninhydrin reagent, and boiled for 10 minutes. Amino acids present if a blue color forms.

- **Biuret test:** 1 ml of 10% sodium hydroxide solution was added to 3 ml of the extract solution and heated. Then adding a drop of the 0.7% copper sulfate solution to the solution. Proteins present if purplish violet color develops.

Detection of saponin glycosides

- **Foam test:** 1 ml of the extract solution was to 2-3 ml of distilled water for getting . a mixture which was vigorously shaken. If foam persists for 10 minutes, it indicates a presence of the saponins.

-**Froth test:** Diluting 1 ml of the extract solution with distilled water to be 20 ml and shacked using a graduated cylinder for 15 minutes. If 1 cm layer of the foam persists for 15 minutes, it indicates a presence of the saponins.

Exposure of the Crude Chloroform Extract to the Laser Radiation

An amount of the chloroform crude extract were dissolved in DMSO to be a solution Containing 50 mg / ml and irradiated to the 450 wavelength and 50 mw power of the semiconductor laser system. The solution was placed in a plastic tube and laser radiation directed on the tube vertically for 20 min. (Fig. 2).

Fig. 2. Illustration of the used laser radiation on the chloroform crude extract produced from leaves of *Eucalyptus* spp.

Antifungal Activity Test of the chloroform crude extract Produced from Leaves of Eucalyptus species

Aseptically, amounts of the chloroform crude extract either non- laser exposed chloroform extract and laser exposed chloroform one, were dissolved in dimethyl sulfoxide (DMSO) to get concentrations were 50 mg / ml and discs of the ketoconazole was also used as a positive control. In addition, Petri dishes of Sabouroad dextrose agar (SDA) were prepared and each dish was inoculated with active inoculum of *Candida albicans*, *C. parapsilosis*, *Aspergillus fumigatus*, *Microsporum canis*, and *Trichophyton cains*. After few minutes, two wells (6 mm in a diameter) were done in each dish and 100 µm of each concentration were loaded into a well. The dishes were incubated at 25 ˚C for 2 days in a state of *Candida* species while 10 days for the dermatophytes. After completing incubation, inhibitory zones (IZs) were measured by mm [23].

Gas chromatography–mass spectrometry Analysis

Both chloroform crude extract, non-laser exposed and laser exposed ones, were submitted to the GC-MS device using Agelint , 7820A, USA GC Mass Spectrometer. The conditions of this analysis were injection Volume: 1µl, GC inlet line temperature: 250 ˚C, pressure: 11.933 psi, injector temperature: 250 ˚C scan range: m/z 25-1000, analytical column: agelint HP-5ms ultra lneit in which 30 m length x 250 µm internal diameter x 0.25 µm film thickness, aux heaters temperature:300 ˚C, service Gas: helium 99.99 %, and , injection type: splitless , and oven application temperature had Ramp 1: 60 ˚C for 3 min., Ramp 2: 60 -180 ˚C for 7 ˚C/min, Ramp 3: 180˚C - 280 8 ˚C/min, and Ramp 4: 280˚C for 3 min.

Results

Antifungal Activity Test of the Chloroform Crude Extract Produced from Leaves of Eucalyptus species

Laser exposed chloroform extract revealed inhibitory zones against *C. parapsilosis* and *M. cains* and it did not give any effect on *A. fumigatus*, *C. albicans*, and *T.mentrophygates*. Contextually, non-laser exposed chloroform extract produced a positive result against *C. parapsilosis* only while the ketoconazole inhibited *C. albicans*

TABLE 1. The values of the IZs were obtained Laser and non-laser traded extracts, and ketoconazole against five pathogenic fungal species.

Tested fungal species	IZs (mm)		
	A	B	Ketoconazole
Candida albicans	NT	NT	20
Candida parapsilosis	10	15	NT
Aspergillus fumigatus	NT	NT	18
Microsporum canis	NT	10	NT
Trichophyton mentrophygates	NT	NT	NT

A: Non- laser exposed extract. B: laser exposed chloroform extract. NT: No inhibition.

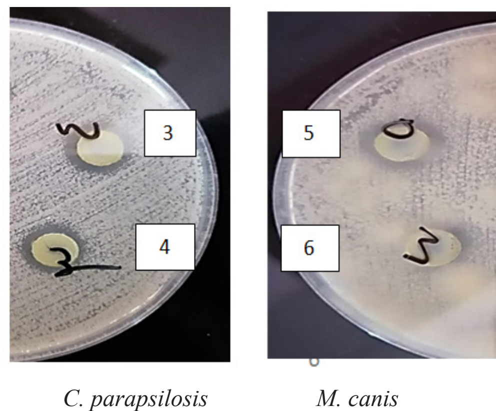

Fig. 3. Antifungal activity of the crude chloroform extract produced from leaves of *Eucalyptus* species. 3: IZs resulted in laser exposed chloroform extract and 4: IZ of non- laser exposed chloroform extract effect. 5: IZ produced from laser exposed chloroform extract while 6 : IZ of non-laser exposed chloroform extract effect.

and *A. fumigatus* only (Table1 and Fig. 3).

Phytochemical Colored Tests

The colored tests showed that the crude chloroform extract appeared terpenoids and alkaloids while it could not be dissolved in the solutions of other tests. Table 2.

GC-MS Analysis

GC-MS analysis showed different retention times (RTs) and three similar compounds of both extracts were (methanesulfonylacetic acid), (pyrimidine-4,6(3H,5H)-dione, 2-butylthio-), and (hexanedioic acid, bis(2-ethylhexyl) ester). (Tables 2, 3).

Discussion

Although some fungal diseases, even if they are contagious, can be self-cured in animals [24]. Some research indicated the possibility of using special medicines as a preventive measure to reduce the spread of infectious fungal infections [25]. Many types of pathogenic fungi have virulence factors that increase their pathogenicity and represent an obstacle in the course of the therapeutic process [26].Researchers seek to find materials that combat the growth of pathogens and manufacture many pharmaceutical drugs, recently stating about 74% out of 119 plant-derived pharmaceutical compounds used in modern medicine [27]. Solvent selection is suggested a one important step that a plant extract can be given because the extract content depends on the affinity between the solvent and plant. Related to this context, essential oils of a plant can be obtined using an absolute petroleum ether [28], This is supported by the results obtained by Raji and Raveendran [29] in which the theory explained differences of some plant extracted substances as an antifungals depending upon the solvent used, this may be due to ability of absolute petroleum ether to dissolve lipid contents in Eucalyptus [30] . Unlike other materials, it gives better results by using water as a solvent, as in a pumpkin leaves [31]. Most antimicrobial compounds within plant extract have medium molecules in their sizes because of their aromatic delocalized π-electrons. Another hand, these compounds are polar substances leading to have high affinity for polar solvents such as water and absolute methanol [32]. Numerous studies on laser therapy over the last 20 years provided positive results into clinical practice to stimulate wound healing, treat inflammation, and reduce pain [33,34]. Thus a basis for the introduction of this technology is experiment with the impact of laser on the chloroform crude extract of Eucalyptus plant and study its effect as antifungal comparing that before and after exposure to the laser, the present study can show why the non- laser exposed chloroform crude extract did not result in inhibitory values against the fungal species except for *C. parapsilosis* (Table 1). Kumoro et al. [35] referred to that quality recognized human and animal pathogenic yeast *Candida* species showed good sized sensitivity to *Eucalyptus* species oil extract with MIC approx. 0.5%. additionally [36], clarified the recreation of Eucalyptus Methanol leaf and bark extracts displaying good sized exercise towards dermatophytes: 0.8–1.6 mg/mL in opposition to *Microsporum* species, 0.125–1.6 mg/mL in opposition to *Trichophyton* species,

and 0.2 mg/mL in opposition to *Epidemophyton flocossum*. The negative results of the inhibition may be attributed to the petroleum ether, water, and methanol which were sequentially used in the extraction process before using chloroform may extract a majority of the bioactive compounds in leaves of the *Eucalyptus* species led to give a few compounds were obtained by the chloroform. Therefore, the phytochemical tests were used in this study appeared the chloroform extract had the terpenoids and alkaloids only while researchers [36]. So no need to use other solvents/ just give chance for chloroform to show up its power .Otherwise another experiment should be done to be control for petroleum ether and methanol. Otherwise, another extracts must be tested in the same experiment

TABLE 2. Results of the colored tests showing a content of the chloroform crude extract.

Test type	Results
Sterol /terpenoids	
Salkawski test	Positive
Libermannn test	Positive
Copper acetate	Negative
Alkaloids	
Wagner's test	Positive
Dragendroff's test	Positive
Carbohydrates	
Molisch test	No dissolve the plant extract in test
Benedict test	No dissolve the plant extract in test
Glycosides	
General alkaline test	No dissolve the plant extract in test
Keller Kiliani test	No dissolve the plant extract in test
Legal test	No dissolve the plant extract in test
Coumerine glycosides	
Alkaline test	No dissolve the plant extract in test
Phenolic compounds	
Flavonoids	
Alkaline test	No dissolve the plant extract in test
Lead acetate	No dissolve the plant extract in test
Acid test	No dissolve the plant extract in test
Ferric chloride test	No dissolve the plant extract in test
Tannin test	
Gelatin test	No dissolve the plant extract in test
Ferric chloride test	No dissolve the plant extract in test
Lead acetate test	No dissolve the plant extract in test
Proteins and amino acids	
Xanthoprotic test	No dissolve the plant extract in test
Ninhydrine test	No dissolve the plant extract in test
Biuret test	No dissolve the plant extract in test
Saponone glycosides	
Foam test	No dissolve the plant extract in test
Forth test	No dissolve the plant extract in test

TABLE 3. GC-MS analysis of the non-laser exposed chloroform extract.

Compounds	Peaks	RTs	Areas %
Dimethyl Sulfoxide	1	5.302	39.77
Dimethyl sulfone Methanesulfonylacetic acid	2	5.475	40.62
Dimethyl Sulfoxide	3	5.702	1.87
Dimethyl Sulfoxide	4	7.633	0.78
Dimethyl Sulfoxide	5	8.474	1.47
Dimethyl Sulfoxide	6	9.855	0.60
Dimethyl Sulfoxide	7	11.765	0.50
Dimethyl Sulfoxide	8	12.002	0.75
Dimethyl Sulfoxide Dimethylsulfoxonium formylmethylid	9	12.811	2.20
Dimethyl Sulfoxide	10	12.930	1.29
Dimethyl Sulfoxide	11	13.070	0.58
Dimethyl Sulfoxide Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	12	14.721	0.46
Dimethyl Sulfoxide	13	16.738	0.46
Pentadecanoic acid, methyl ester Hexadecanoic acid, methyl ester	14	16.943	2.09
Dimethyl Sulfoxide	15	17.591	0.66
2-Chloroethyl oleate 9-Octadecenoic acid (Z)-, methyl ester 9-Octadecenoic acid, (E)-	16	18.874	2.81
Methyl 8-methyl-nonanoate Methyl stearate Hexacosanoic acid, methyl ester	17	19.112	0.87
Hexanedioic acid, bis(2-ethylhexyl) ester Hexanedioic acid, mono(2-ethylhexy l)ester	18	21.679	1.23
Dimethyl Sulfoxide	19	23.880	0.54
Dimethyl Sulfoxide	20	24.603	0.46

In addition, the laser- exposed chloroform crude extract of the *Eucalyptus* species leaves which was used in the current study gave accepted inhibitory results somewhat compared with non-laser chloroform crude extract (Table1) and (Fig. 2). In this study, the compounds may lead to facilitating their fabrications when they were exposed to laser radiation. This may be attributed why laser-exposed crude extract by chloroform appeared a number of the compounds (Table 4) differ from those in the non-laser exposed chloroform one (Table 3), however; three similar compounds of both extracts were detected using GC-MS analysis.

As well as, this study used chloroform crude extract but no pure compounds that the composition of the crude form may not give inhibitory outcomes due to inert compounds in this extract on the antimicrobial ones, however, plant possess bioactive substances especially if they are fractioned separately. And as a recent experiment, based on theories related to the positive effect of laser on antifungal compounds we used Eucalyptus plant which have this effect on some fungal pathogenic species show a difference in the extracted materials before and after exposing them to the laser and in vitro evaluate the results.

TABLE 4. GC-MS analysis of the laser exposed chloroform extract.

Compounds	Peaks	RTs	Areas %
Dimethyl Sulfoxide	1	7.147	32.11
Dimethyl sulfone Methane sulfonylacetic acid	2	7.266	39.23
Dimethyl Sulfoxide	3	7.460	1.74
Dimethyl Sulfoxide	4	7.719	1.23
Dimethyl Sulfoxide	5	8.517	0.81
Dimethyl Sulfoxide	6	12.628	0.96
1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-,[1ar-(1a.alpha.,4a.alpha.,7.beta., 7a.beta.,7b.alpha.)]- (-)-Spathulenol	7	12.811	5.49
Globulol (-)-Globulol Viridiflorol	8	12.930	3.76
Dimethyl Sulfoxide Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	9	13.070	1.19
Dimethyl Sulfoxide	10	13.189	0.74
Dimethyl Sulfoxide	11	13.901	0.82
Dimethyl Sulfoxide	12	14.505	0.67
Dimethyl Sulfoxide	13	14.732	1.31
Dimethyl Sulfoxide	14	15.562	0.74
Dimethyl Sulfoxide Pyrimidine-4,6(3H,5H)-dione, 2-but ylthio-	15	15.929	0.90
Dimethyl Sulfoxide	16	16.749	1.01
Dimethyl Sulfoxide	17	17.569	1.59
Dimethyl Sulfoxide	18	18.842	0.67
Dimethyl Sulfoxide Pyrimidine-4,6(3H,5H)-dione, 2-but ylthio-	19	19.554	1.96
Hexanedioic acid, bis(2-ethylhexyl) ester	20	21.690	3.07

Conclusions and Recommendations

This study concluded that the chloroform extract produced from *Eucalyptus* species leaves has little antifungal activity and laser irradiation increases on some spp. Although this conclusion, it needs further studies such separation of the crude compounds and test them against fungi separately and then chemical characterization of each fraction must be done. In addition, other *in vitro* and *in vivo* studies must be performed for evaluating the bioactive fractions.

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Conflict of interest

The authors declare no conflict of interest.

Ethical statement

Ethical approve OR data collection permits UM.VET.2022.030

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التأثيرات المخبرية لإشعاع الليزر على النشاط المضاد للفطريات لمستخلص أوراق نبات اووكالبتووس بواسطة الكلوروفووم

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بعض النباتات تعتبر مصادر مهمة يمكن من خلالها تحضير الأدوية بما في ذلك العوامل المضادة للميكروبات. هدفت هذه الدراسة إلى تقييم تأثير أشعة الليزر على مستخلص الكلوروفورم الخام المنتج من أوراق نباتات أوكالبتوس. تم اختبار كلا المستخلصين، مستخلص الكلوروفورم الخام المعرض بالليزر والمستخلص غير االمعرض بالليزر في المختبر ضد نمو بعض الأنواع الفطرية المعزولة من الحيوانات والتي تم الحصول عليها من بنك العزلات في مختبر الاحياء المجهرية /كلية الطب البيطري/جامعة الموصل، وشملت

Candida albicans, Candida parapsilosis, Aspergillus fumigatus, Microsporum canis, and *Trichophyton cains*. حيث أظهرت نتائج هذه الدراسة أن مستخلص الكلوروفورم الخام المعرض بالليزر أنتج مناطق مثبطة ضد داء *C. parapsilosis* و *M. cains* بأن المناطق المثبطة الملحوظة كانت ١٥ و ١٠ مم ، على التوالي ، لكن المستخلص غير المعرض لليزر نتج عنه ١٠ مم ضد *C. parapsilosis* فقط. في هذا السياق ، تم استخدام الكيتوكونازول أيضًا كعنصر تحكم إيجابي ومثبط لنمو *C. albicans* و *A. fumigatus* فقط اعطى تثبيط ٢٠ و ١٨ ملم ضد هذه الفطريات ، على التوالي. ظهر كلا المستخلصين في محتوياتهما باستخدام GC-MS التي أظهرت ثلاثة مركبات متشابهة فيها وهي حمض الميثان سلفونيل أسيتيك ، بيريميدين -٤ ، ٦ (٣H ، ٥H) - ديون ، ٢-بوتيلثيو- ، وحمض هيكسانديويك ، ثنائي (٢-إيثيل هكسيل) استر. يجب الانتباه إلى زيادة النشاط المضاد للفطريات لهذه المركبات بعد التعرض لإشعاع الليزر. على الرغم من عدم وجود نتائج مثبطة ملحوظة على الفطريات الأخرى ، إلا أن الآلية الدقيقة كمضاد للفطريات غير واضحة وتحتاج إلى توضيح في المستقبل.