Tirmania (Zubaidi) and *Terfezia* (Khallasi) Fungi Preparation Method Modulates Body and Testicular Weights and Blood and Testicular TestosteroneConcentration in Albino Rats

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Abstract: The current study aimed at investigating the effects of administering various recipes of a mixture of *Tirmanianivea*and*Terfeziaclavervi* on the body weight, testes weight and serum and testicular testosterone in albino male rats.Tirmania and Terfezia fungi were purchased from Saudi local markets during their harvesting season. Mimicking the natural human consumption for these fungi in the Gulf communities, recipes were prepared by boiling (B), dryness (D), aqueous extraction (A) and extract residues (R). Seventy fiveprepuberal male albino rats were randomly allocated into five treatments (n = 15 rats/group). Group 1 animals served as control (C) given the normal pellets diet (18% CP) and drinking water, group2 animals (A) were given the normal diet in addition to fungal aqueous solution as a replacer for drinking water, group 3 animals (D) were given dried fungi and drinking water, group 4 animals (R) were given the solid residues of fungal aqueous extraction (Tefl) and drinking water and group 5 animals (B) were given boiled fungi and drinking water. The experiment lasted 28 days. Animals were acclimatized for one week before the commencement of the experiment during which a blood sample was taken out of 3 animals within a treatment (i.e. 12 animals were tested for treatments) for the determination of pretreatment testosterone. Each group of animals were housed in a wire cage in a well ventilated room. Results exhibited significant (P<0.05) decreases in testes weight of rats given the solid extract residues (3.02±0.11 g) and those given the boiled fungi (2.69±0.24 g) as compared to control (3.14±0.13 g). Meanwhile, there were no obvious differences (P>0.05) in testes weights due to either aqueous extract (3.35±0.09 g) or dried fungi (3.14±0.11 g). On the other hand, feeding rats with either boiled fungi or solid residues decreased (P<0.05) body weight than control and other treatments. Testosterone levels in both blood serum and testicular tissue extract were highest (P<0.01) only in rats given the aqueous extract (A) and lowest in rats given boiled fungi (B). Dry fungi (D) and extract residues (R) resulted in lower levels of testosterone in serum and testes than in control rats.In conclusion, ingesting *Tirmania* and *Terfezia* fungi must be cautioned by the recipe or cooking method by which these plants are prepared for human consumption.

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Key words:*Tirmania, Terfezia*, rats, body weight, testicular weight, testosterone.

1.Introduction

The desert truffles are a group of subsoil tubers that grown in different areas of the world. They are a type of an obligate hypogeous as comycetesectomycorrhizal fungi formed in association with host roots of *Helianthemum spp.* and the soil inhabiting fungi *Terfezia*or*Tirmania spp.*[1]. The type of mycorrhizalfungus ramifies through the soil, absorbing nitrogen andother minerals, which is transported back to the host plant [2].Desert truffles are seasonal and socio-economically importantfungi. The truffles usually appear in the deserts followingthe rainy season between February and April in many Gulf states [3;4]. They aredistributed naturally from North Africa (Morocco, Tunisia, Algeriaand Egypt) to the Middle East (Saudi Arabia, Kuwait, Iraq, Iran,Lebanon, Syria and Jordon). The people in the Middle East region areconsidered the largest truffle consumers [5], and thetruffle commodity is regardedas a costly delicacy. In these countries,no data is available on total production and consumption.Among the various known edible desert truffle varieties, onlytwo species of the dark brown color truffles belonging to the genus*Terfezia,* locally called khlasi (*Terfeziaclaveryi* and *Terfeziaboudieri*) and one species of the white color truffles belonging tothe genus *Tirmania,* locally called Zubaidi (*Tirmanianivea*) arefound on the Arabian Peninsula [5].Khlasi is ovoidwith a black skin and a pinkish-ivory interior with a nut-like flavor.Zubaidi is cream-colored with a more delicate flavor and is usuallymore expensive. The truffles of the desert are not so stronglyflavored, compared with the European truffles [6]. Nevertheless, the truffle is a type of tuber highlyprized for its unique musky aroma and flavor[7].The popularity of truffles is believed to be due to theirnutritional value and delicious taste. Wild edible fungi not only addflavor to bland staple foods but they are also valuable foods in theirown right. As such, desert truffles are a rich source of protein,amino acids, fatty acids, minerals and carbohydrates [8;9;10].

Some truffles emit the odor of certain mammalian steroids, including androstenol (androst-16-en-3-ol), a chemical that occurs in the black truffle tuber melanosporum and in the testicles of swine. In fact, it is found in the saliva and breathe of rutting boars, where it serves as a pheromone to overcome the sexual inhibitions of young female pigs. This probably explains the natural lust and talent sows have for truffle hunting. The same substance is found in the underarm perspiration of men and in urine of women, and truffles have been used for an aphrodisiac. The sexual role of truffles in humans has not been clearly established, however, men rating photographs of normally dressed women for sexual attractiveness generally give higher marks while sniffing androstenol. This chemical is already being added to certain cosmetics designed to attract potential mating partners. Androstenolhas similar nucleus like the male and female sex hormones (i.e. testosterone and progesterone).

Since truffles are demandable in the gulf region especially for men who believe on the existence of aphrodisiac effects of these fungi. Therefore, this motivated the researcher to highlighten this phenomena by mimicking the recipes of the human consuming the plant and testing the effects of feeding these preparations to male rats on their live body weight, testicular weight and serum and testicular contents of testosterone.

2.Material and Methods

A) Fungus Collection and Preparation

Samples (about one kilogram of each a specie within a location) were purchased on their own raw nature from different locations in Saudi Arabia (Qassim, Arar, Tabouk and Aljouf). Fungi were washed by tap and distilled water until dust and mud were removed and left on absorbing paper to be dried at room temperature overnight. Samples were categorized according to the specie (khalasi*vs*zubaidi). Mimking what is done by the peoples in Gulf region to ingest such fungi,mainly four preparation methods were applied; drying, aqueous extract, boiling and solid residues(Tefl). Samples were dried at 65ºC for 6 hours for the purpose of proximate analyses. For aqueous extraction 250 grams of dried fungi were submerged by one liter of distilled water and left at 90ºC for two hours. After cooling at room temperature the aqueous phase was separated by filtering through sterilized gauze and used as aqueous extract. However, the solid residues precipitated was dried and used as Tefl. Boiling was done by submersing the fresh clean fungi (500 g) in one liter boiled (> 100ºC) distilled water for 10 minutes.

B) Experimental Design

Seventy five adult Wister albino rats were obtained from the experimental animal unit of the Faculty of Pharmacy, King Saud University. Animals were treated according to the animal ethics guideline. Animals were housed in a well ventilated room in galvanized wire cages supplied with feeders and drinkers. Animals were acclimatized for the experiment condition for one week. Animals were randomly allocated into five treatments (n= 15 rats/treatment).Three rats out of each treatment were sacrificed at the commencement of the experiment, blood was collected and sera were harvested for subsequent testosterone determinations. First group served as control (C) animals were given the commercial pellets for rabbits (18% crude protein) and water was provided as a free choice, group 2 animals (A) were provided with pellets plus fungi aqueous solution as a replacer to drinking water, group 3 animals (D) were given dried fungi plus drinking water, group 4animals (R) were given the solid residues of aqueous extract (Tefl) and group 5 animals (B) were given boiled fungi plus drinking water. The experiment lasted 28 days. At the conclusion of the experiment the animals were weighed and sacrificed. Testicles were removed,weighed and kept in normal saline (0.9% NaCl) for further analyses.Blood samples were cooled refrigerated for 2 hours before they were centrifuged (3000 rpm/15 minutes), sera were harvested and kept frozen (- 20ºC) until analyzed for testosterone concentrations.

C) Testosterone Determinations in Serum and Testicles

C-1) Testosterone in Serum

A commercial ELISA kits (HUMAN®,Germany) was used to determine testosterone in rat' sera according to Marcus and Durnford[11]. The procedure complies with the kit insert instructions. Intra- and inter assay coefficient of variations were 3.7 and 5.2%, respectively.

C-2) Testosterone in Testicular Tissues

The whole testis was sonicated (Q700 Sonicator, USA) and tissue homogenate was taken and subjected to hormone extraction in 70% methanol by the incubation of 1g of the homogenate with 5 ml methanol in a shaking water bath (37ºC) for 2 hours. The suspension was cooled, centrifuged (3000 rpm/15 minutes). The supernatant was quantitatively transferred to a labeled tube. A second cycle of the extraction was carried out on the precipitate using another 5ml methanol, shaken in water bath for 2 hours and centrifuged. The first and second supernatants were combined and subjected to a weak stream of nitrogen gas over a warm (30ºC) block until complete dryness. The dried residues were reconstituted in phosphate buffer saline (PBS) and testosterone concentrations were quantified.

D) Determinations of total phenolics, total tannins and antioxidant activity

Powdered fungi were subjected to extraction with 70% acetone (w/w). One gram of powdered sample was shaken with 250 ml of the solvent for 12 hrs, using shaking incubator at room temperature. Solids were separated by centrifugation and filtration. Antioxidant activity, total phenolics and total tannins, were then determined in the extracts.

D-1) Determinations of total phenolics and total tannins

Total phenolics was determined using the Folin-Ciocalteu reagent and gallic acid as a standard [12], and expressed as gallic acid equivalents (mg gallic acid g^{-1} dry matter). Tannin phenolics were separated from non-tannin phenolics using polyvinylpolypyrrolidone (PVPP) as described byOsoro*et al.*[13]. Non-tannin-phenolics were determined using the Folin-Ciocalteu reagent according to a previously described method. The differences between total phenolics and non tannin-phenolics values represented the total tannin contents, which was expressed as mg gallic acid g^{-1}DM.

D-2) Determination of antioxidant activity

1-2,2-Diphenyl-2-picrylhydrazyl(DPPH) radical-scavenging activity.

The DPPH assay was performed according to the method ofOsoro*et al.*[14]with some modifications. The stock solution was prepared by dissolving 24 mg 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 100 ml absolute methanol and then stored at −20 °C until needed. The working solution was obtained by mixing 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.1±0.02 units at 515 nm using the spectrophotometer. Fungi extracts (750 µl) were allowed to react with 1500 µl of the DPPH solution for 5 min in the dark. Then the absorbance was recorded at 515 nm. The standard curve was linear between 25 and 800 µmolTrolox. The results are expressed in µmolTroloxequevalent per 100g DM.

E) Determination of flavonoids and phytoestrogen by HPLC

Flavonoids and phytoestrogens in fungi were hydrolyzed and determinedaccording Ewald *et al.*[15] as follows: one gram of fresh fungus was homogenized using mortar and pestle and subjected to hydrolysis in 40ml of 62.5% aqueousmethanol with 2mg/ml of BHT and 10 ml of 6 M HCl at 90 ^{O}C with reflux for 2 h. Methanol was added to all samples to 100 ml after hydrolysis, and the samples were finally sonicated for 5 min to remove oxygen before being subjected toanalysis by HPLC. The samples were separated with reversed phase column (250 × 4.6 mm) using LC-20 Shimadzu (Japan). The mobile phase consists of 30% of acetonitrile in 0.025 M potassium dihydrogen phosphate (pH 2.4) with a flow rate of 1 ml/min. The compounds were detected by a UV detector set at 290 nm. The eight flavonoids used as standard were quercetin, kaempferol, apigenin, leteolin, myrectein, naringin, neringenin and isorhmantin as well as two phytoestrogen compounds genistein and daidzein. The standards were dissolved in hydrolysis solution.

F) Statistical Analysis

Data were analyzed by the general linear model least square analysis of variances [16] with mean comparisons by Duncan's Multiple Range Test (DMRT)[17]. Significance differences were considered at $P<0.05$.

The following statistical model was applied;

$Y_{ijk} = \mu + T_i + e_{ijk}$

Where;

μ = Overall mean.

Y_{ijk} = Observation on ijkth hormone concentration.

T_i = Cooking method (boiling, aqueous extract, dryingandtefl).

e_{ijk} = Random error.

3. Results

As shown in Table 1, organic matter percentage was higher ($P<0.05$) in Zubaidi (93.3%) than Khallasi (82.8%). Moisture percentages were about 29.6% and 27.8% in Khallasi and Zubaidi, respectively. Percentage of soluble carbohydrates was higher ($P<0.05$) in Zubaidi (57.1 %) than Khallasi (48.4%). Conversely, ash (17.3%) and fats (3.7%) were higher in Khallasi than in Zubaidi (6.7 and 2.8%, respectively). Protein and fibers percentages were not different ($P>0.05$) between the two species.

Both Phenolics and tannins (Table 2) were significantly ($P<0.01$) higher in zubaidi (31.3 and 20.4 mg/g DM, respectively) than khallasi (18.15 and 11.65 mg/g DM, respectively). Likewise, antioxidant activity, as measured by the micromoles Trolox per 100 g DM, was higher ($P<0.01$) in zubaidi (2232)thankhallasi (1452).

Phytoestrogens (Quercetin and Diadzein) concentrations showed (Table 3) opposite trend in zubaidi and Khallasi fungi. The magnitude of increase of Quercetin concentration in fresh Khallasi (2386 mg/kg) was 45 times that found in zubaidi (52.57 mg/kg) fungus. However, this magnitude increased to 82 folds when fungi were dried (10940 vs132.8 mg/kg for khallasivszubaidi). Contrariwise, fresh zubaidi contains about 4 folds higher ($P<0.01$) concentration of Diadzein (25.32 mg/kg) than khallasi (6.38 mg/kg). This magnitude of increase approached 2.2 in case of dry matter.

Table 4 presents effect of cooking/preparation method on rats body and testicular weights. Boiled fungi and extract solid residues exhibit significant ($P<0.01$) decreases by about 25% in body weight than control, however other treatments did not change body weight than control.

Testes weight (Figure 1) significantly (P<0.01) decreased in rats given boiled fungus (2.69 g) as compared with control (3.14 g), however other treatments did not change testicular weights than control.

As depicted in Figure 2, mean testicular tissue testosterone (T) concentration (7.74±0.10 ng/g) was significantly (P<0.01) higher by 4.2 folds than in blood serum (1.85±0.63 ng/ml). Surprisingly none of the treatments have raised testosterone concentration in blood serum, but rather the boiled, dried and solid residues fungi significantly (P<0.01) decreased blood testosterone. The only treatment that maintained similar blood testosterone content was fungal aqueous extract. Corresponding blood testosterone values were 2.7±0.05, 2.2±0.16, 1.8±0.15, 1.3±0.19 and 0.8 ±0.16 ng/ml for control, aqueous extract, dried, tefl and boiled fungus, respectively.

Similar trend was found in testicular tissue testosterone concentrations (Figure 2). There were no an increase in testicular testosterone due to any of the tested treatments. Aqueous extract maintained similar T concentration (10.03±1.08ng/g) as that in control testes (10.59±1.43ng/g). Otherwise, other treatments significantly (P<0.01) decreased T levels in testes.Mean values of testicular T were ; 10.6±1.4, 10.03 ±1.1, 7.2±1.0, 4.4±1.1 and 2.4±1.0, ng/g for control, aqueous, dried,tefl and boiled fungus, respectively. The sharp reduction of T in testis approached 60 and 78% in rats given tefl and boiled fungus, respectively as compared with control rats.

Table 1. Proximate analysis of*Terfeziaclavervi* (Khallasi) and *Tirmanianivea*(Zubaidi) fungi (based on dry matter)(Mean±SEM)

Ingredient	Khallasi	Zubaidi
Organic matter %	82.75 ± 2.15[a]	93.3 ± 3.22[b]
Carbohydrates %	48.36 ± 1.92[a]	57.1 ± 2.21[b]
Crude protein %	18.15 ± 0.67	17.51 ± 0.92
Ether extract %	3.74 ± 0.12[a]	2.8 ± 0.24[b]
Fiber %	12.5 ± 1.1	15.9 ± 2.15
Ash %	17.25 ± 2.75[a]	6.7 ± 1.87[b]

Means in the same row with different superscripts are significantly different (P<0.05).

Table 2. Differences between*Tirmania*(Zubaidi) and *Terfezia* (Khallasi) fungi in phenolics, tannins and antioxidant activity (Mean±SEM)

Fungus	Phenolics (mg/g DM)	Tannins (mg/g DM)	Antioxidant Activity (µ molTrolox/100 g DM)
Khallasi	18.15±0.9[a]	11.65±1.1[a]	1452±65[a]
Zubaidi	31.3± 1.20[b]	20.4±1.38[b]	2232±88[b]

Means in the same column with different superscripts significantly differ (P<0.01).

Table 3. Differences between*Tirmania*(Zubaidi) and *Terfezia* (Khallasi) fungiinQuercetin and Diadzein(mg/kg) (Mean±SEM)

Fungus	Fresh Fungus		Dried Fungus	
	Quercetin	Diadzein	Quercetin	Diadzein
Khallasi	2386± 223[a]	6.38 ± 0.67[a]	10940 ± 435[a]	28.93 ± 4.53[a]
Zubaidi	52.57 ± 5.12[b]	25.32 ± 2.13[b]	132.8 ± 15.22[b]	63.93 ± 5.77[b]

Means in the same column with different superscripts significantly differ (P<0.01).

Table 4. Effect of preparation method of *Tirmania* (Zubaidi) and *Terfezia* (Khallasi) fungi on body and testes weight of albino rats (Mean ± SEM)

Fungi Preparation Method	Post Treatment Body Weight (g)	Post Treatment Testes Weight (g)
Control	265.75 ±16.34[a]	3.14±0.25[a]
Fungi Aqueous Extract	267.01 ±7.86[a]	3.48±0.32[a]
Boiled Fungi	199.43 ±11.12[b]	2.69±0.11[b]
Dried Fungi	240.55 ±12.86[a]	3.14±0.21[a]
Tefl (Solid extract residues)	195.90 ±6.78[b]	3.02±0.09[a]

Means in the same column with different superscripts significantly differ (P<0.01).

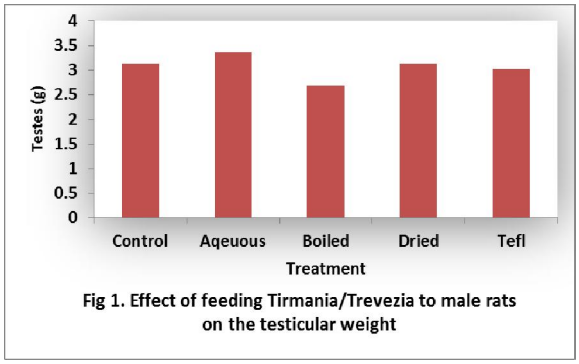

Fig 1. Effect of feeding Tirmania/Trevezia to male rats on the testicular weight

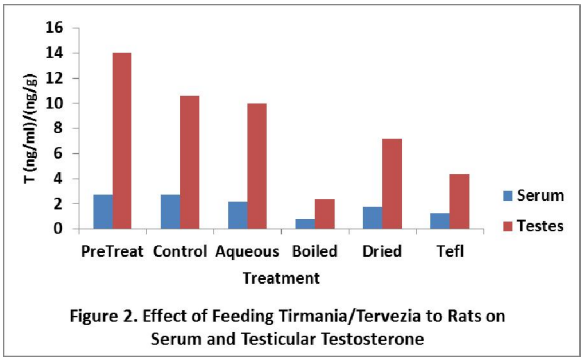

Figure 2. Effect of Feeding Tirmania/Tervezia to Rats on Serum and Testicular Testosterone

4.Discussion

Truffles have long been known as an aphrodisiac. Like all natural medicines their efficiency in increasing sex drive is contested by the medical community.

Many believe it to be a treatment for impotence, acting as an aphrodisiac in both men and women [18].The current study aimed to testing whether ingesting desert truffles (i.e. *Tirmania* and*Terfezia*) would enhance the sexual activity of males which might be due to testosterone levels. most desert and Gulf inhabitants believe that such truffles owe androgenic effects and as a consequence the people ingest these fungi either in the fresh or cooked pattern. The present study highlighted the most common ways of truffle ingestion in the Middle Eastern regions. To associate various compounds in the truffles, two main species of such truffles were obtained from local markets after the thunder season in 2012, i.e. zubaidi (*Tirmania*) and khallasi (*Terfezia*). The intake of the fungi in various recipes revealed the variability on body as well as testicular weights. Boiled and solid residues of aqueous extract gave lower body weights which might be attributed to the fewer intakes and the palatability of these two preparations. Concurrently, there found about 14% reduction in testes weight in rats given boiled as compared with control rats; however none of the other treatments resulted in a change in testicular weight. This decrease in testicular weight paralleled the decrease in body weights of the rats given boiled fungi. Up to the best of our knowledge little, if none, research efforts have been done to monitor the effects of recipes by which these two species of truffles could impact the sexual activity of either male or female mammals. Ingesting boiled fungi or aqueous solid residues (Tefl) resulted in reductions of testosterone concentrations in testicular tissues by about 58 and 78%, respectively of their corresponding value of control rats (10.6, 4.4 and 2.4 ng T/g testis, for control, Tefl and boiled fungi, respectively). Fungal aqueous extract has given similar testicular T levels to that found in control rats. It has been found that these mushroom-like fungi are rich in a weak androgen-like compound,i.e. androstenol, a steroid excreted in underarm sweat and used as a pheromone for promoting sexual activity. It is a sex pheromone in pigs, possessing a musk-like odor. It is found in large quantities in boar saliva, but also in smaller quantities in human sweat glands. It is analogous to sex hormones yet has minimal or no androgenic activity. Androstenol is secreted by the adrenal gland into systemic circulation in humans[19].

Pheromone influences GnRH through direct rhythm of hypothalamic function. These effects are of key importance with respect to humans sexual activity [20] and pheromones remain a factor in properly timed human reproductive sexual behavior[21;22]. Other studies have demonstrated that the smell of androstadienone, maintains higher levels of cortisol in females [23], and that the compound is detected via the olfactory mucosa [24]. The ability of this compound to influence the endocrine balance of the opposite sex makes it a human pheromonal chemo-signal.Preclinical data on the effects of *C. sinensis*on mice showed sex-steroid-like effects [25; 26]. Human clinical trials have similarly demonstrated the effectiveness of *Cordyceps*(wild fungus)in combating decreased sex drive and virility [27; 28].

Concurrent with the present finding of getting best hormonal response with the aqueous extract, mice treated with cyclophosphamide, which suppresses immune function, also treated with the same hot water fungal extract, exhibited their immune function returning to normal, as measured by the IgM and IgG response and macrophage activity [29]. The existence of the very high levels of Quercetin per kg DM of these fungi ,i.e.ZubaidiandKhallasi could serve as a potent antioxidant for body cells maintaining the normal body and organs growth and functionality. Khallasi fungus contains the highest, so far, Quercetin concentration as compared with other edible fruits and vegetables.Quercetin has a higher reduction potential compared with curcumin at three different pH settings and is comparable to Trolox at pH 7-9.5. Its total antioxidant capacity (TAC) is 3.5 fold higher than curcumin. It also reduced lipid peroxides (LPS)-induced ROS to near normal levels and reduced LPS-induced NO production [30].Quercetin is also a phytoestrogen, or a plant hormone that mimics the effects of estrogen in the human body.Phytoestrogens are able to bind to estrogen receptors thus activating several estrogenresponsivegenes[31]. In particular, quercetin has been found to exhibit both estrogenic and antiestrogenic actions *in vitro* thus suggesting different potential effects on reproductive function. Recent evidence [32] suggested that quercetin affects porcine granulosa cell function by interfering with steroidogenic activity and redox status. Moreover, phytoestrogens are thought to act as chemopreventive agents via changing hormone concentration. There are three possible ways of interfering with the complex human endocrine system. First, phytoestrogens might mimic endogenous hormones at the hormone receptor, exerting agonistic orantagonistic effects; second working at key enzymes of hormone metabolism, affectingthe level of active steroids and third phytohormones might have diverse non-hormonal effects[33].Due to the existence of these flavonoids in the form of glycosides, they possess the water solubility, this might explains why the aqueous extract of the fungi in the current study maintained the similar to control testosterone concentrations. Conversely, the boiled and tefl might have lost a relatively large amounts of such water-soluble compounds in the boiling or extracting water. The very high percent of querectin in khallasi fungus might be considered, rather than the testosterone, as the source of the fungal nutritive privilege. Also, the existence of androstenol

as a weak androgen might serve as a precursor for testosterone.

As for daidzein, the concentration was found to be higher in zubaidi than khallasi (about two folds), this also might plays a vital role in modulating testosterone synthesis. When the rats were examined at adulthood, [34] observed that those animals treated with a medium (20 mg/kg) or high (100 mg/kg) dose of daidzein, but not with a low dose (2 mg/kg), showed lower plasma testosterone levels and attenuated erectile parameters, including apomorphine-induced erections and intracavernous pressure concomitant with markedly decreased expression of estrogen receptor beta in the corpora cavernosa. However, the penis still grew to its normal size, as in controls. Thus, these results suggested that exposure of juvenile rats to daidzein in a relatively large amount could adversely affect penile erection in adult hood. In a study on goats [35] found that phytoestrogen-fed goat male kidsexhibited lower plasma and testicular testosterone concentrations.

In conclusion, ingesting truffles (i.e. *Tirmania*and*Terfezia* spp.) as aphrodisiacs for men must be cautioned by the way of cooking, the amount ingested and the stage of ripening, as their contents of glycosides might work as antagonists rather than agonists for male sex hormones, i.e. testosterone. Also, we must confirm that the misbelieve that these fungi are androgenic might be attributed to psychological effects of its scent of androstenol which attracts the opposite sex.

Further studies are requested to monitor various preparations/recipes of these fungi on the subsequent male reproductive performance.

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