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In vitro and *in vivo* safety evaluation of *Terfezia claveryi* ruiting bodies extract

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Abstract

Desert truffles are edible economically important fungi with well known nutritional and therapeutic values. The purpose of this study was to evaluate the safety of aqueous extract of Terfezia claveryi fruiting bodies on growth of some probiotic bacterial strains (Lactobacillus jensenii, Streptococcus thermophilus, Lactobacillus casei and Lactobacillus bulgaricus) in vitro. The result showed that the truffle extract has no adverse effect on the growth of all strains of bacteria compared with control counterparts. The study also was extended to investigate the safety of the truffle extract via systemic four weeks repeated dose toxicity study in rats. Male rats were administered orally different doses of T. claveryi extract daily for 4 weeks (0, 250, 500 and 1000) mgkg^{-1}, respectively. The rats were visually inspected for changes in behavior, food, water consumption and appearance during the experiment period. At the end of the experiment, clinical biochemical serum analysis, hematological, and histopathological examination were carried out. The results demonstrated that no one of the tested doses -induced abnormalities in rats as observed by clinical signs, serum biochemistry, hematology and histopathology.

***Conclusion:** The results suggest that T claveryi aqueous extract is safe and may be considered as a natural effective drug in clinical applications with a wide therapeutic index.*

Key words: *Desert truffles, extract, safety, biochemical analysis, probiotic bacteria*

Introduction

Desert truffles are edible socio-economically important fungi. They are grow wild in many Gulf States [1] and distributed naturally from North Africa (Morocco, Tunisia, Algeria and Egypt) to the Middle East (Saudi Arabia, Kuwait, Iraq, Iran, Lebanon, Syria and Jordon). The people in the Middle East region are considered the largest truffle consumers [2]. The truffles are popular to their well known nutritional value and potential health benefits [3]. They are a rich source of protein, amino acids, fatty acids, minerals and carbohydrates [3-5]. In addition to their nutritional value, truffles was found to be alternatives and unlimited source of therapeutic compounds with anti-inflammatory, antioxidant, antimicrobial, antimutagenic, anticarcinogenic, anti-malarial, anti-tuberculoid and hepatoprotective properties [6-12]. The antioxidant capacity of different truffles may be related to that the truffles contain various chemical constituents such as vitamin C, carotenoids and many phenolic compounds [13-14]. There are many species of edible desert truffle, among of them are the dark brown color truffles, *Terfezia claveryi* (Family,

Terfeziaceae) [2]. *T. claveryi* was reported to exhibit a higher oxidative inhibition on lipid peroxidation, and deoxyribose [14] and has the ability to scavenge nitric oxide radical [15]. Also, it was reported that aqueous and methanolic extracts of *T. claveryi* possesses antimicrobial activities [8-9]. Beside, Janakat and Nassar [9] found that the aqueous extract of *T. claveryi* has a very powerful hepatoprotective activity against CCl4. The protective flora in the gut is usually stable, but it can be affected by some dietary and environmental factors causing alteration in the gut ecosystem [16]. So, safety evaluation of the fungi, particularly which used as valuable foods by many people and accepted as natural remedies, is important in implementing safety measures for public health. Thus a crucial standardization and toxicological evaluation of their safety in pre-clinical models could be relevant. The goal of this study was to evaluate in vitro the safety of *T. claveryi* aqueous extract on growth of some probiotic bacterial strains (*Lactobacillus jensenii*, *Streptococcus thermophilus*, *Lactobacillus casei* and *Lactobacillus bulgaricus*) which have a beneficial effect on human health. This study will determine if the used truffle extract has no any adverse effect on the growth of these beneficial bacteria The study also investigated the safety of the extract when administered orally to rats at concentrations providing doses of 0, 250, 500 or 1000 mg/kg body weight daily for four weeks. The following parameters were monitored: clinical chemistry and hematology markers in blood and histological examination of selected organs.

Material and methods

Chemicals

All chemicals used were of high analytical grade, product of Sigma and Merck companies. Kits used for the quantitative determination of different parameters were purchased from Biogamma, Stanbio, West Germany.

Desert truffle

The desert truffle, *Terfezia claveryi* fruiting bodies (dark brown red, rounded and small in size), were purchased from the local market of Riyadh in Saudi Arabia during summer .

Truffle aqueous extract preparation

Five hundred game of *T. claveryi* fruiting bodies were washed and homogenized in distilled water (1:3 w/v), using a household blender. The homogenates were refrigerated overnight, filtered through cheesecloth and then centrifuged at 4000 rpm for 15 min. The supernatants were then dried using rotary evaporator [15].

Probiotic bacteria

Lactobacillus jensenii, *Streptococcus thermophilus*, *Lactobacillus casei* and *Lactobacillus bulgaricus* were obtained from National Research Centre, Egypt. They were cultured on standard MRS media supplemented with 0.05% L-cysteine HC1 (Sigma Chemical Co., St. Louis, Mo) for enrichment of bacterial strains. The bacterial different stains were grown in anaerobic conditions using anaerobic Jar and anaerobic gas generating kits (Oxoid, Hampshire, England).

Preparation of bacterial suspension

Bacteria were enriched in specific media agar (for each strain) and inoculated in the broth at 37˚C for 24 h. Bacteria were then activated by transferring one full-node of the needle to 9 ml media broth and incubated anaerobically at 37˚C for 24 h. The number of bacteria was adjusted by measuring the absorbance to obtain equal number for each strain (10^6 living cell/ml). The number of bacteria in each strain was determined colorimetric using spectrophotometer (Spectro 23, Labomed, Inc.) at 620 nm.

Safety of aqueous extract of *T claveryi* on growth of some probiotic bacterial strains

Safety of of *T. claveryi* extract on growth of the tested probiotic bacteria different strains (*Lactobacillus jensenii*, *Lactobacillus casei*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) after activation for 24 h was studied. Truffle extract was dissolved in sterile distilled water (at a concentration of 25%) and 2ml of it was added to MRS broth inoculated with 1 ml of the selected strains of probiotic bacteria (10^6 CFU/ml) each alone. A control sample for each bacterial strain without truffle extract was also included in the experiment. All bacterial strain samples were incubated an aerobically using anaerobic Jar at 37°C for 72 h, then the growth of each strain was determined colorimetric using spectrophotometer at 620 nm.

Animals

Forty healthy male albino rats (120–150 g) were supplied by the Experimental Animal Center, King Fahad Medical Research Center, Jeddah, King Abdelaziz University. Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the College of Science, King Abdelaziz University. Animals were kept in special cages and maintained on a constant 12-h light/12-h dark cycle with air conditioning and a controlled temperature of 20°C to 22°C and humidity of 60%. Rats were fed a standard rat pellet chow with free access to tap water *ad libitum* for 1 week before the experiment for acclimatization. The animals were randomly divided to 4 groups, ten rats each. Group 1, control; Group 2, animals treated with 250mg / Kg body weight truffle aqueous extract; Group 3 animals treated with 500 mg/Kg body weight truffle aqueous extract; Group 4, animals treated with 1000 mg/Kg body weight truffle aqueous extract.

Clinical examination

The rats were observed for signs of toxicity, mortality behavioral changes and consumption of food and water once a day throughout the study. At the end of the experiment (four weeks), blood samples were collected from all animals, then sacrificed under ether anesthesia. Internal organs (heart, liver, kidneys) were removed, washed in cold phosphate buffered saline and used for gross findings and histological examination.

Clinical biochemistry

A part of blood collected from each animal of all groups was transferred into sterilized tubes for serum separation (Sarstedt, Germany). Serum was separated by centrifugation at 3000 rpm for 10 min. Total proteins, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma- glutamyltransferase (GGT), alkaline phosphatase (ALP), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), uric acid, creatinine, urea, glucose, cholesterol, low density lipoprotein (LDL-C), high density lipoprotein (HDL-C) and triglycerides (TGs) were determined in the blood serum using an automatic serum analyzer (model 7060; Hitachi, Tokyo, Japan). Sodium, potassium, calcium were measured with mineral analyzer (Bayer 644).

Hematology

3mL of collected blood were transferred into a CBC bottle (EDTA 3K; Sewon Medical, Busan, Korea) for white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet analyses using an autohematoanalyzer (ADVIA120E; Bayer, New York, NY, USA).

Histological examination

Liver, heart and kidney specimens were fixed in 10% formalin and then embedded in paraffin and 5–6-µm thick sections were cut on a rotary microtome. The sections were stained with hematoxylin-eosin and examined by light microscopy.

Statistical analysis

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± SE. Significant differences among values were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test post-ANOVA.

Results

Effect of aqueous extract of *Terfezia claveryi* on growth of probiotic bacterial strains

Results presented in Table (1) revealed that treatment of selected different strains of bacteria with *T. claveryi* extract showed non-significant change in growth of all strains compared with its control counterpart after 72h.

Table 1. Effect of aqueous extract of *T. claveryi* on growth of probioticbacterial strains in the MRS broth in a concentration of 25% (mean of replicates ±SE)

Probiotic strains		Growth
lactobacillus jensenii	control	0.033 ±0.010
	Treatment	0.04 ± 0.009
Streptococcus thermophilus	control	0.023±0.006
	Treatment	0.025 ±0.008
lactobacillus casei	control	0.056±0.033
	Treatment	0.053±0.011
lactobacillus bulgaricus	control	0.053±0.005
	Treatment	0.043±0.01

Data are presented as mean ± S.D. of 4 replicates for each bacterial strain. Statistical analysis using T-test showing non- significant change in growth of bacteria different strains treated with truffle extract compared with untreated control

Mortality and clinical signs

No animals died in all groups treated with different doses of *T. claveryi* extract during the experimental period (4 weeks). Non significant differences in food and water consumption were also observed between the treated groups and the control group (data not shown).

Serum biochemical parameters

The levels of serum biochemical markers (liver, heart and kidney functions, serum glucose and lipid profiles) in control and *T. claveryi* extract -treated groups are depicted in Tables 2, 3 and 4 respectively. Non-significant changes in the tested serum biochemical parameters were observed between control and *T. claveryi* extract -treated different animal groups.

Table 2 . Influence of different doses of aqueous extract of *Terfezia claveryi* fruiting bodies on serum liver and heart function biomarkers

Parameters	Normal	*T. claveryi* -treated animals with different doses		
		250mg/ kg	500mg/kg	1000mg/kg
Total proteins (g/dL)	7.5±0.3	7.6± 0.15	7.4±0.2	7.5±0.22
Albumin(g/dL	4.86±0.35	4.9±0.17	4.7±0.3	4.86±0.21
ALT (U/L)	36.8±1.4	37.7±1.0	37.8±0.9	38.6±1.66
AST(U/L)	17.9±0.56	18.5±0.65	18.7±1.0	18.3±0.35
GGT(U/L)	8.2±0.33	7.56±0.21	7.86±0.15	8.3±0.32
ALP(U/L)	77.93± 2.3	78.2±1.2	80.0±1.3	79.3±1.9
CPK(U/L)	125± 7.6	130.5± 8.3	132.9± 10.4	135.3± 12.7
LDH(U/L)	175.8 ± 11	177.8± 10.6	180.7± 12.7	179.3± 9.7

Data are presented as mean ± S.D. from 8 rats. Statistical analysis using ANOVA followed by Bonferroni as a post-ANOVA test showing that non significant changes in the different biomarkers of both liver and heart damage between animals treated with different repeated dose of *T. claveryi* aqueous extract and normal untreated animals.

Table 3. Influence of different doses of *T. claveryi* fruiting bodies aqueous extract on serum kidney function biomarkers and some minerals

Parameters	Normal	Trufle-treated animals with different doses		
		250mg/ kg	500mg/kg	1000mg/kg
Uric acid	1.73±0.21	1.8±0.15	1.88±0.19	1.9±0.22
Creatinine (mg/dL)	0.61±0.025	0.62±0.036	0.66±0.041	0.65±0.032
mg/dL)(Urea	23.1±1.65	24.8±0.9	24.9±1.2	25.3±2.3
Sodium(mmol/L)	144.9±4.9	145.4±5.3	143.6± 6.4	146.56±4.8
Calcium(mmol/L)	9.7±0.56	9.86±0.52	9.4±0.28	9.5±0.29
Potassium (mmol/L)	3.96±0.31	4.1±0.32	4.06±0.42	4.2±0.21

Data are presented as mean ± S.D. from 8 rats. Statistical analysis, showing that no significant changes in the different parameters between animals treated with different repeated dose of *T. claveryi* aqueous extract and control untreated animals.

Table 4. Influence of different doses of *T. claveryi* fruiting bodies aqueous extract on serum glucose and lipid profiles

Glucose(mg/dL)	95.26±4.7	94.4±3.1	95.96±2.9	97.7±4.7
Cholesterol(mg/dL)	85.6±4.2	82.1±5.4	80.6±2.7	80.2±3.1
LDL-C(mg/dL)	34.1±1.55	30.43±3.9	32.6±1.9	33.3±1.3
HDL-C(mg/dL)	48.5±3.6	50.2±2.9	49.3±4.9	49.8±3.7
TGs(mg/dL)	42.36±2.77	41.4±2.5	40.23±3.1	38.3±3.5

Data are presented as mean ± S.D. from 8 rats. Statistical analysis, showing that non significant changes was observed in the tested parameters between animals treated with different repeated dose of *T. claveryi* aqueous extract and normal untreated animals.

Hematological parameters

The levels of different hematological parameters in different experimental groups are illustrated in Table (5). Non significant changes were found in the hematological parameters between control and *T. claveryi* extract -treated different animal groups.

Table 5. Influence of different doses of *T. claveryi* fruiting bodies aqueous extract on the levels of some hematological parameters in rats

Parameters	Normal	Trufle-treated animals with different doses		
		250mg/ kg	500mg/kg	1000mg/kg
WBC (x103mm^3)	6.0±0.9	6.4±0.65	6.89±0.45	6.5±0.52
RBC(x10^{6}mm^3)	7.65± 1.3	7.4± 1.5	7.89±1.8	8.1±1.4
Hb (g/dl)	14.7± 1.8	15.2±1.6	15.5±0.8	16.8±2.3
MCH (pg)	19.2 ±1.8	18.7±1.5	18.1±1.9	18.4±1.7
MCV (fl)	74.9 ±3.2	78.4±5.3	60.3±6.7	62.7±7.9
HCT(%)	57.3± 6.4	52.5±7.8	53.6±5.7	58.5±4.5
MCHC (g/dl)	32.7 ±3.4	29.9±2.8	31.5±1.5	29.3±2.4
Platelets (x10^3)mm^3	928. 8± 100.4	1200.7±200	1050±150	1123±120

Results are expressed as mean ± SD of 8 animals. WBC = White blood cells, RBC= Red blood cells, Hb= Haemoglobin concentration, MCH= Mean Corpuscular Haemoglobin, MCHC= Mean Corpuscular Haemoglobin Concentration, MCV= Mean Corpuscular Volume, HCT = Hematocrit .non significant changes was observed between different groups treated with *T. claveryi* fruiting bodies water extract and normal groups.

Discussion

Worldwide, different medicinal natural product derived drugs have been widely used as primary therapeutics or supplements for treating various human diseases [17,19]. However, the safety of many of these natural remedies has not been scientifically validated.

In the present study, the safety of the aqueous extract of *T. claveryi* fruiting bodies was investigated in vitro and in vivo. In vitro study showed the effect of the truffle extract on the growth of some strains of probiotic bacteria which have important role in gastro-intestinal function. There is good evidence that the complex microbial flora present in the gastrointestinal tract of all warm-blooded animals is effective in providing resistance to disease. However, the composition of this protective flora can be altered by dietary and environmental influences, making the host animal susceptible to disease and/or reducing its efficiency of food utilization [16]. The current study showed that exposure of some probiotic bacteria (*Lactobacillus jensenii*, *Streptococcus thermophilus*, *Lactobacillus casei* and *Lactobacillus bulgaricus*) to the aqueous extract of *T. claveryi* fruiting bodies showed no adverse inhibitory effect on the growth of all strains compared with control un-exposed counterparts. These results may indicate the safety of the natural compounds present in the used truffle extract. Typical results were obtained with the extracts of some plants [3,20].

In vivo study, the safety of aqueous extract of *T. claveryi* fruiting bodies was evaluated on rats. The used extract was ingested orally to rats using different repeated- dose (250mg, 500mg or 10000mg/Kg body weight) daily for 4 weeks. The study revealed that the tested different doses of the truffle extract did not show any adverse effects or any mortality up to the maximum dose (1000mg/kg). During the 4 weeks repeated-dose (250mg, 500mg and 10000mg/Kg body weight of *T. claveryi* extract) study, no notable changes in clinical signs were observed in any of the *T. claveryi* extract dosage groups compared to the control group. Non significant changes in serum clinical biochemistry markers were observed in all *T. claveryi* extract dosage groups in relation to the control group. These markers include liver function biomarkers (total proteins, albumin, ALT, AST, GGT and ALP), heart tissue damage markers (CK and LDH) and kidney function biomarkers (uric acid, creatinine and urea) as well as sodium, potassium and calcium. This biochemical results were confirmed by histological studies. These studies revealed that no changes in the corresponding histomorphological pictures of related organs were observed. Moreover, no marked alterations in serum glucose, lipid profiles as well as in hematological parameters were

noticed in *T. claveryi* extract dosage groups versus control one. This finding may indicate that the phytoconstiteuents of the used extract at the tested different doses and up to 1000mg/Kg body weight have no any adverse toxic effects on the studied biomarkers and related organs.

The current results are supported by some authors who studied of the composition and nutritional value of desert truffles in countries where they are known and appreciated. The studies demonstrated that the truffles consists of protein, fat (unsaturated and saturated fatty acids); crude fiber; carbohydrates; ascorbic acid, carotenoids and many phenolic compounds [10,13 and14]. High levels of potassium and phosphate and significant amounts of iron have been reported [21-22]. No known toxic compound has been detected [23]. It was reported that the beneficial impact of truffle extracts against the eye disease trachoma has a scientific basis [24] and evidence of a rather broad antibiotic activity of truffle extracts has emerged [8 and 15].

The use of natural remedies worldwide raises the demands for standardization and scientific evidence of the safety of such products [25]. Some studies have reported adverse effects of natural products such as hepatotoxicity and nephrotoxicity [26-27]. Natural compounds-induced toxicities mainly originate from misidentification, incorrect preparation, heavy metals overload, and adulteration [28]. The adverse reactions due to the interaction of some natural compound ingredients with Western medicines were also reported [29-30]. Good manufacturing practices and scientific evidence are needed to ensure the safety and efficacy of natural products used as medicine. The standardization of this product is very an important issue for toxicology. Because the amount of ingredients of this product is different according to region, harvested season, preparation condition, age, etc. [31-33].

It is concluded that the used *T. claveryi* fruiting bodies extract is safe to gastrointestinal flora as proved by in vitro study. In vivo study proved that the different doses of the extract (250, 500or 1000mg/Kg) were well tolerated in the rats with no evidence for systemic toxicity. The results suggest that the used fungal extract is safe and could be used as an effective natural formulation in clinical applications with a wide therapeutic index.

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