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# *Rhodotorula glutinis* and Its Two Mutants Ameliorate Hepato-Renal Dysfunction Induced by Ochratoxin A on Rats

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author WH and all authors designed the study, followed up the practical work and wrote the final version of the manuscript. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aim:** This study was designed to investigate the possible curative effect of *Rhodotorula glutinis* (*R. glutinis*) and its two mutants (Col-1R1 and Col-1R3) against hepatorenal toxicity induced by ochratoxin (OA) in rat.
**Methods:** The strains of yeast Col- 1R1 and Col- 1R3 have been genetically improved and isolated from *R. glutinis* after colchicines treatment. OA was produced and determined from *Aspergillus ochracus* isolate from Egyptian corn. Experimental design: Five groups of rats were treated as follows: group 1, was the control group orally given 4 ml / Kg 0.1 M $NaCOH_3$; group 2 treated with OA (1.7 mg /Kg).Groups 3, 4 and 5 orally administered the *R. glutinis* and its two mutants (50 $X10^6$ colony forming unit (cfu) / 10 ml saline / kg body weight) prior 1hr of OA -treatment for 15 successive days.
**Results:** The studied autoploidy strains showed significant increase in caratenoids level,

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protease, β-1, 3-glucanase and chitinase activities when compared with the parental strain. Biochemical results revealed that OA significantly decreased serum total antioxidant capacity (TAC) and it caused elevation inserum transaminases (AST, ALT), creatinine, uric acid, nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and carcinoembryonic antigen (CEA) ($P < 0.05$) as compared with the control group. The three tested yeasts significantly decreased the elevated values toward the normal levels and improved the pathological feature in liver and kidney tissues. Moreover, *R. glutinis* and the two mutants significantly reduced hepatorenal damaged arias, increased optical density of DNA and alleviated ochratoxin A-induced caspase-3 activation. The resultant effect of the two mutant strains had more powerful effect more than the wild strainto ameliorate hepatorenal dysfunction in ochratoxicosis-rat.

**Conclusion:** Col-1R3 was more effective than Col-1R1 may be due to its higher contents of carotenoids, glucane and chitine, which act as antioxidants.

*Keywords: Rhodotorula glutinis; mutants; ochratoxin a; hepatorenal damage; antioxidant; oxidative stress.*

## 1. INTRODUCTION

*Ochratoxins* are fungal secondary metabolites that contaminate grains, legumes, and dried fruits [1]. Several mycotoxins in agricultural products cause health hazards to people and animals and economical problem. Dangerous mycotoxins are naturally present in foods, feeds and our environment. They are pathologically classified as hepatotoxins, nephrotoxins, vomitoxin and neuro-musculotoxin, some of which are potentially carcinogenic and mutagenic.Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest grains, toxinogenic fungi can be divided into three groups: (a) field fungi; (b) storage fungi; and (c) advanced deterioration fungi. The first category includes species of plant pathogenic fungi, namely, genus *Fusarium,* e.g. *F. moniliforme, F roseus, F. tricinctum and F. nivale.* The "storage fungi" are principally the general *Aspergillius and Penicillium*, e.g. *A. flavus* and *A. parasiticus* [2].

The decreasing efficacy of many fungicides used to control plant diseases, as well as risks associated with fungicide residues on the leaves and fruit, have highlighted the need for a more effective and safer alternative control measures. Development of economically and environmentally sound control strategies through developing indigenous antagonistic yeasts effective against pathogens involves an effective, accurate and reproducible method to identify and characterize the promising isolates, instead of classical identification methods.

Recently, it has been proposed to reduce Ochratoxin A producing *Asperigillus niger* by means of biocontrol yeast, *Rhodotorula glutinis* Harrison [3]. Several isolates of *R. glutinis* reduced sporulation and established pathogen and disease severity [4]. Several yeast species are known to accumulate carotenoid pigments as secondary metabolites. In these microorganisms, carotenoid synthesis is associated with growth. Maximum carotenoid accumulation is observed in stationary phase in relation with cell ageing, and is probably a general mechanism of defense against oxidative stress [5]. The synthesis of torularhodin, torulene, and b-carotene is common in several genera of Rhodotorula, Sporobolomyces, and Cystofilobasidium [5]. In these microorganisms, carotenoids may contribute to preserve the viability of ageing cells by quenching oxygen radicals, possibly compensating for their lack of antioxidant enzymes. It is well known that some of economically important yeasts are high

ploidy. The increment of ploidy leads to a larger cells than that of the wild strain. Colchicine treatment of cells is an easy and simple method for breeding a microorganism possessing a high ploidy [6]. The aneuploidy can confer selective advantages by increasing the copy number of beneficial genes and protecting against lethal or deleterious mutations [7]. Many trails were conducted for minimizing the effects of toxins on human and animal health as well as increasing the animal productivity and performance. In animals, many additives such as specific types of clay could be added to their food to bind or reduce the harmful effects of toxins [8]. This study was designed to evaluate the possible curative effect of the wild strain and its two mutants against hepatorenal toxicity induced by ochratoxin (OA) in rat.

## 2. MATERIALS AND METHODS

### 2.1 Types of Used Yeasts

The strains of yeast G2 (Col- 1R1) and G3 (Col- 1R3) have been genetically improved and isolated from the wild type yeast strains *Rhodotorula glutinis* (G1) after colchicines treatments [9]. Briefly, wild type yeast isolates were cultured in flasks containing Yeast Nitrogen Base (YNB) liquid medium supplemented with 5% glucose and incubated at 30ºC for 18 h. Then, colchicine was added to the yeast cell suspensions to give final concentrations (0.2%). The treated cell suspensions were incubated for further 19 h. The treated cells were inoculated on YMPG medium plates and incubated for 2 weeks at 30ºC. A glance on colonies grown on the agar plates showed that large colonies were always composed of large cells than that of small colonies. Consequently, large cells from about 270 initial large colonies of *R. glutinis* were selected.

### 2.2 Carotenoid Analysis

Culture was prepared by inoculating one loop of a slant culture in 50 ml of YM broth (Difco) in a 150 ml Erlenmeyer flask, incubating at 25ºC in a shaker at 200 rpm for 72h. Fermentation experiments were carried out in 500ml Erlenmeyer flasks containing 200 ml of YM broth. Each flask was inoculated with starter culture (5% v/v) and incubated at 25ºC for 5 days at 200 rpm without illumination. After cultivation, the cells were harvested by centrifugation at 16,000 x g for 20 minutes, washed twice with deionized water and centrifuged again. The pigments were extracted and quantified according to Rodriguez-Amaya [10]. The total carotenoids were determined spectrophotometrically, and expressed as b-carotene. For high performance liquid chromatography (HPLC), the extract was concentrated in a rotary evaporator, dried under nitrogen, and redissolved in 1ml acetone with the aid of sonication. An aliquot of 10 µl was then injected into the liquid chromatography. The column was C18, 5 µm, 4.6 mm x 250 mm. The mobile phase for *Rhodotorula* strains was tetrahydrofuran: H2O: methanol from 15:4:81 to 30:0:70 in a linear gradient in 40min, maintaining this proportion until the end of the run. The flow rate was 0.5 ml/min.

### 2.3 Enzymes Activities

Activities of hydrolysis enzymes were assessed in culture filtrates of the tested yeast isolate 7 days using a Lab systems Uniskan II microtiter plate spectrophotometer. Protease activity was measured in dimethylcasein (5 mg/ ml in 20 mM phosphate buffer pH 7.0) as substrate using auto analyser. The release of alanine was measured and used as a basis for the expression of protease activity (I U = 1 µmol alanine/ min/ g) [11]. Exo-glucanase activity was detonated using glucose oxidase-O-dianisidine reaction (Sigma Chemicals, glucose

determination kit 510-A) which specifically measures glucose produced from laminarin hydrolysis. ß-1,3-Glucanase activity was assayed by incubating 0.2 ml of culture filtratein 50 mM potassium acetate buffer (pH 5.5) with 50µl of enzyme solution appropriately diluted in the same buffer. Reaction mixtures were incubated at 37°C for 30 min and were stopped by boiling for 5 min. One unit of ß-1,3-glucanaseactivity was defined as the amount of enzyme that releases 1 µmolof reducing sugar equivalents (expressed as glucose) per min under the standard assay conditions [12]. Protein concentrations were determined by the method of Bradford, with bovine serum albumin as the standard [13].The activity of exo-chitinase, was measured as the release of N-acetyl glucoseamine from chitin and one unit (U) of enzyme activity was defined as the amount of enzyme that release 1 µmol of reducing groups/ min/ ml of the filtrate [13].

## 2.4 Production and Determination of Ochratoxin A (OA)

The OA production was carried out by *Aspergillus ochracus* isolate from Egyptian corn. OA was produced and determined following the methodology described by [14]. The strain was grown in stationary cultures using 200 ml quantities of YES medium (2% yeast extract, 15% sucrose) in 1000 ml Erlenmeyer flasks and were inoculated with 1 ml of dense conidial suspensions and incubated at 30ºC for 10 days in darkness. After incubation, 1 ml portions of this culture media were then mixed with 1 ml of chloroform and centrifuged at 4000 *g* for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and dissolved in appropriate amounts of the mobile phase (57% acetonitrile, 41% water and 2% acetic acid) and OA was detected on a HPLC isocratic system. The HPLC apparatus used for detection was a Hewlett-Packard chromatograph (Waters Company) with a loop of 100 µl, equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a $C_{18}$ column (Supelcosil LC-ABZ, Supelco, Belleforte, PA, USA; 150 × 4,6 mm, 5 *µ*m particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4,6 mm, 5 *µ*m particle size). The mobile phase, was pumped at a rate of 1 ml $min^{-1}$. Extracts were considered positive if they yielded a peak at retention time identical to that of standard OA. The standard solution was prepared as described by AOAC [15] using OA obtained from Sigma (St Louis, MO, USA). The lowest limit of detection was 1 ng $ml^{-1}$.

## 2.5 Kits and Chemicals

Total antioxidant capacity (TAC), carcinoembryonic antigen (CEA), Alanine transaminase (ALT), Aspartate transaminase (AST), uric acid and creatinine kit were purchased from Diagnostic Products Corporation Co., USA. Tumor necrosis factor alpha (TNF-a) kit was produced by Diaclone Research Co., France. Nitric oxide (NO) kit R & D system was purchased from Gmbtt, Germany. All other chemicals, used throughout the experiment were of the highest analytical grade available.

## 2.6 Experimental Animals

Adult male Sprague–Dawley rats weighing 120-130 g were obtained from the animal house, National Research Centre (Cairo, Egypt) and fed a standard laboratory diet and tap water *ad libitum*. Experimental animals were housed in an air-conditioned room at 22–25ºC with a 12-h light/dark cycle. All animals received animal care and the study protocols were in compliance with institutional guidelines for the use of laboratory animals.

## 2.7 Experimental Design

Animals were divided into 5 equal groups (6 rats each) and treated as follows: group 1 was the control group given 0.1 M $NaHCO_3$ (4ml / kg, oral gavage); group 2, treated with OA (1.7 mg/kg, intraperitoneally, i.p.) dissolved in 0.1 M $NaHCO_3$ [16]; groups 3,4, and 5 orally administered $50^6$ colony forming unit (cfu) / 10 ml saline / kg body weight) for each strain which equal 100 µg cuf/ml [17], respectively, daily for 15 successive days 1hr prior OA gavage. At the end of experimental period, blood samples were withdrawn from the retro-orbitalveinus plexus from each rat under light anesthesia. Serum was separated to determine the biochemical analysis.

### 2.7.1 Histopathological and histochemical studies

Livers and kidneys were dissected from all animals and portions from these tissues were fixed in 10% neutral buffered formalin solution**,** and then embedded in paraffin wax. Sections of 5µm in thickness were cut and stained with hematoxylin and eosin (H & E) to be prepared for histopathological examination [18]. While other set of slides were stained with Feulgen reaction for DNA quantitative measurement.

### 2.7.2 Immunohistochemistry of activated Caspase-3 in liver and kidney tissues

Sections of 5 µm thickness were deparaffinized and incubated with fresh 0.3% hydrogen peroxide / methanol for 30 min at room temperature. The specimens were then incubated with anti-caspase-3 antibody (1:100 dilution). The specimens were counterstained with H and E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

### 2.7.3 Image analysis of area damage

Liver and kidney damaged areas were directly determined using Qwin Leica image processing and analysis system 2(Cambridge, UK) on slides stained by H & E.

The percentage of damaged areas was measured and the degeneration was estimated as a percent of the total section area. The average measurements from each tissue section were 15 random fields /section was statistically analyzed as means± standard error of the means.

### 2.7.4 Image analysis of quantitative of DNA

Quantitative measurements were obtained using Leica Qwin 500 Image Analyzer Computer System (England). Ten fields were chosen in each specimen, and then statistically analyzed as means± standard error of the means.

## 2.8 Statistical Analysis

In the present study, all results were expressed as mean ± standard error of the mean. Data were analyzed by one-way analysis of variance (ANOVA) at $P<0.5$. When the variation among groups was proved significant, the least significant difference (LSD) test was performed to compare significance between groups.

## 3. RESULTS

The pigmented yeast of the *Rhodotorula wild type and its polypoliday* have the capacity to produce carotenoids. Selected autoploidy strains showed that strain Col-1R1andCol-1R3 produced the largest amount of caratenoids (Table 1).The extracellular enzymes production of the selected autoploidy strains showed that strain Col-1R1 and Col-1R3 produced the largest amount of extracellular protease, β-1, 3-glucanase and chitinase when compared with their wild type strains (Table 1).

**Table 1. Changes in cell properties of the wild type yeast *Rhodotorula glutinis* compared with some of large cell strains isolated after colchicine treatments**

| Yeast Isolates | Total carotenoid (g yeast)-'] | Protease | β-1, 3 glucanase | Chitinase |
|---|---|---|---|---|
| | | μ $g^{-1}$ cells dry weight | | |
| *R. glutinis* (WT) | 170±0.012 | 1.41±0.01 | 1.26±0.01 | 0.88±0.002 |
| Col-1R1 | 213±0.42 | 4.62±0.03 | 5.31±0.03 | 3.4 1±0.02 |
| Col-1R3 | 265±0.32 | 4.71±0.03 | 5.6 2±0.04 | 3.9 4±0.03 |

Ochratoxin A (OA) significantly decreased serum TAC level, and significantly elevated in serum of TNF-α, CEA and NO values comparing with the control or all treatment groups (Table 2). Serum liver enzymes (ALT & AST) increased significantly in the group treated with OA comparing with any other treatment groups. OA had similar effect on serum uric acid or creatinine to that of ALT and AST elevation.

Concomitant treatment with OA and Col-1R1 or Col-1R3 exhibited significant increase in TAC level when compared with OA- treatment group; their values did not significantly different than control group. While *R. glutinis*+ OA caused significant decrease in TAC on comparing with OA alone but its TAC values still decreased significantly than control group (Table 2).

**Table 2. Effect of *R. glutinis* and its two mutants on serum TAC, tumor markers (tumor necrosis alpha and CEA) and nitric oxide (NO) in serum of rats administered Ochratoxin A (OA) after 15 days of daily treatment. (Means ± SE; number= 6)**

| Parameters | Control | OA | OA + *R. glutinis* | OA + Col-1R1 | OA + Col-1R3 |
|---|---|---|---|---|---|
| TAC | A | B | C | A | AC |
| mmol/L | 1.32 ± 0.034 | 0.75 ± 0.022 | 0.95 ± 0.021 | 1.31 ± 0.018 | 1.14 ± 0.023 |
| TNF-α | A | B | A | A | C |
| pg/ ml | 56.3 ± 1.23 | 98.4 ± 2.31 | 57.8 ± 1.21 | 55.6 ± 1.45 | 50.4 ± 1.84 |
| CEA | A | B | C | D | E |
| ng/ml | 0.79 ± 0.041 | 3.14 ± 0.081 | 2.44 ± 0.032 | 2.01 ± 0.076 | 1.56 ± 0.09 |
| NO | A | B | C | D | E |
| μmol/ L | 30.51 ± 1.24 | 70.3 ± 1.43 | 49.25 ± 1.98 | 41.53 ± 2.42 | 35.19 ± 1.38 |

*ANOVA –one way, at P<0.05. The different capital letters are significantly different between groups*

There is significant decrease in CEA and NO values in the groups treated with OA + *R. glutinis*, OA + Col-1R1 or OA + Col-1R3 when compared with OA or control groups; whereas, OA + Col-1R1 the resultant effect of Col-1R3 more effective than Col-1R1 than *R. glutinis* in decreasing the elevated values of CEA and NO by OA- administration. Liver enzymes

showed significant reduction in ALT value of OA- treatment group; only Col-1R1 normalized serum ALT level (insignificantly different from the control level) as illustrated in Table 3. *R. glutinis*, Col-1R1 and Col-1R3 treatments caused significant inhibition in AST value compared with OA alone, while *R. glutinis* and Col-1R3 non-significantly different than control group; moreover, Col-1R1 treatment significantly decreased AST than control value (Table 3). Generally, kidney function tests decreased significantly by *R. glutinis*, Col-1R1 and Col-1R3 administration when compared with OA-group. Uric A value significantly decreased in the group treated with Col-1R1 when compared with the other treatment groups (Table 3). All treatment groups had significant decrease in creatinine level but did not exceeded the normal value (Table 3).

**Table 3. Effect of *R. glutinis* and its two mutants on liver enzyme markers (ALT and AST) and kidney function tests (uric acid and creatinine) in serum of rats orally administered Ochratoxin A(OA) after 15 days of daily treatment. (means ± SE ; number= 6)**

| Parameters | Control | OA | OA + *R. glutinis* | OA + Col-1R1 | OA + Col-1R3 |
|---|---|---|---|---|---|
| | A | B | C | D | A |
| ALT U/L | 28.3 ± 0.43 | 74.2 ± 1.34 | 45.0 ± 1.11 | 38.8 ± 1.90 | 27.2 ± 1.02 |
| AST | A | B | A | A | C |
| U/L | 46.2± 1.57 | 110 ± 2.10 | 44.8 ± 1.35 | 42.3 ± 1.45 | 39.2 ± 1.23 |
| | A | B | C | D | A |
| Uric acid mg/ dl | 0.45 ± 0.014 | 1.36 ± 0.024 | 0.65 ± 0.036 | 0.56 ± 0.016 | 0.48 ± 0.022 |
| Creatinine | A | B | C | C | D |
| mg/ dl | 0.46 ± 0.012 | 1.67 ± 0.07 | 0.88 ± 0.034 | 0.83 ± 0.021 | 0.63 ± 0.024 |

*ANOVA –one way, at P< 0.05. The different capital letters are significantly different between groups*

## 3.1 Histopathological Study of Liver

The control livers showed normal hepatic architecture with distinct hepatic cells, nucleus, sinusoidal spaces and a central vein (Fig. 1A). In comparison with the control group, the following histopathological damages in liver were seen with the OA-treated group: cell degeneration of the liver cells, proliferation of bile duct in some portal areas, enlargement of periportal areas with mononuclear cell inflammatory infiltration, with mild degree fibrous tissue proliferation and necrotic paranchimal liver cells. The central veins and sinusoids of livers in the OA-treated group were dilated and engorged with blood, and this was associated with small areas of haemorrhages, with pyknotic and apoptotic nuclei (Fig. 1 B & C). The effect of *R. glutinis* on the histological alterations induced by OA was substantial on rats pretreated with *R. glutinis* compared to OA treated rats. Many parts of the liver sections showed normal architecture structure of the hepatic tissues, distinct hepatocyte strands except few pyknotic nuclei with dilated sinusoids (Fig. 1D). On the other hand, the group treated with Col-1R1 showed normal histological appearance of the liver tissues with mostly elaborated hepatic architecture and hexagonal hepatocytes with normal nuclei except for the presence of a few activated kupffer cells (Fig. 1E). Meanwhile, in the group treated with Col-1R3 showed mild ameliorative effect. With few histological alterations including congestion, dilatation of the sinusoids nuclear pyknosis were observed (Fig. 1F).

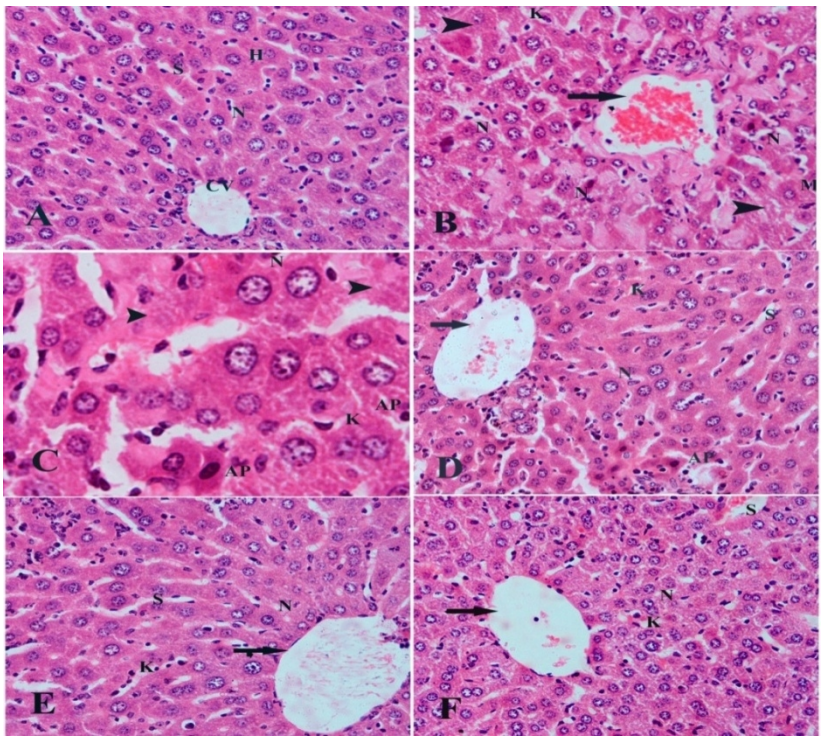



**Fig. 1A. Liver section of a control rat showing normal strands of hepatocytes (H), nuclei (N), sinusoids(S) and central vein (CV) (H&EX400)**
**B. Liver section of OA-treated rat showing degenerated of hepatocytes (arrow head) with pyknotic nuclei (N), mitotic figure (M) as well as congested of central vein (arrow) and activated Kupfffer cells. (K) (H&EX400)**
**C. Liver section of OA-treated rat showing degenerated of hepatocytes (arrow head) with pyknotic nuclei (AP), and polymorpholism nuclei (N). Note activated Kupfffer cells. (K) (H&EX1000)**
**D. Liver section of *R. glutinis* and OA-treated rat showing normal hepatic tissue pattern with normal appearance of most of the hepatocytes and the central vein (long arrow) with nuclei (N). Note inflammatory cells, dialated sinusoids(S) and few pyknotic nuclei (AP) (H&EX400).**
**E. Liver section of Col-1R1 + OA-treated rat showing near to normal architecture of the liver tissue with distinct hepatocyte strands. Normal central vein (long arrow) with normal nuclei (N) except for the presence of few dilated sinusoids(S) and activated Kupfffer cells.(K) was noticed (H&EX400)**
**F. Liver section of Eh2 + OA-treated rat showing near to normal architecture of the liver tissue. Normal central vein (long arrow) with normal nuclei (N), except few degenrated hepatocyte, congested sinusoids(S) and activated Kupfffer cells was noticed (K)(H&EX400)**

## 3.2 Histopathological Study of kidney

The Kidney of control rats had normal renal structure of cortex which showed a normal structure of renal glomeruli, proximal convoluted tubule and distal convoluted tubules. The tubules have a relatively regular distinct lumen (Fig. 2 A). In the OA-treated kidney tissues showed marked destruction and distortion of renal tubule cells and degenerated with cytoplasmic vacuolation, necrosis, tubular epithelial cell proliferation, as well as cells seemingly undergoing apoptotic, karyomegaly in some epithelial cell nuclei, glomeruli revealed shrinkage and widening of capsular space peritubular and periglomerular

lymphocyte infiltration and few fibrous tissue and thickening of tubular basement membranes (Fig. 2 B & C). In comparison with the OA-treated group, the reducing effects of *R. glutinis* treatment were observed in kidney tissues, except that mononuclear cell infiltration (Fig. 2 D).Light microscopic examination in the liver of rats treated with Col-1R1 and OA revealed marked reduction of the toxic effect on the kidney tissues and almost similar to control rat kidney (Fig. 2 E). However, in the group treated with Col-1R3 and OA revealed reduction toxic effects of OA except mild some degenerative changes in the tubular epithelium were observed (Fig. 2 F).

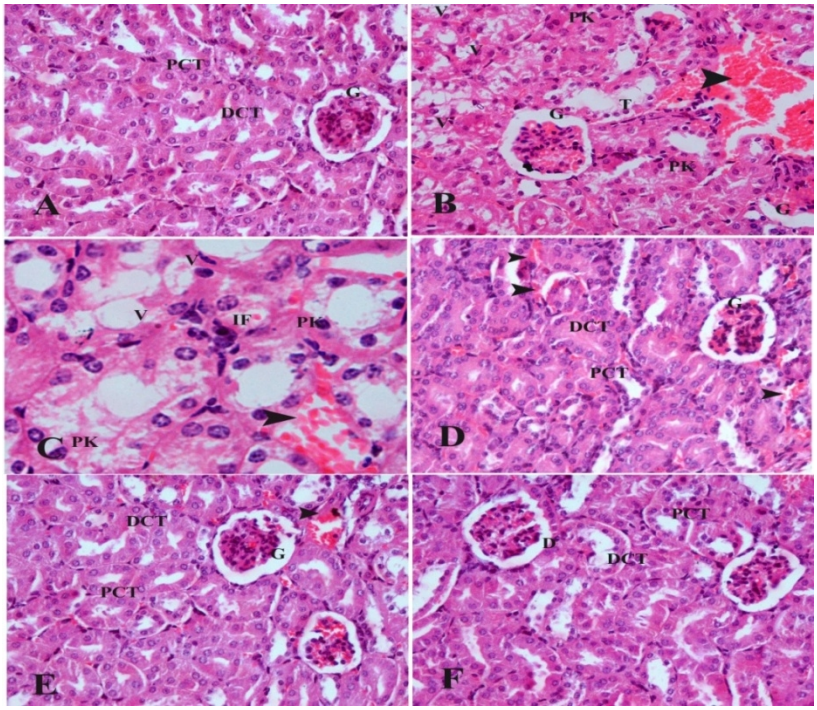
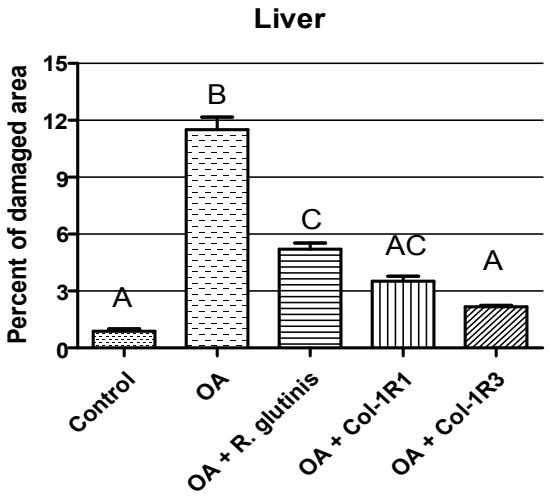



**Fig. 2A. Kidney section (cortical part) of a control rat showing normal renal glomeruli (G), the proximal (PCT) and with distal (DCT) convoluted tubules (H&EX400)**
**B. Kidney section of OA-treated rat showing some degree of mesengial proliferation of glomeruli (G), marked vacuolation of cytoplasm (V) of the epithelial lining with destruction of the epithelial lining of some tubules (T), pyknotic nuclei (PK) and interstitial congestion(arrow head) (H&EX400)**
**C. Kidney section of OA-treated rat showing some degree of degeneration and vacuolation of the epithelial lining with destruction of the epithelial lining of some tubules (V), pyknotic nuclei (PK) and interstitial congestion(arrow head). Interstitial inflammatory cells (IF) was noticed (H&EX1000)**
**D. Kidney section of *R. glutinis* and OA-treated rat showing normal kidney tissue with normal appearance of glomeruli (G), moderate tubular degeneration of the proximal (PCT) and with distal (DCT) convoluted tubules and few interstitial congestion(arrow head)(H&EX400)**
**E. Kidney section of Col-1R1 and OA-treated rat showing normal kidney tissue with normal appearance of glomeruli (G), mild tubular degeneration of the proximal (PCT) and with distal (DCT) convoluted tubules and few interstitial congestion(arrow head)(H&EX400)**
**F. Kidney section of Col-1R3 and OA-treated rat showing normal kidney tissue with normal appearance of glomeruli, mild tubular degeneration of the proximal (PCT) and with distal (DCT) convoluted tubules. Slight dilated of Bowman's space (D) was noticed(H&EX400)**

### 3.3 Analysis of Liver and Kidney Area Damage

Quantitative analysis of the area of damage in liver and kidney showed marked observed in the ochratoxin group. After *R. glutinis* and two mutants treatment; area of damage showed highly decreased. In the group of *R. glutinis* Slight improvement in, however in the two munats (Col-1R1&Col-1R3) more obvious improvement was observed (Histograms 1 & 2).

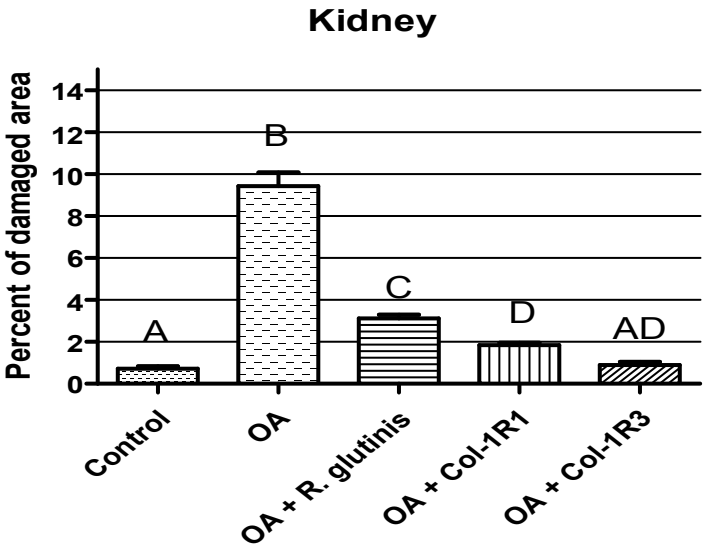



**Histogram 1. Effect of *R. glutinis* and its two mutants on area damage of liver after 15 days of Ochratoxin A(OA) oral administration (means ± SE ; number= 6)**

*ANOVA –one way, at P< 0.05. The different capital letters above columns are significantly different between groups*

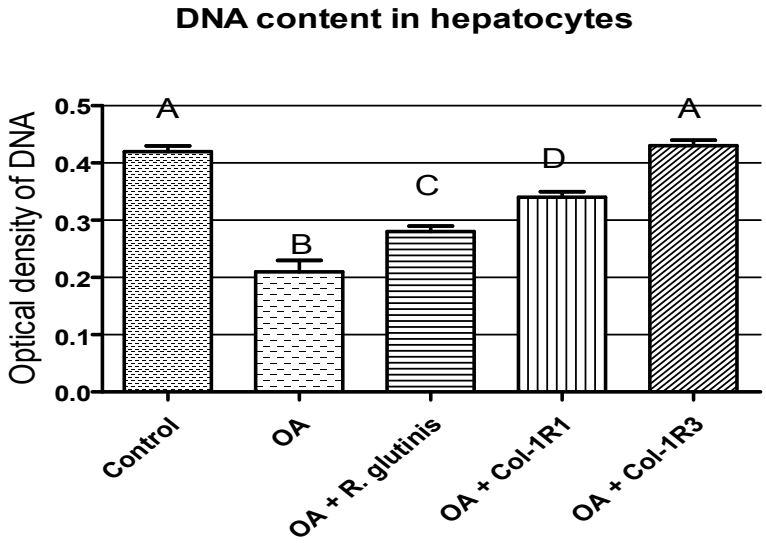
### 3.4 Image Analysis of Quantitative DNA in Liver and Kidney

The normal distribution of DNA was detected in the liver and kidney of control rat DNA content in liver and kidney cells of the rats injected with OA showed significant decrease ($P < 0.05$) as compared to control group (Histograms 1 & 2). Highly significant increase in DNA content ($P < 0.05$) was observed in the rats treated with *R. glutinis* and two ( Col-1R1&Col-1R3) mutants with ochratoxin group, this increase more pronounced in the mutants two as compared to ochratoxin group (Histograms, 3 and 4).



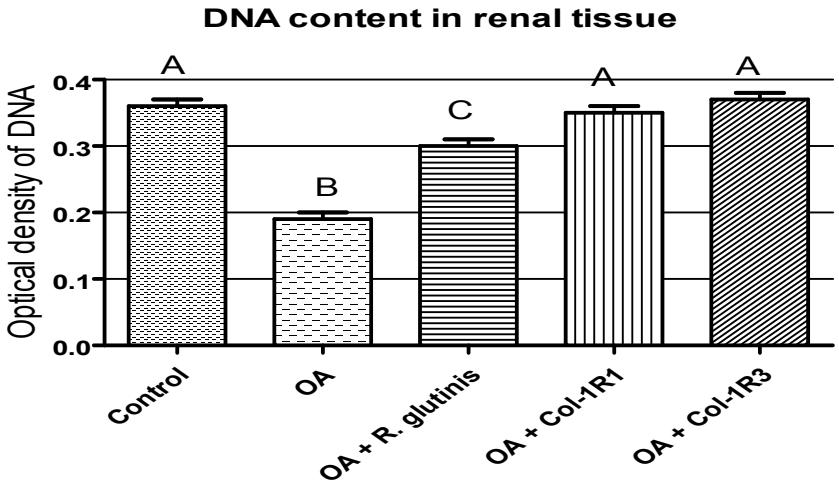

**Histogram 2. Effect of *R. glutinis* and its two mutants on area damage of kidney after 15 days of Ochratoxin A(OA) oral administration (means ± SE ; number= 6)**

*ANOVA –one way, at P< 0.05. The different capital letters above columns are significantly different between groups*




**Histogram 3. Effect of *R. glutinis* and its two mutants with on DNA –optical density of liver after 15 days of Ochratoxin A (OA) oral administration (means ± SE ; number= 6)**

*ANOVA –one way, at P< 0.05. The different capital letters above columns are significantly different between groups*




**Histogram 4. Effect of *R. glutinis* and its two mutants with on DNA –optical density of renal tissue after 15 days of Ochratoxin A(OA) oral administration. (means ± SE ; number= 6)**

*ANOVA –one way, at P< 0.05. The different capital letters above columns are significantly different between groups*

## 3.5 Immunohistochemistry of Caspase-3inliver

Immunohistochemical staining was used to analyze the expression of caspase-3 proteins in the liver tissues. Positive expression of caspase-3 was rarely detected in liver tissues from the control group with very week staining (Fig. 3A). However, the OA group exhibited a strong expression of caspase-3 in the liver tissues as compared with controls group. Hepatocytes and/or infiltrating inflammatory cells within the liver tissues were the major cell types expressing caspase 3 (Fig. 3B). Compared to the OAtoxicated group, the expression of hepatic caspase-3 revealed reductions in the group treated with *R. glutinis* and two mutants (Col-1R1&Col-1R3) (Fig. 3 C, D &E).

## 3.6 Immunohistochemistry of Caspase-3 in Kidney

The immune histochemical studies revealed that no detectable caspase-3 staining was observed in the renal cortices of control rats (Fig. 4A). Immunohistochemical evaluation showed excessive intense of caspase-3 expression in OA-treatment rats when compared with the control group. The number of caspase-3–positive cells was seen in all the proximal and distal tubules. *R. glutinis* and two mutants (Col-1R1&Col-1R3) were able to inhibit caspase-3 activity (Fig. 4 C, D &F). As shown in Fig. caspase-3 activation was decreased in kidneys with as compared with OA group. Low caspase-3 expression was observed in the glomeruli and tubules in group treated with Col-1R1 and OA (Fig. 4 D).

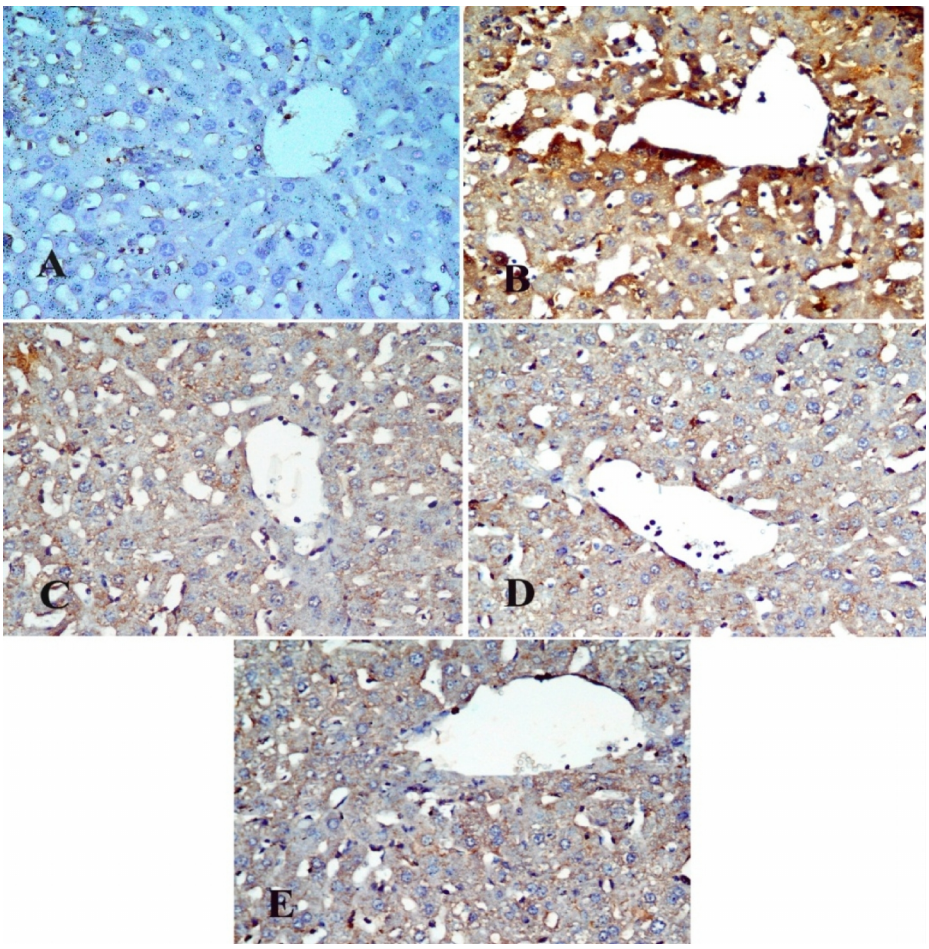



**Fig. 3A. Caspase-3 immunohistochemistry of a liver from a control rat counterstained with Hematoxylin. Caspase-3-immunolabeled cells were rarely present in the liver of control rats.**
**B. Caspase-3 immunohistochemistry of a liver from a rat treated with LPS identified by brown staining and counterstained with Hematoxylin. A slightly increased number of caspase-3 immunolabeled hepatocytes were observed around central veins compared to control animals, suggesting a slight increase in apoptosis**
**C. Caspase-3 immunohistochemistry of a liver from a rat treated with *R. glutinis* Caspase-3-immunolabeled cells were slight decrease compared to control rats.**
**D. Caspase-3 immunohistochemistry of a liver from a rat treated with Col-1R1 Caspase-3-immunolabeled cells were slight decrease compared to control rats.**
**E. Caspase-3 immunohistochemistry of a liver from a rat treated with Col-1R3 Caspase-3-immunolabeled cells were slight decrease compared to control rats.**
**(Caspase-3 immune Staining, x400). Brown color indicates immunopositivity**

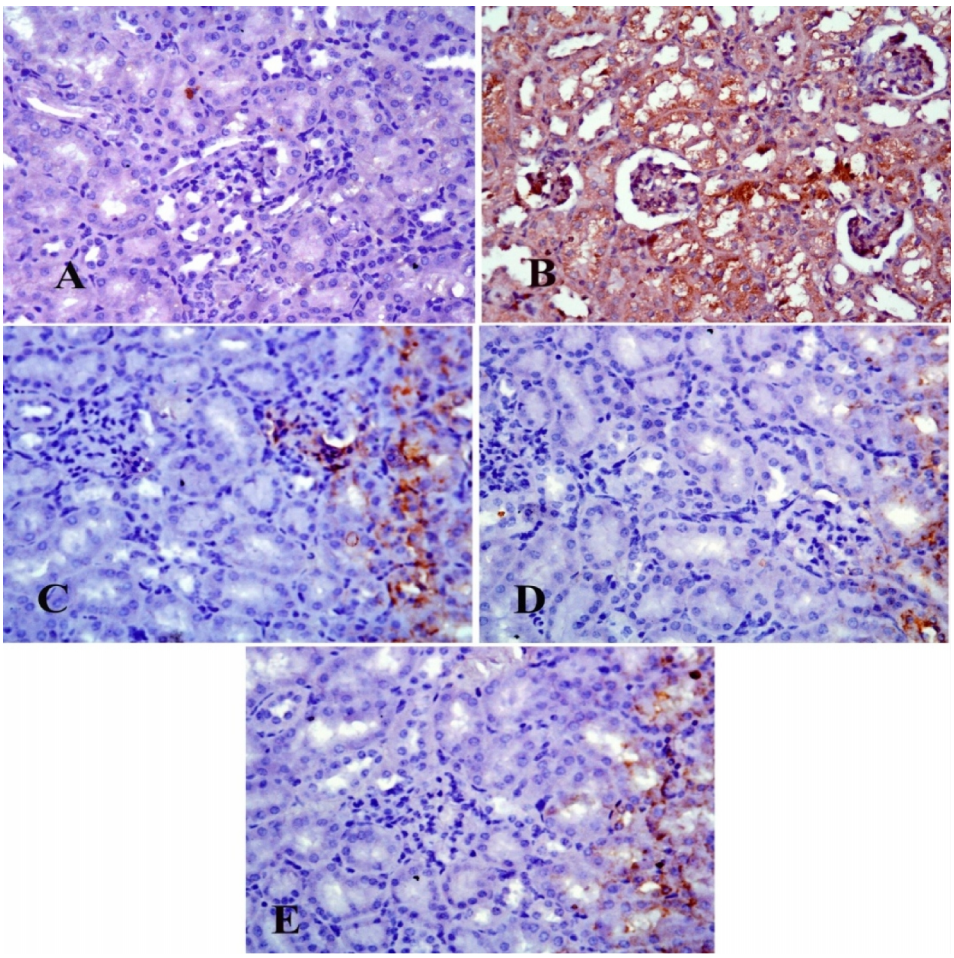



**Fig. 4A. Caspase-3 immunohistochemistry of a liver from a control rat counterstained with Hematoxylin. There is no expression of caspase-3 in the cortical regions of kidney**

**B. Kidney section of OA administration increased strongly caspase-3 expression in cortical areas especially in the proximal convoluted tubules, whereas in the glomerular structure there was less caspase-3 expression.**

**C. Kidney section of *R. glutinis* and OA group showing partial inhibition of caspase-3 expression as evidenced by weak immune staining in the distal tubules in the cortical regions**

**D. Kidney section of Col-1R1 and OA group showing decrease of caspase-3 expression as evidenced by weak immunostaining in the distal tubules in the cortical regions**

**E. Kidney section of Col-1R3 and OA group showing decrease of caspase-3 expression as evidenced by weak immunostaining in the distal tubules in the cortical regions (Caspase-3 immune Staining, x400). Brown color indicates immunopositivity**

## 4. DISCUSSION

Various strategies have been developed to prevent OA – food contamination. Some microorganisms have been proven to prevent the growth of ochratoxigenic fungi and OA production. They could be used as natural control material. Our results revealed that OA-treatment group caused serious hepatorenal damage. Rat treated with OA-alone showed significant decrease in serum TAC, and significant elevation in NO, CEA, TNF-α, liver enzyme markers (ALT & AST) and kidney function tests (Uric A. & creatinine). OA- treatment alone showed diffuse fatty infiltration in hepatocytes, scattered necrotic foci, vacuolated hepatocytes, congestion, hemorrhage, mild fibrous tissue proliferation, interstitial and periportal inflammation throughout the liver parenchyma [19,14,20]. In the present study, renal tissue of OA- treated rats showed cytoplasmic degeneration, swelling of tubular epithelial cells, necrosis, and desquamous tubular basement membrane of the proximal convoluted tubules [21].

We demonstrated that *R. glutinis* and its two mutants inhibit caspase-3 activation and prevent DNA depletion as discussed by Seegers et al. [22] who suggest that OA induced apoptotic necrosis of renal epithelial cells is a late event.

In the present study, *R. glutinis* and the two mutants had direct free radical scavenging and indirect antioxidant activities; which quench the free radicals and singlet oxygen species. The mutants had powerful antioxidant effect more than the wild form. Our results revealed that, the groups of rats treated with OA and *R. glutinis* species exhibited significant reduction in TAC, TNF-α, CEA, and NO values. They had ameliorative effect in hepatorenal tissues in variable degree (*R. glutinis*< Col-1R1 < Col-1R3). All protective treatment groups had remarkable inhibitory effect on caspase-3 expression and DNA damage, reduced apoptosis and damaged area- optical density in hepatorenal tissues as discussed in the results before. The possibility of the curative mechanisms may be due to *R. glutinis* enriched with antioxidants and chelating agents (caratenoids, protease, β-1,3-glucanase and chitinase); moreover, autoploidy strains produced higher content of their metabolites than the wild type strains Several antagonistic yeasts have been reported to reduce the growth of ochratoxigenic fungi as well as OA production [3].

It has been suggested that extracellular enzymes and metabolites produced in the growth medium may play an important role in the inhibitory activity of microorganisms and competition at their surfaces [23].

In previous study, we found that *R. glutinis* as protective agents against toxic effect of aflatoxin B1 in mice [17]. These are used in the manufacturing of fermentation of the active pharmaceutical compounds, such as the antifungal, antiviral, anti-cancer, agents of immune suppression, insecticides, weed killers [24]. Because yeasts can produce many bioactive substances, such as protein, amino acids, vitamins, polysaccharide, fatty acid, phospholipid, polyamine, astaxanthin, carotenoid, trehalose, glutathione, superoxide dismutase, chitinase, amylase, phytase, protease, killer toxin and so on, they have been receiving much attention for many decades. This distinctive colour is the result of pigments that the yeast creates to block out certain wavelengths of light that would otherwise be damaging to the cell. Colony colour can vary from being cream coloured to orange/red/pink or yellow. Several antagonistic yeasts have been reported to reduce the growth of ochratoxigenic fungi as well as OA production [24]. It has been suggested that extracellular enzymes and metabolites produced in the growth medium may play an important role in the inhibitory activity of microorganisms and competition at their surfaces. OA degradation was also due to yeasts and filamentous

fungi. For example, *Phaffia rhodozyma* degraded more than 90% of OA [25]. Microorganisms are also able to remove OA by adsorption onto the cell surface components. The biological characteristics of these anti-aflatoxigenic compounds are under investigation. Biosorption is generally based on a set of chemical and physical mechanisms leading to the immobilization of a solute component on the microbial cell wall components and is not dependent on metabolism [26].

## 5. CONCLUSION

Col-1R3 was more effective than Col-1R1 may be due to its higher contents of carotenoids, glucane and chitine, which act as antioxidants.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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