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Production, Purification and Characterization of Terpenes Isolated From Two New Strains of Yeasts in Iraq and Study of Their Antiparasitic Activity

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ABSTRACT

The current study was included isolation and identification of yeasts from five sediments soil samples from Al-Faw and Shatt al-Arab in Basrah provinces, Iraq. The soil samples were collected during fallow period 1-1-2021 to 1-6-2022. In the primary isolation dilution method used for cultured soil samples, two-crud colonies filtration was used. Two types of terpenes (T1, T2) were purified from culture filtrates of both species *Cystobasidium benthicum* and *C .minutum* separately. The thin layer chromatography and column chromatography were used in the first step of purification. Cytotoxicity test revealed no toxic effect for both terpenes at 0.2 gm / ml concentration. The two terpenes were identified using ultra violet and infra-red spectroscopy as well. Therefore, the results showed the terpenes nature of both toxins and determination of important active groups in the structure of the two isolated terpenes. The current study aimed to search for more treatments that limit the vitality of the larval stages of the parasite, so it was done antiparasite activity for both terpenes were performed against *Echinococcus granulosus* the results showed the T1 more activity against parasite compared with T2.

1. Introduction

Significant improvements have been achieved over the previous 25 years to use terpenes, the upgrading of extracting specific pigment from yeast remains a challenge. causing it to be used as an antiparasitic drug against special strains of parasites in vivo and in vitro, Infected with Echinococcus were shown in a variety of ways, depending on the timetables of recent developments in processing and manufacturing technology. microbe's pigments, and there's room for improvement yet (Barabadi et al., 2017). The results of treating *Echinococcus granulosus* protoscolex with terpense at several concentrations suggested a strong impact on destroying protoscolex in vitro. Scientists believe that the yeast's natural pigment has significant medical potential. Together with other species, *Cystobadium benthicum and C. minutum* of the genus Basidiomycota are responsible for producing this pigment. Reaction to bacterial infections, Fungi such as *Rhodotorula, Saccharomyces monasticus, Yarrowia, Phaffia*, and so on are the primary sources of natural colors such as carotenoids, flavonoids, and terpenes. Antioxidant or maybe tumor-inhibiting carotenoids, anthraquinone, and chlorophyll have been agents with broad medicinal potential (Narsing Rao, Xiao, & Li, 2017) Yeast, mushrooms, bacteria, and algae are only a few natural sources for a wide range of products. The biological nature of microbial pigments has sparked a fresh interest in these compounds, which has led to a rise in demand for both natural and synthetic terpenes. Plants have medicinal and nutritional potential; the challenges of regional and seasonality variation (Marrez & Mohamad, 2020).

The terpene component combination was analyzed using several numbers of procedures, chromatography being one of them. It is distinguished by a high level of sensitivity and specificity in determining findings, incorporating high-

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performance liquid chromatography (HPLC) and thin-layer gas-liquid chromatography (PC) [10] GC-13 [gas chromatography using a standard pressure and temperature] spectroscopy with selectivity (Shelor, Yoshikawa, & Dasgupta, 2021) The research aims to extract and purify terpenes from colored yeasts and test their effectiveness only on parasite.

2. Materials and methods

2.1. Samples Collection

Yeasts samples were collected from sediment in Al-Faw and Shatt Al-arab in Basrah /Iraq. Each sample was collected by spoon and placed in a clean labeled bag and transported to the Microbiology Lab. / Department of Biology / College of Science / Uni.of Basrah. Immediately. All samples were cultured directly on isolation media with 250 mg / L chloramphenicol to prevent bacterial contamination, the cultured samples incubated at 25 C ° for 30-45 days.

The culture media were examined under the microscope and the pure isolates were cultured is slants for morphological and biochemical identification according to (Kurtzman, Fell, & Boekhout, 2011). In addition, a molecular Identification by *ITS1- 5.8S - ITS2 rDNA* Gene. Two yeast isolates refreshed colonies on potato dextrose agar for 48 hrs. at 25 C ° , used for DNA extraction According to…name of kit … protocol (Geneaid,Taiwan).

The PCR performed according to (Mirhendi, et al., 2006) to amplify the internal transcribed spacer (ITS1-5.8S ITS2) region and 2 universal primers to amplify ITS1 and ITS4 , the total volume for the reaction as follow 2 µl each primers , 10 µl g DNA , ….Master Mix (Bioneer, Korea) and 36l µl Nuclease free water. The thermal cycler ((Applied Biosystem, USA) condition was 94 C° for 5 min. followed by 94 C° for 30 sec. (25 cycles) 56 C° for 45 sec. and 72 for 1 min, the final extension was done at 72 C° for 7 min.

The PCR product was visualized by agarose gel electrophoresis (2 % agarose , 25 ml TBE buffer and 0.2µl green gel stain) , 100 bp DNA ladder were used (Bioneer, Korea). ITS1-ITS2 ergion r DNA gene sequencing PCR product (20 µl) for ITS1-ITS2 ergion r DNA gene were sent to Macrogen company for purification and sequencing. All yeasts isolates identified using National Center for Biotechnology Information (NCBI) Blast.

2.2. Fermentation

2.2.1. Yeast strains

The yeast strains *Cystobasidium benthicum* and *C. minutum* were isolated from soil –Basrah-Iraq.

2.2.2. Culture Media

Yeast malt extract broth (YMB) with : Yeast extract 3 g Malt extract 3 gm , Peptone, 5 gm , Glucose 10 gm , distilled water 1000 ml

2.3. Purification of terpenes (T1, T2)

2.3.1. Fermentation medium

During this study, the best fermentation conditions were selected (medium of producing killer toxins), which is the medium of Yeast malt extract broth (YMB) (7) with the optimum conditions 30 C° for 3-5 days and rotated at 150 rpm.

2.3.2. Purification of terpenes

Used Thin layer chromatography with column chromatography to purification terpenes according to (Harborne, 1984). Fractions (1 ml/tube) were collected at a flow rate of 1 ml/min. with a fraction collector. The fraction diagnosis by UV and IR. UV absorbance Spectrum of terpenes. The ultraviolet absorbance spectrum of terpenes was recorded on PG T90U UV-Visible spectrophotometer using conventional quartz cell having an optical path length of 1 cm at 298 k. in an aqueous solution with double beam spectrophotometer at ambient temperature 30 °C. Fourier Transformer Infrared Spectroscopy(FTIR) Analysis of terpenes. A mixture of sample and KBr (5% sample, 95 %KBr) was pass into a disk for FTIR (Shimadzu, USA) measurement. The spectra were recorded in the frequency range 4000 cm $^{-1}$ to500 cm $^{-1.}$ Disks were prepared in triplicate to obtain a constant spectrum. Cytotoxicity on red blood cell performed according to (Abu-Mejdad, 2019).

2.3.3. terpenes anti parasite activity

The extracts T1and T2 with the drug of albendazole were added to the culture bottles with a cap in concentrations (200-400-600 mg/ml container identical amounts of hydatid cyst fluid containing the protoscolex and left an untreated control group with five replicates for each group and tested the effect of the extract and treatment by measuring. The viability of the protoscolex (Fakhar, et al., 2015).

2.3.4. Statistical analysis

To evaluate the difference among activities terpenes against parasite using SPSS, P≤ 0.05 were considered as statistically significant.

3. Results and Discussion

3.1. Molecular identification

ITS1-ITS2 5.8S rDNA gene is gold *standard to identify* yeast isolates. Its quick reliableness technique in a very comparison with biochemistry ways, additional more providing a formation regarding the evolutionary relationships.

3.2. Identification of yeast isolates

3.2.1. ITS1-ITS2 5.8S rDNA gene

ITS1-ITS2 5.8S rDNA gene of 2 yeast isolates were shown on agarose gel electrophoresis under UV transilluminator at the postion 500 bp by comparing with standard DNA ladder (Figure 1).

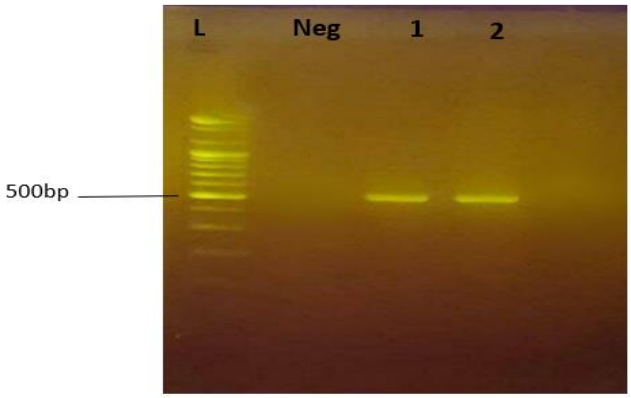

Fig. 1. Agarose gel electrophoresis 2 %of PCR products for internal transcribed spacer ITS1 – ITS2 regions (including 5.8S rDNA gene) : Lane L: (100 bp)DNA ladder, Lane 1: negative control , Lane 2: *Cystobasidium minutum* (500 bp) , Lane 3: *Cystobasidium benthicum* (500 bp) for Basidiomycetes yeasts isolates.

3.2.2. Thin Layer Chromatography

Analysis of the results of purified terpenes on thin layer chromatography were shown sharp band (T1) for *Cystobasidium benthicum* presence at rf= 0.8 band Figure (2).

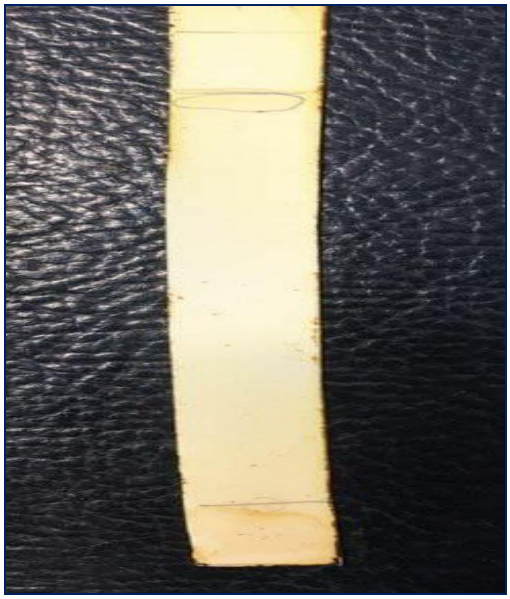

Fig. 2. Thin Layer Chromatography for terpenes component T1 with rf=0.8

3.2.3. UV-Visible spectroscopy

Figure 3 shows the UV absorption spectra in distilled water. One strong absorption bands at lower wavelengths appeared at 244, 251, 270, 312 nm for T1 these absorption bands correspond to π–π* transitions that appear due to groups like c=c. At longer wavelengths, the absorption spectra showed weak third bands within 480,423 in T1 UV , which could be correlated to n–π* transitions that appear due to groups like for oxygen ion pairs.

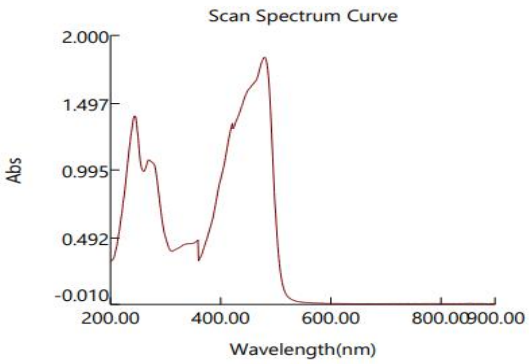

Fig. 3. UV-visible spectra of terpenes component T1 showing on peak

Compound	π- π *	n-π*
T1	244,251,270,312	480,423

π- π * : for c=c

n-π* : for oxygen ion pairs

3.2.4. Infra red spectra

The identification of the major carotenoid fractions extracted from *Cystobasidium* was based on absorption.as shown in FT-IR spectra (Fig.4). Similar results were also reported by (Wang, Xu, & Zhan, 2017) in *R. glutinis*. (Nurfitriyana, Fithri, & Yanuarti, 2022) reported that FT-Infra red spectrum of freshly isolated terpenes bands around 2930, 1720, 1450 and 1370 cm-1 respectively. An attempt was made to see the enable of the using pigment in different medical application.

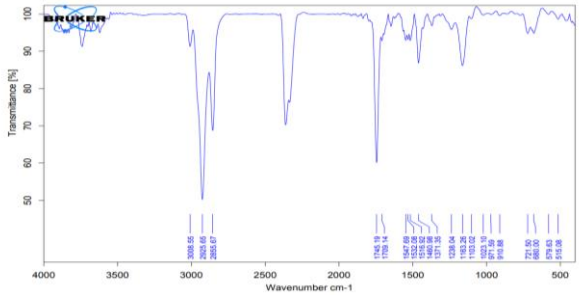

Fig. 4. Infra-red spectra of terpenes component T1

Table 1

The FT-IR functional groups of purified T1

Wave number (cm $^{-1}$	Assignment
3008	C-H aromatic stretching
2825	C-H aliphatic stretching
1746	C=O stretching
1460	C=C stretching
1163	C-O stretching
721	C=C bending

3.2.5. The cytotoxicity test of terpenes T1 and T2

The results showed that killer toxins had no toxicity against the human red blood cells with the concentration

0.2 gm / ml by using positive control tap water and negative control phosphate buffer saline(PBS) .The results revealed (T1,T2) non hemolytic and non toxic on human blood . as illustrated in Table (2) Figure (5)

Table 2
cytotoxicity test of killer toxins in concentration 0.2 gm/ ml

Compound	Toxicity against RBC
TK	-ve
WK	-ve
Tap water	+ve
PBS	-ve

-ve 0% lysis, +ve lysis 100%

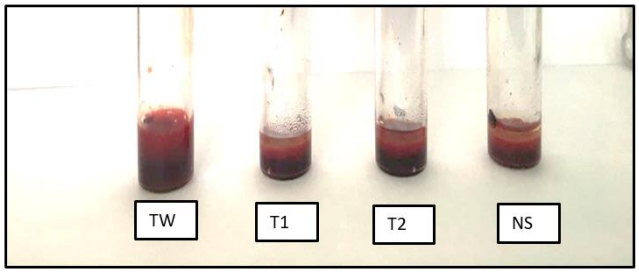

Fig. 5. The cytotoxicity test of two terpenes. TW: Tap water (positive control), T1: terpenes component1, T2: terpens component2, PBS: phosphate buffer saline (negative control)

3.2.6. Antiparasit activity

The results showed the effect of T1-T2 compounds and the standard albendazole treatment approved for the treatment of hydatid cyst disease on the ex vivo primary sarcoids with a significant difference compared with the control groups. Comparison with standard albendazole Fig. (6).

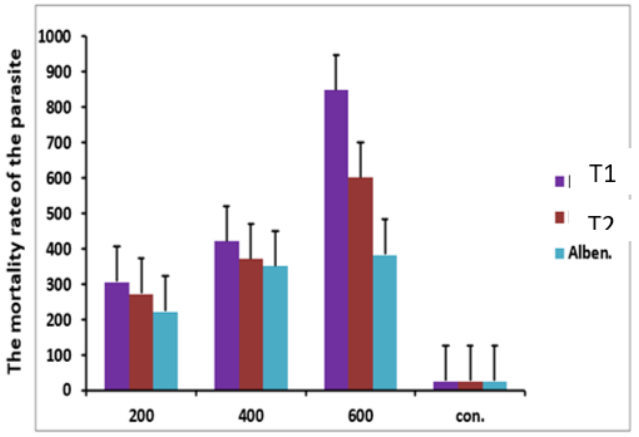

Fig. 6. Effect of T1-T2 compounds and albendazole treatment on In Vitro protozoa

Terpenes are a kind of fungus extract that has been utilized for thousands of years, primarily in the areas of food preservation, medicines, alternative medicine, and natural remedies. Several volatile oils (monoterpenoids and sesquiterpenoids) have been shown to have antibacterial and anticancer action, and fungal extraction has been utilized to treat a wide variety of illnesses and infections in recent years (Jiang, et al., 2020).

Various research has revealed terpenes have an impact on helminths when tested in vitro. In addition, the anthelmintic action of yeast terpenes was shown to be rather strong in laboratory tests. This work is the first detailed account of the in vitro influence of several terpenes on the proliferation of E. granulosus larval cells, Tetraterpenes' anthelmintic activity found, Different treatments consistently produced different results compared to the control condition; however, unlike terpene treatments, there was no evidence of dose-dependent effects. Maybe it is because they utilized weaker concentrations. Earlier research has looked at the antiparasitic action of terpenes on trypanosomatids, with doses ranging from 20 to 150 g/mL being reported (Kuhnert, et al., 2015).

Whereas the method of action of terpenes is poorly understood now, the wide range of their components suggests that they operate on a variety of targets and by several number of processes in various animals. Terpenes from Mentha pulegium (mostly piperitone oxide), Mentha piperita (primarily isomenthol), and Rosmarinus officinalis (primarily beta-myrcene) were employed in this study, A piperitone oxide inhibitory action against Staphylococcus aureus, Aspergillus flavus, and Enterobacteriaceae has been found. However, multiple writers have shown that menthol (the isomer of isomenthol) has antimicrobial effects. And beta-antioxidant myrcene's and antibacterial benefits were established (Ntie-Kang et al., 2016).

4. Conclusion

We conclude from the current study that the compounds extracted and purified from colored yeasts were highly effective in inhibiting or killing parasites and can be used as therapeutic alternatives.

Competing Interests

The authors have declared that no competing interests exist.

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