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Efficacy of Crude Alcoholic Extracts from Culture Broth of Desert Truffles against Some Clinical Microbial Isolates

Jamal M. Khaled[1,2]*, Naiyf S. Alharbi[1] and K. Shine[1]

[1]Department of Botany and Microbiology, College of Science,
King Saud University, Riyadh, Saudi Arabia.
[2]Department of Biotechnology and Food Technology, Thamar University, Yemen.

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This work amid to study the microscopic and macroscopic features of desert truffles cultivated on potato dextrose agar and broth. Also evaluation of crude alcoholic extracts of primary and secondary metabolisms against some clinical microorganisms isolates. The white truffles (Al-kama'a or Al-faga'a) locally known as "Zubaidi" collected from Riyadh markets. Isolation of ascospores and purity of ascospores test were performed after that the ascospores of truffles was cultivated on PDA and PDB. The microscopic and macroscopic were studied. The fermentation was carried out by four several conditions. After extraction by ethyl acetate and ethanol, the extracts were evaluated as anti-microbial agents. The results indicated that the desert truffles ascospores grew on PDA and PDB and the mycelia and macrospores were produced. The colony was white cottony with bloc in center without any pigment in front or reverse plate. A hyaline septate hyphae, conidiophore on both sides of hyphae, slightly ellipsoid macro-conidia and like of chlamydospores at end of some conidiophore were observed. The extracts [Ethyl acetate (CEA) and ethanol (CE)] from cultures that were grown in 12 hours light and 12 hours dark without shaker had higher effective against *S. mutans*, *Shigella* spp., *Cr. neoformans* and *C. albicans*. The results show that all extracts had not any activity against *A. niger*. The work suggested that primary and secondary metabolisms extracts of desert truffles grown on laboratory could be renewable resource of a many of biological compounds.

Key words: desert, truffles, ascospores, crude, anti-microorganisms.

Recently, the number of resistant of microorganisms are increasing and accompanied by rise in infectious diseases which were caused by those microorganisms. There has been a globalincrease in infectious diseases caused by micro-organisms resistant to many antimicrobial agents.This, in turn, led to increasing in the mortality and healthcare costs.These dire needs for new drugs demanded the hunt for new compounds from natural sources such as fungi. Fungi like truffles are an obligate hypogeous ascomycetesec to mycorrhizal fungus (Mandeel and Al-Laith, 2007).A term of desert truffle can be useful to a several edible hypogeous fungal fruiting bodies growing in arid regions throughout the world (Moreno *et al*. 2014). Arabs considered desert truffles one of the oldest traditional foods. It has nutritional value, especially when cooked with meats (Bokhary and Parvez, 1988). And used as a substitute for meat in their diet. (Al-Delaimy and Abu-Ghraib, 1970). Desert truffles are traditionally used in folkloric medicine for treatment of eye ailments in some Arab countries like Saudi Arabia (Janakat *et al*., 2004). These truffles are grown wild in different part of Saudi Arabia. The

* To whom all correspondence should be addressed.
Tel.: +966 553073732. Fax: +96614675806;
E-mail: jkhaled@ksu.edu.sa, jmak202@gmail.com

truffles grow and appear in the period from fifteenth of October to sixteenth of December (rainy season). It is called Al-kama'a or Al-faga'a. (Al-Rahmah , 2001 , Al-Ruqaie 2002). In the Arabian Peninsula, Ikhlasi is a local namefor*Te. claveryi and Te. boudieri* that have dark color, while truffles with white color, locally called Zubaidi include *Ti. nivea.* (Al-Rahmah, 2001).Rahma (2001) reported that the desert truffles grow on potato dextrose agar and produce the fungal hyphae. Truffles are mostly untouched source of new pharmaceutical products. They have antimicrobial, antioxidant, anticancer and immunomodulatory activity, such as bioactive polysaccharides and bioactive proteins such as lectins, fungal immunomodulatory proteins and ribosome inactivating proteins as reported in Xu *et al*., (2011).

Although, the desert truffles are very useful and contain several vital compounds such as anti-microbial, anti-oxidant and anti-cancer agents but themain disadvantages in this resource are the seasonal, scarcity of production in the nature and the high price which could be up to 100$ per 1 kg. In addition, until this timethe researchers not able to cultivate the fruiting bodies desert truffles in laboratories or greenhouses. For this reason, this work amid to produce the hyphae of desert truffle in artificial media and extract the bioactive compounds that have ability as anti-bacteria or anti-fungi from primary and secondary metabolites. This search will open the door for renewable resource of important bioactive extracts.

MATERIALS AND METHODS

Collection of desert truffles

The white truffles (Al-kama'a or Al-faga'a) locally known as "Zubaidi" collected from Riyadh markets. Immediately, the truffles were transferred to microbiology laboratory in Botany and Microbiology Department, King Saud University.The surface of sampleswere thoroughly washed usingdistilled water, and followed by 70% ethanol for 3 minutes and 5% sodium hypochlorite for 5 minutes (Kour *et al*., 2008) after that the truffles were washed three times by sterilized physiological saline solution (Nacl 0.89 %), then part of truffles were preserved in sterilized bottles at – 40 °C and the other part at 4 °C. The work was performed inside safety biological cabinet near the flames.

Purity of ascospores test

Very small pieces ofinner tissues were made by sterilized scalpel. Some pieces randomly were tested by microscope to confirm a purity of truffle ascospores and absence any another fungal elements or spores. The observation indicated that the truffle ascospores were pure from any another spore or fungal structures.

Cultivation on mycological media

One very small pieces were cultivated on potato dextrose agar (PDA) (Merck, Germany) [Typical composition (g/liter): Potato infusion 4 (infusion from 200 g. potatoes), D(+) Glucose 20, agar-agar 15]. Submerge fermentation was carried out in 4 erlenmeyer flasks (500 ml) containing sterilized 250 ml potato dextrose broth (PDB) (PDA without agar-agar). One gram of inner tissue was add each flask, after well mixing the flasks were incubated at 22 °C as following: in dark, 12 hours Light: 12 hours dark, shake(200 rpm) in dark and 12 hours Light: 12 hours dark with shaking (200 rpm). The fermentation continued for 18 day then the flasks were stored at 4 °C. Wet biomass and dry biomass were estimated by centrifugation methods at 12000 rpm for 15 minutes according to Asha-Augustine *et al*. (2006).The macroscopic and microscopic characteristics were examined on PDA and under light microscope with digital camera (Motic, China)

Extraction of crude alcoholic extracts

The crudealcoholic extracts from broth was perform from stored filtrated broth according to Barrow (2006) with a slight modification as the following scheme

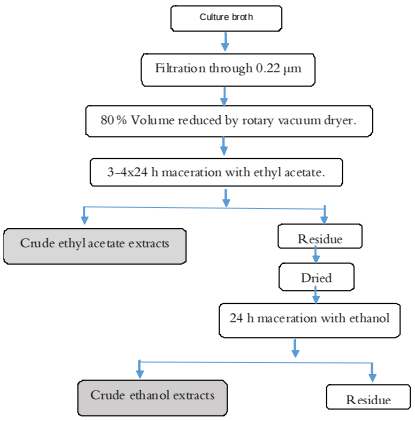

Fig. 1. Extraction scheme of crud alcoholic extracts

Biological activity of crude alcoholic extracts.

Crude extracts from culture broth were tested for their ability to inhibit growth of clinical microbial isolates. The test microorganism (*Streptococcus mutans*, *Shigella* spp., *Candida albicans*, *Cryptococcusneo formans* and *Aspergillu sniger*) were obtained from medical microbiology laboratory, king Khaled University Hospital. Before the test, all the microorganisms were activated in suitable medium at least three times. The disks (6 × 1 mm) were made from filter paper and sterilized by autoclave at 121 °C for 15 min inside sealed test tube. The net weight of crude extracts was estimated in crude extracts dried by rotary vacuum dryer then the concentered solutions (4 mg/ml) were prepared by methanol.After that, 20 µl of concentered solution was added to disk (4 mg/disk) and the disks were kept for 24 hours inside UV- safety biological cabinet to ensure evaporation of alcohol. Disk diffusion assay to determine antibacterial and anti-fungal activity were carried out according to EUCAST (2013). Mueller-Hilton agar (Oxoid, UK) was prepared according to the manufacturer's instructions (Approximately level depth was 4 mm) and was used to test bacteriawhile PDA was used to test fungi (Yeasts and Mold). The microbial suspensions in sterilized saline solution were prepared from activated colony of bacteria or yeasts whereas the spore suspensions of *A. niger* was prepared from spore phase of mold (The inoculum suspensions were standarized to McFarland 0.5 standard). 100 µl of each test microorganism was spread on surface of suitable medium (Bacteria on Mueller-Hilton agar and fungi on PDA) then the test disks and standard disks were gently putted on the surface of medium. The bacteria and yeasts were incubated at 37 °C for 24 hours while the mold was incubated at 22 °C for 3 days. After incubation the inhibition zones were measured.

The Minimal Inhibitory concentration for crude extraction were evaluated by micro-dilution methods according to Yanez *et al.*, (2014) with modifications. To perform this test, the test microorganism (above-mentioned), sterile round-bottom 96 well plates, dimethylesulfoxide DMSO solution (5 %), suspension of extract (4 mg/ml in DMSO) and *p*-iodonitro-tetrazolium violet (*p*-INTV) reagent (0.04%, w/v) were required. The test microorganism suspensions were prepared in the appropriate medium broth (The total count was 5×10^6 Colony Form Unit/ml).100 µl of microbial suspension was added in all well except negative control wells which served as sterilized saline solution (0.89% Nacl).The 100 µl of suspension of extract were added in first well then were mixed after that the 100 µl from first well was transferred into second well. The transfer and mixing was continued to a final well. From the final well, 100 µlwas thrown out plate in airtight lock flask containing 70% ethanol.For bacteria, the plates were incubated at 37 °C for 18 h whereas for fungi 22 °C for 48 h. To determine the MIC, the 20 µl of *p*-INTV reagent was added to all wells after that the plates were incubated for 30 min at 37 °C for bacteria or at 22 °C for fungi. After the incubation the change in color yellow to pink was observed. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC (Mothana *et al.*, 2013).

RESULTS

The microscopic characteristics of desert truffles (locally called Zubaidi) were studied by light microscope. In this work an oval asci with 8 hyaline global ascospores with dual-wall were observed. In a magnification power 1000x, a length of ascus was 519.3 µm and its width reached 350 µm while a diameter of oval globular ascospore was 126 µm (Figure 2). Figure 3 shows colony features produced by ascospores of Zubaidi truffles on PDA at 22 °C for 4 day that appear as white cottony colony with bloc in center and without any pigment in front or reverse plate. The colony requests to 14 days to cover all surface of PDA plate.The microscopic features of colony were illustrated by light microscopic micrograph in figure 4. A hyaline septate hyphae, conidiophore on both sides of hyphae, slightly ellipsoid macro-conidia and like of chlamydospores at end of some conidiophore were seen.

Growth of desert truffle ascospores on PDB varied depending on fermentation conditions that studied in this work. The wet biomass reached11.284% when the fermentation was carried out with shaking (200 rpm) 12 hours light and 12 dark while in above conditions without shaking was 7.6%. In cultivation without light, the wet

Table 1. Efficacy of crude ethyl acetate (CEA) and ethanol extracts (CE) extracted from culture broth of Desert truffles on potato dextrose broth against clinical microorganisms isolates

Growth condition		Extracts or Standard Antibiotics	Test microorganisms, diameter of inhibition zone (IZ) and minimal inhibitory concentration (MIC)				
			Streptococcus mutans	*Shigella* spp	*Candida albicans*	*Cryptococcusneo formans*	*Aspergillus niger*
D	CEA	IZ (mm)	-	-	-	-	-
		MIC (mg/ml)	-	-	-	-	-
	CE	IZ (mm)	-	-	-	-	-
		MIC (mg/ml)	-	-	-	-	-
LD	CEA	IZ (mm)	13	12	14	11	
		MIC (mg/ml)	0.0625	0.0625	0.0156	0.0625	
	CE	IZ (mm)	15	13	15	12	-
		MIC (mg/ml)	0.0156	0.0625	0.0156	0.0625	
SD	CEA		-	-	-	-	-
	CE		-	-	-	-	-
SDL	CEA	IZ (mm)	9	10	9	10	
		MIC (mg/ml)	0.25	0.25	0.25	0.25	
	CE	IZ (mm)	10	11	11	11	-
		MIC (mg/ml)	0.125	0.125	0.0625	0.0625	
Cephalothin 30 µg/disk		20mm	-	Not tested	Not tested	Not tested	
Ampicillin 25 µg/disk		10mm	18mm	Not tested	Not tested	Not tested	
Norfloxacin 10 µg/disk		22mm	19mm	Not tested	Not tested	Not tested	
Nitrofurantion 300 µg/disk		23mm	30mm	Not tested	Not tested	Not tested	
Nalidixic acid 30 µg/disk		-	-	Not tested	Not tested	Not tested	
Mecillinam 33 µg/disk		-	-	Not tested	Not tested	Not tested	
Gentamicin 10 µg/disk		20mm	8mm	Not tested	Not tested	Not tested	
Cotrimoxazole 25 µg/disk		20mm	-	Not tested	Not tested	Not tested	
Caspofungin 16 µg/disk		Not tested	Not tested	12mm	20mm	25mm	

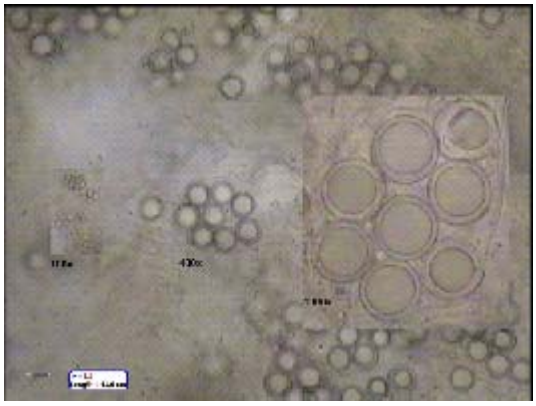

Fig. 2. Microscopic characteristics of desert truffle asci and ascospores

biomass reached 9.7% whereas the percentage reduced to 7.2% without shaking.

The crude alcoholic extracts extracted from free cell broth by ethyl acetate and ethanol were tested as renewable resource of antimicrobial agents. The table 1 shows that some extracts had ability to inhibit some clinical microbial isolates more than another extracts. The tested extracts from cultures that were grown in dark either with shaking (SD) or without shaking (SD) had not any activity against test microorganisms. The extracts [Ethyl acetate (CEA) and ethanol (CE)] from cultures that were grown in 12 hours light and 12 hours dark without shaker (LD) had higher effective against

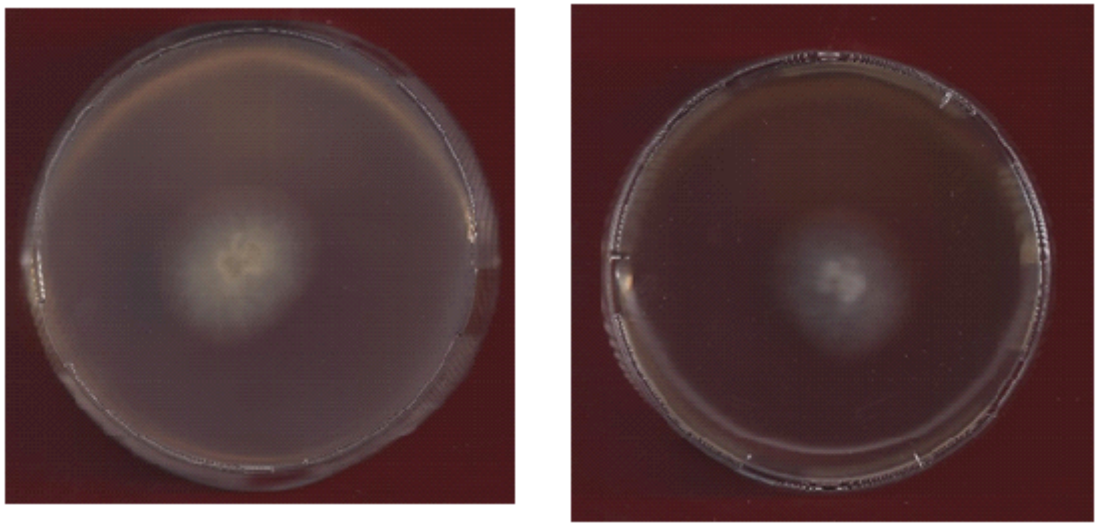

Fig. 3. Macroscopic characteristics of colony produced by desert truffle ascospores on PDA at 22 °C for 4 days. Reverse (left) and colony texture (right)

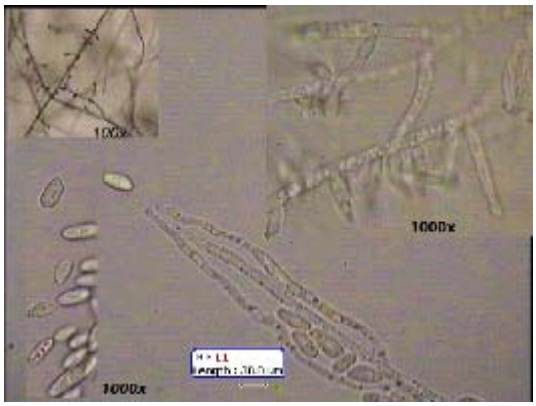

Fig. 4. Microscopic characteristics of hyphae, conidiophore and macro-conidia produced by desert truffle ascospores on PDA at 22 °C for 7 day.

S. mutans, *Shigella* spp., *Cr. neoformans* and *C. albicans*.The results in table 1 show that all extracts had not any activity against *A. niger*. The activity of CEA extract against *S. mutans* and *C. albicans* had more than Ampicillin (anti-bacterial agent)andCaspofungin (anti-fungal agent).

DISCUSSION

In generally, the desert truffles grow in complex and an extreme environment also they are associated with many of another microorganisms such as fungi andbacteria, or with some plantae such as *Helianthemum*spp where it forms mycorrhizas with a range of flowering plants (Al-whaibi, 2009). The similarity in the microscopic

characteristics of asci or ascospores in this study with standard characteristics of *Tirmanianivea*was clear. Most the study reported that *Ti. nivea* have ellipsoid to obovoid, amyloidasci containing eight hyaline, unattached with ascus, bluish, thin-walled ascospores(Kagan-Zur and Roth-Bejerano (2008); Bradai *et al*., 2014).

Most of the ancient or modern studies focusedon biological compounds extracted from fruiting bodies of the several truffles and evaluate of their ability as anti-microbial, anti-oxidant and anti-cancer agent (Janakat *et al*., (2004)Xu *et al*., (2011);Stojkovic *et al*., 2013;). The cultivation of truffleascospores researches on artificial media and extraction some biological extracts from primary or secondary metabolites are still rare. Probably, the reason could be due to a difficulty of cultivation the desert truffle fruiting bodies in Laboratories.In this work, the light and agitation affected the biomass produced by truffle ascospores on PDB. Determination of biomass is a simple measure for efficient growth of the microorganisms and it is definitely an important key variable because it indicate to rate of growth and/or product formation contain biomass as amost important state variable (Sonnleitner, 1992). This research did not aim to study the effect factorson the biomass (there are many factors and their interaction) but it focused on cultivation of truffle ascosporeson PDA and demonstrating that the produced mycelia could be considered a renewable resource for a several biological compounds.We have not found any study about microscopic and macroscopic of desert truffles grown on PDA in laboratory. The observed features in this work were similar to *Fusariumoxysporum*. That means one of two possibilities, either the isolates of desert truffles are endophytic fungi (*Fusariumoxysporum*) or are anamorphic phase of *Ti. nivea.*

The work in this study includeda cultivation of ascospores desert truffle on PDB and evaluation of the crude alcoholic extracts as anti-microorganisms. All those toconfirm that primary and secondary metabolitesare renewable recourse for biological compounds. Efficacy of ethanol and ethyl acetate extracts against tested clinical microorganisms isolatesrelated withnet biomass that associated with light and agitation. The results indicated that all extracts from culture broths which incubated in 12 hours light: 12 hours darks with shaking or without shaking had efficiency against tested clinical bacteria and yeasts but did not have any efficacy against *A. niger*.Although the crude extracts inhibited the test microorganisms less than some standard anti-bacteria or anti-fungi but they inhibited a nalidixic acid-, mecillinam-resistant *S. mutants*and cotrimoxazole-,nalidixic acid-,mecillinam-resistant *Shigella* spp**.**Almost the clinical bacterial or yeast isolates which are resistant to antibiotics such as mecillinam, nalidixic acid or cortimoxazolewill be candidate for causing a serious diseases. For example, increase in mecillinam-resistant shigella related to difficulty of treating shigellosis in Bangladesh and other developing countries (Hossain *et al*., 1998).The role of *C. albiacns* co-accumulation with *S. mutans*during adherence to dental surfaces was confirmed by a numerous of researches. Understandingthe homeostatic synchrony between *C. albicans*and *S. mutans*, and an inhibiting biofilm-induced caries have become decisive (Metwalli *et al*. 2013). In this results, the crude extracts inhibited both *C. albicans* and *S. mutans* that is meaning that the crude extracts could be became renewable resource for some pure drugs using to treat teeth loss in both children and adults. This work **concluded** that mycelia of desert truffles could be produced on artificial media in laboratory and the primary and secondary metabolites in liquid medium are considered renewable resources for biological compounds.

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