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Study the therapeutic effect of truffles on *Rhodotorula* spp. in *vivo*

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Abstract

The current research spots the light on the antimicrobial activity of *Terfezia claveryi* which was evaluated *in vivo*. The laboratory animals were divided to four sets, each set contains (10) mice. The sets were as follows: First set of mice had (two ml/kg) / body wt. of normal saline as a control. As for the second set, they had (two ml/kg) / body wt. of fungal broth (*Rhodotorula spp*), third set had (two ml/kg) / body wt. of aqueous extract of *T. claveryi*. While the fourth set had (two ml/kg) / body wt. of fungal broth and also treated with (two ml/kg) / body wt. of aqueous extract of *T. claveryi*, daily for two weeks orally using gavage tube. The therapeutic effect of aqueous extract of *T. claveryi* was tested by measuring the serum level for AST, ALT, and ALP. Also, histopathological sections for liver and kidney were examined. The findings of the activity of liver enzymes showed that the statistical analysis of the ALP serum level displayed a substantial increment at the level of probability ($p<0.05$) in this parameter in the collection of fungal broth treated (71.40±6.25) IU / ml and serum GOT (231.00±9.42) IU / ml compared to the other three sets. Additionally, non-significant differences at the level of probability ($P>0.05$) in serum GPT level across all four experimental sets were recognized. As for Renal function tests results displayed a substantial increment at the level of probability ($P<0.05$) of B. urea in fungal broth treated set (28.51±3.10) mg/dl when compared with other three sets. In conclusion, the aqueous extract of *T. claveryi* was found to have an antimicrobial effect versus the infectious *Rhodotorula spp.*

Keywords: Truffle, Antimicrobial activity, Edible mushroom, *Rhodotorula spp*., Therapeutic effect.

Introduction

The yeast *Rhodotorula* is a widely distributed species, it can exist in water and soil habitat. In addition, this yeast can be isolated from human infections. This species belongs to the phylum Basidiomycota (Larone, 1995), with colored colonies (pink – red) (Miceli *et al*., 2011). This genus consists of 8 species, *R. mucilaginosa*, *R. glutinis* and *R. minuta* are infectious and known to cause diseases, since it was isolated from humans and several types of animals (Tuon and Costa, 2008; Wirth and Goldani, 2012). (Baran, 1998; Kurnatowska and Kurnatowski, 2006) have regard that these fungi may invade the mucous membranes of gastrointestinal tract, urinary ducts, vagina and skin, and in the immunosuppressed patients (Krzyściak *et al*., 2011). In addition, the possibility of isolating these types of yeasts has been known from birds, bird droppings and animals and found in different ecosystems (Cafarchia *et al*., 2008; Al-Temimay and Hasan, 2016). (Krzys´ciak, 2010) have evidenced the presence of some fungal virulence factors in tested *Rhodotorula* strains. While some of them have appeared resistance to antifungal drugs.

Recently, most of researches have been focused in identifying various different or reciprocal medicines to treat infections caused by the drug resistant microbial. Truffles are taken into account as good alternatives to synthetic chemicals. They are fungi that completely live underground associated with the roots of some trees, mostly oaks. In spite of over than 20 species of truffles are known in Europe, only a few have cooking valence. Truffles spend their life stages under the ground and must be eaten by animals to set free their spores, especially mammals which attracted to its strong aroma (Truffles. 2008).

Besides to truffles nutritional importance, they performed a very wide and broadly unexplored source of therapeutic compounds such as antimicrobial, anti-mutagenic and anti-carcinogenic properties so far (Murcia *et al*., 2003; Janakat *et al*., 2004). The recent uses of mushrooms are quite diverse of the early days of civilization, because of doing many applied researches based on the effectiveness of the natural chemical components

found in mushrooms, which resulted in the possibility of using mushrooms as food and treatment at the same time fighting many diseases (Rai, 1994). Truffles extractions, fundamentally of *T. claveryi, T. nivea*, and *T. boudieri*, was used for the purpose of treating skin diseases and eye infections in the Middle East countries (Mandeel and Al-Laith, 2007; Patel, S. 2012). Recently, specialists and nutritionists focused attention on the healing and vital properties of truffles, such as their antioxidant, antimicrobial, antiviral, anticancer, anti-inflammatory activities, hepatoprotective and anti-mutagenic (Hussain and Al-Ruqaie, 1999; Shavit and Efrat, 2014). The leading objective of current research is to evaluate the therapeutic effect of aqueous extract which was tested on both liver and kidney functions after they were infected by an inoculum of yeast *Rhodotorula spp*. by an experimental model of female albino mice, although most previous studies were *In Vitro* and against different species of bacteria; so, this study is the first one in Iraq.

Material and Methods

Sample's collection: Samples of bird's droppings were collected from around chicken eggs, chicken fields and cages of domestic birds. During the period that confined between September 2018- Jun 2019, samples were cultured and examined by dilution plate method. The growth of yeasts colonies (*Rhodotorula* spp.) was appeared on both Sabouraud's Dextrose Agar medium (SDA) and Potato Dextrose Agar medium (PDA), at (25°C) as an optimum temperature, the identification occurred according to the colonial morphology and microscopic characteristics (Mandeel and Al-Laith, 2007). Samples of dropping were collected in sterile tubes before the daily cleaning of the cages, about (1gm) of dropping was placed in each tube and diluted in 5ml of sterile water, then tubes were centrifuged at (3000 rpm) for ten min., 0.1ml of inoculum was placed on both SDA and PDA media with (0.05) mg/ml of Chloramphenicol in sterile condition. All plates were incubated at room temperature (25 ±1°C) for 2- 3 days depending on the growth rate (Patel, S. 2012).

Isolation and identification of fungi: Sub-culturing was applied to get pure cultures then the growth cultures were microscopically examined to check for fungi colony. The purified fungal isolates were subjected to taxonomic classification up to species level as far as possible by a detailed examination of their colonial characters and microscopic morphology in lactophenol cotton blue, and compared their characteristics with the descriptions of the species in the standard books and manuals (Hussain and Al-Ruqaie, 1999).

Preparation the aqueous solution of *T. claveryi*: In order to prepare the aqueous solution, 50 g of fresh truffle fruiting bodies were broken into fine fragments with a blade, and then held for 24 hours in 1:3 D.W (w / v). The mixture was then homogenized for 1 min. in a house hold blender. The solution resulting from mixing was placed in a very soft two-layer cloth and squeezed vigorously, then it was separated by a centrifuge at 4^{o}C for 10 min. at (3000 rpm). Then the supernatant was filtered via sterile filter (0.45 µm) (Miceli *et al*., 2011).

Experimental animal: For the purpose of doing this experiment, 40 female albino mice were selected, and their weights ranged between 30-35 g. All experimental mice were cared for and incubated in standard laboratory conditions (12 h /12 h) light-dark programmed cycle, at (22 °C – 25 °C) room temperature in the animal tissue laboratory \ Biotechnology Research Center \ Al-Nahrain University.

Experimental design: The total number of mice was separated into four sets, every set had (10) mice as follows:

1st set: mice were received (2) ml / kg/ body wt. of normal saline and considered as a control.

2^{ed} set: mice were treated (2) ml / kg/ body wt. of fungal broth without treatment

3rd set: mice were treated (2) ml/kg/ body wt. for aqueous extract of *T. claveryi*.

4th set: mice were treated with both (2) ml / kg/ body wt. of fungal broth and (2) ml / kg/ body wt. for aqueous extract of *T. claveryi*

*All sets received the treatment orally using gavage tube and daily for two weeks.

Collection of Blood and Organ Samples: After two weeks mice were subjected to fasting for the whole night, then they were anesthetized with chloroform, later, they were sacrificed.

As for blood samples, they were obtained using syringe and needle via puncture of the heart. After taking a blood sample from each mouse separately, these samples were placed in dry tubes for clinical chemistry analyzes and other tubes containing EDTA for hematology tests.

The tubes containing the blood samples were left vertically at laboratory temperature for 20 min. for the purpose of clot formation, then they were placed in a centrifuge at a speed of 3000 rpm for 5 min. Sera samples kept in -20°C until used for biochemical tests. Mice were dissected and the

organs (liver and kidney) were carefully removed, excised and washed with 0.9 % of normal saline (NaCl), after that, they were placed in plates with fixative solution (neutral buffered 10% formalin) for histological examination (AL-Taie, 2019).

Biochemical and histological tests: The histological sections were made due to the aforementioned method of work by (Luna, 1968), then all the sections were stained with Hematoxylin-eosin staining (H and E stain 20x) and subjected to a microscopic examination for the purpose of studying the histological variations under 40 times of magnification.

A- The function of liver was examined by the rating of:

1. Serum aspartate aminotransferase (AST/GOT) (Kit: Biomaghreb, Tunis). 2. Serum alanine aminotransferase (ALT/ GPT) (Kit: Biolabo Reagents, France).
3. Serum alkaline Phosphatase (ALP) (Kit: BioMerieux, France).

B- The function of kidney was examined by the rating of:

1. Both serum creatinine and urea using enzymatic methods (Kit: BioMerieux, France) (Burtis and Ashwood,1999).

Statistical analysis: The data obtained was statistically analyzed by means of the analysis of variances at a probability level (P 0.05), all variances were expressed as the mean ± standard error (SE) using the software program SPSS (Basher, 2003).

Results and Discussion

Liver enzymes activity: The results of liver enzymes activity of the four experimental sets included in this study is illustrated in (Table-1). Statistical analysis of the giving data detected that there were no significant differences at probability level (P>0.05) in GPT serum level among all four experimental sets. A substantial raise at probability level (P<0.05) was found in GOT serum level in the fungal broth treated set (231.00 ± 9.42) IU/ml when comparing it with others; while, there were no significant differences at probability level (P>0.05) among the rest.

Concerning the serum ALP level, statistical analysis revealed that there was a substantial raise at probability level (p<0.05) in set of mice that were treated with fungal broth (71.40 ± 6.25) IU/ml when comparing it with others. While, there were no significant differences at probability level (P>0.05) between other three sets.

Table (1): Serum enzymes activity in the experimental sets of the study

Mice sets/ treatments	Serum Enzymes Activity (means± SE)		
	ALT GPT (IU/ml)	AST GOT (IU/ml)	ALP (IU/ml)
Control	73.86±5.08 [a]	203.01±8.44 [b]	65.47±4.62 [b]
Fungal broth (2 ml/kg)	74.51±4.91 [a]	231.00±9.42 [a]	71.40±6.25 [a]
Aqueous extract of *T. claveryi* (2 ml/kg)	73.83±5.05 [a]	203.00±8.41 [b]	65.44±4.61 [b]
Fungal broth (2 ml/kg) +Aqueous extract of *T. claveryi* (2 ml/kg)	73.98±5.06 [a]	206.72±9.22 [b]	66.21±4.37 [b]

*** The mean of similar lowercase letters indicates no significant difference at probability level (P>0.05).**

***The mean of different lowercase letters indicates significant difference at probability level (P<0.05).**

Renal function tests: Renal function tests of the four experimental sets in current experiment are indicated in (Table- 2). The findings showed a substantial raise at probability level (P<0.05) concerning B. urea level in mice set that were treated with fungal broth (28.51±3.10) mg/dl when comparing it with others. Also, no significant difference at probability level (P>0.05) was detected in mice set that were treated with *T. claveryi*'s aqueous extract only, and the set that were treated with *T. claveryi*'s aqueous extract + fungal broth.

Regarding the S. creatinine, statistical analysis of resulting data reveled the presence of significant raise at probability level (p<0.05) in fungal broth treated set (0.93 ± 0.08) mg/dl when compared with other sets. On the contrary, no significant differences at probability level (P>0.05) between each of the aqueous extract of *T. claveryi* treated set as well as the fungal broth + aqueous extract of *T. claveryi* treated set when compared with the control set.

Table (2): Renal function tests in the experimental sets of the study

Mice sets/ treatments	Renal function tests (mean± SE)	
	B. urea (mg/dl)	S. creatinine (mg/dl)
Control	20.22±3.28 [b]	0.74±0.06 [b]
Fungal broth (2 ml/kg)	28.51±3.10 [a]	0.93±0.08 [a]
Aqueous extract of *T. claveryi* (2 ml/kg)	20.20±3.27 [b]	0.73±0.07 [b]
Fungal broth (2 ml/kg) + Aqueous extract of *T. claveryi* (2 ml/kg)	21.12±3.22 [b]	0.78±0.06 [b]

*** The mean of similar lowercase letters indicates no significant difference at probability level (P>0.05).**
***The mean of different lowercase letters indicates significant difference at probability level (P<0.05).**

Histological Study: Histological examination of the liver from control mice set, Figure (1), showed a typical structure of liver tissue. The lobule of the liver has a hexagonal shape and the central vein is located in the middle of it. As for the hepatocytes, they are arranged in the form of a single rope-like layer. Histological assessment of liver tissues from the fungal broth treated mice set revealed that there were histological changes on great scale, Figures (2 and 3), such as degenerative cells and apoptotic cells, in addition to heavy hemorrhage was evident; section also showed inflammatory cell infiltrations and sever necrosis. In mice treated with aqueous extract of *T. claveryi*, liver sections showed normal central vein, normal hepatocytes and distinct lobulation, in which normal hepatic structure was maintained, Figure (4).

Regarding the fungal broth+ aqueous extract of *T. claveryi* treated mice set, the histological changes of the liver were slight than those in the fungal broth treated mice set. In this set, mild vascular congestion and mild hemorrhage was observed, Figure (5). Light microscopic examination of the kidney sections in the control mice set, Figure (6), revealed normal histomorphologic tissue. In addition to heavy hemorrhage was evident; section also showed inflammatory cell infiltrations and sever necrosis.

Histological evaluation of kidney tissues from the fungal broth treated mice set showed histopathologic signs such as heavy hemorrhage; sever necrosis, and inflammatory cell infiltrations, Figures (7 and 8).

Kidney tissue belongs to mice treated with aqueous extract of *T. claveryi* showed no histological changes, Figure (9). Treatment of the mice with combined fungal broth and aqueous extract of *T. claveryi* produced slight histological changes of the kidney represented by mild vascular congestion and there was better improvement compared to fungal broth mice treated set, Figure (10).

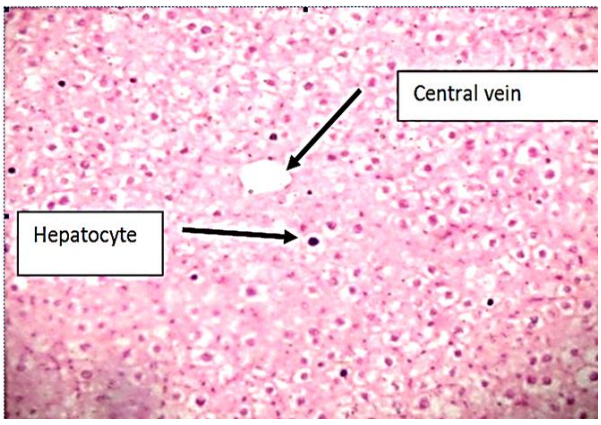

Figure (1): Section of control mice liver showing normal appearance of hepatocyte with central vein Slides stained with (Hematoxylin-eosin staining, 10 X)

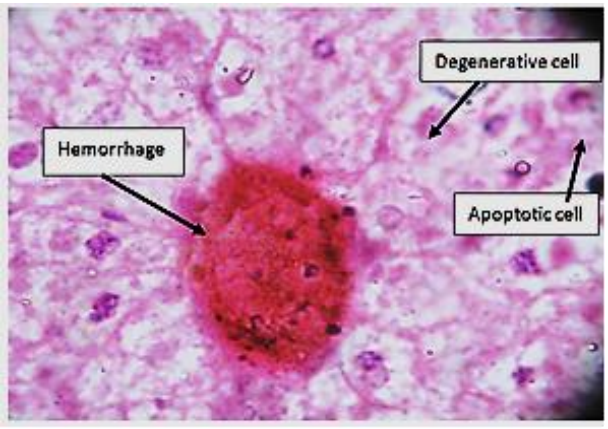

Figure (2): Section of mice liver treated with fungal broth (2 ml/kg) showing Degenerative cells and apoptotic cells with heavy hemorrhage. Slides stained with (Hematoxylin-eosin staining, 100 X)

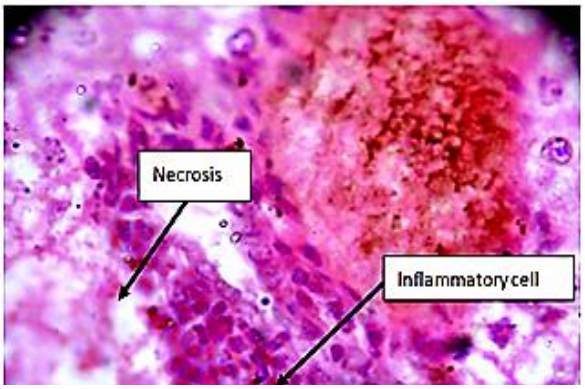

Figure (3): Section of mice liver treated with fungal broth (2 ml/kg) showing inflammatory cells with necrosis Slides stained with (Hematoxylin-eosin staining, 100 X)

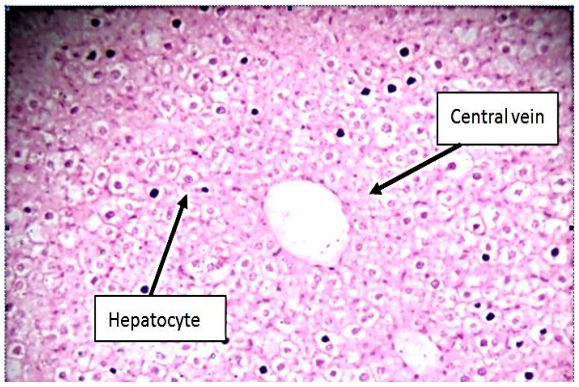

Figure (4): Section of mice liver treated with aqueous extract of *T. claveryi* showing normal central vein and normal hepatocytes with distinct lobulation (Slides stained with (Hematoxylin-eosin staining, 20 X)

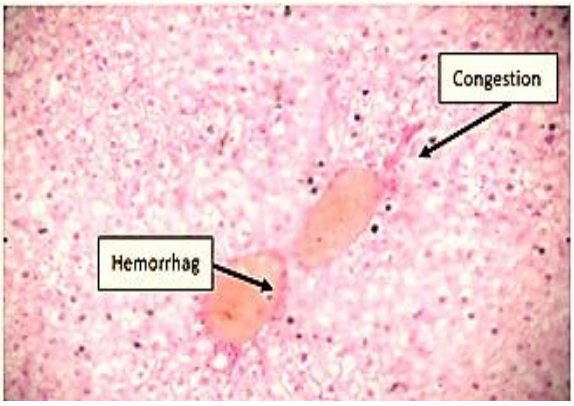

Figure (5): Section of mice liver treated with fungal broth (2 ml/kg) +aqueous extract of *T. claveryi* (2ml/kg) showing mild vascular congestion and mild hemorrhage Slides stained with (Hematoxylin-eosin staining, 10 X)

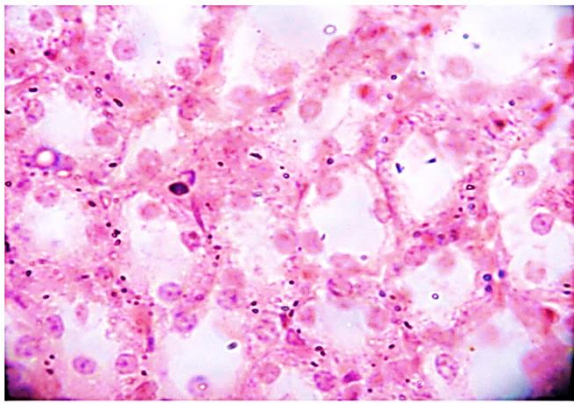

Figure (6): Section of control mice kidney showing Normal histomorphologic tissue Slides stained with (Hematoxylin-eosin staining, 40 X)

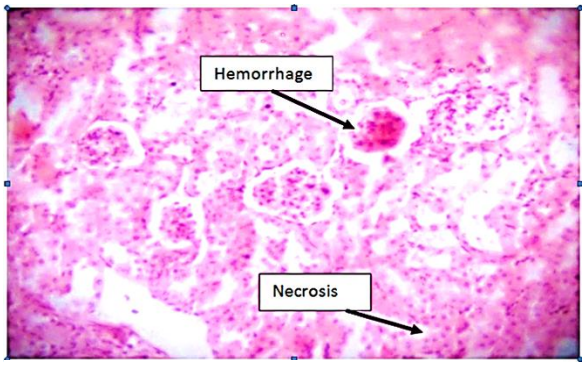

Figure (7): Section of mice kidney treated with fungal broth (2 ml/kg) showing necrosis and hemorrhage Slides stained with (Hematoxylin-eosin staining, 20 X)

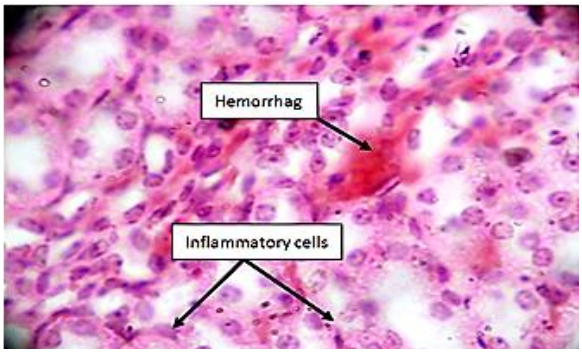

Figure (8): Section of mice kidney treated with fungal broth (2 ml/kg) showing hemorrhage and inflammatory cells Slides stained with (Hematoxylin-eosin staining, 40 X)

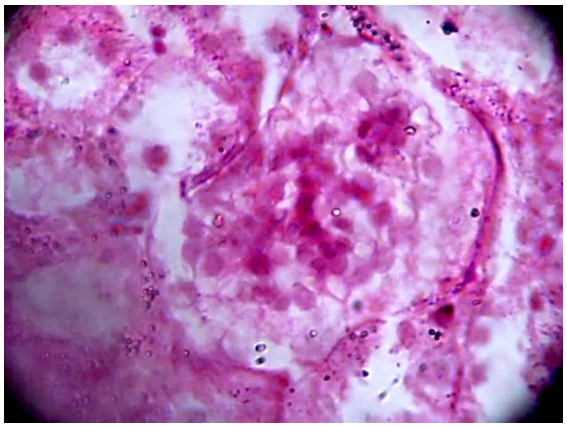

Figure (9): Section of mice kidney treated with aqueous extract of *T.claveryi* showing normal renal morphology Slides stained with (Hematoxylin-eosin staining, 40 X)

Figure (10): Section of mice kidney treated with fungal broth (2 ml/kg) + aqueous extract of *T. claveryi* (2 ml/kg) showing mild vascular congestion Slides stained with (Hematoxylin-eosin staining, 40 X)

The functions of liver were examined by estimating aspartate aminotransferase serum (AST/GOT), alanine aminotransferase serum (ALT/ GPT) and alkaline Phosphatase serum (ALP) activities. In addition, the functions of kidney were examined by estimating both creatinine and urea sera concentrations using enzymatic processes (Al-Jowari and Hussein, 2014). Due to the resulting data, fungal broth treated set has shown a considerable change in both AST and ALT sera activities of liver. Therefore, the activities of both AST and ALT sera are splendid signs of hepatocyte breakdown, moreover, the specificity of ALT serum is higher than that of AST serum in the assessment of liver damage (Radostits, 2007). Through doing some studies and experiments, it was found that the observed changes in serum enzymes indicate their tendency to rise in case of exposure to an infection, and this rise can be considered as reflective evidence of the degree of liver damage (Al-Kaissy, 2010). Such damage leads to modification in metabolism which leads to decrease the synthesis of hepatic protein (Montoya and Liesenfeld, 2004).

Desert truffles' extract, particularly that of *T. boudieri, T. claveryi and T. nivea* species, has been used by many people to treat both ophthalmic infections as well as skin lesions for hundreds of years (Shavit and Efrat, 2014; Janakat, 2005). In the same field, some studies revealed that the desert truffles' peel in particular possesses beneficial nutrients, antioxidents and effective compounds with biological and antimicrobial activity (Patel, S. 2012; Shavit and Efrat, 2014).

(Murcia *et al*., 2003; Janakat, 2005), have been examined the effect of partially purified proteins from both aqueous and alcoholic (methanolic) extracts of *T. claveryi* against *Staphylococcus aureus* and *Pesudomonas aeruginosa*, while (Bekci, 2011) had clearly indicated that acetone compound which was extracted from *T. claveryi* has high efficacy and in varying proportions against many important bacterial species and genera such as *Bacillus cereus, Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus enteritidis*, *Staphylococcus aureus*, *Candida glabrata* and *Candida albicans*. The reason for such anti-microbial activity may be due to the possession of these pathogenic organisms with special receptors that are sensitive to the various chemicals present in the compounds of this fungus (Jeong, 2009; Shavit and Efrat, 2014).

No studies were reported about the antifungal activity of the aqueous extract of *T. claveryi* against the yeast *Rhodotorulla* spp., *In Vivo*, so this study is the first one in Iraq.

Conclusion

According to results of the present experience, it has been shown that the aqueous extract of *T. claveryi* has been found to have an antimicrobial activity against infections caused by *Rhodotorula spp*. on four sets of laboratory mice. The therapeutic effect of aqueous extract of *T. claveryi* was tested by measuring the serum level of liver enzymes for AST,

ALT, and ALP. As well as, the renal function tests for the level of B. urea and S. creatinine besides dissection and study the histological variations of some organs such as the liver and kidneys.

The results obtained were satisfactory and reasonable. It is recommended to use such types of extracts on a larger scale through testing their effectiveness on some infections resulting from pathogenic yeasts such as *Candida spp*. and various bacterial infections.

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