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Stabilized rice bran extract: Acute and 28-day repeated dose oral toxicity with *in vitro* mutagenicity and genotoxicity study

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The toxicity of Egyptian stabilized rice bran extract (RBE) has been evaluated for acute and repeated 28 day oral toxicity with the outcome of genotoxicity. The extract was administered to female white albino rats at 2000 mg kg^{-1} for acute testing and to male and female rats at concentrations of 1000, 500, and 100 mg kg^{-1} for 28 days. Mutagenic potential was evaluated. Chromosomal and DNA damage using standard karyotyping and alkaline comet assay were applied. Acute study showed neither deaths nor gross of pathological abnormalities. In 28 days study, no dose-related changes were observed in body weights, haematological as well as the majority of the serum biochemistry parameters. Results showed neither mutagenic nor genotoxic effects of the RBE. Histological findings showed significant changes in rat groups administering 1000 and 500 mg kg^{-1} doses. The extract proved to be non toxic acutely and the degree of toxicity was noticed to be dose dependent after chronic administration. Thus we suggest that RBE at dose of 100 mg kg^{-1} (equivalent to 1 g oil daily consumption) is safe to be administered as dietary supplement for long term use.

Key words: Stabilized rice bran oil extract (RBE), ames test, alkaline comet assay, chromosomal aberrations, white albino rats.

INTRODUCTION

Rice bran (*Oryza sativa*) is a by-product of the rice milling industry and comprises of 10% of the whole rice grain. Worldwide production of rice bran has reached about 50 to 60 million metric tons per year. The bran is regarded

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as a good source of antioxidants, fatty acids, proteins, carbohydrates, minerals and vitamins in addition to other nutrients (Khush, 1977). Rice bran oil (Barber et al., 1980) is incorporated in food industries whilst the defatted rice bran is used in animal feed. The bran layer contains a number of micronutrient such as γ-oryzanol in addition to tocopherols, tocotrienols (unsaturated analogues of tocopherols Vitamin E), phytosterols and carotenoids which have been considered as potent antioxidants (Xu et al., 2001; Qureshi et al., 2002; Rukimini and Raghuram, 1991; Edward and Radcliffe, 1994). Despite being a very promising, broadly applicable, and deeply investigated material, the use of rice bran in several industries is hindered by its instability. Rice bran tends to become rancid and unsuitable for human consumption a few hours after milling due to the effects of lipase enzyme acids. The later targets the oil content liberating free fatty that got oxidised rapidly, rendering the bran unfit for human consumption. Consequently, it is either discarded or used as an animal feed (Orthoefer, 1996) and thereby pouring its nutritional values and potential medical efficiency down the drain at the expense of a great financial and human utility loss.

Methods for stabilization of rice bran have been reviewed. Heat stabilization method with high temperature short time (HTST) was adopted to deactivate the lipase enzyme and in the same time keep the integrity of rice bran bioactives (Ramezanzadeh et al., 2000; Prakash, 1996). In Egypt, more than 500,000 tons of rice bran is produced annually with relative low market value (LE 1200/MT). This value is expected to be doubled when stabilize rice bran and its extract is developed into functional food and dietary supplements.

Toxicity of rice bran oil and its main components was previously investigated in various studies unfolding its acute toxicity at *in vitro* and *in vivo* levels (De Deckere and Korver, 1996). Rice bran oil had an oral LD_{50} of > 5 g kg^{-1} in white rats which is estimated to be about 56 g in a human weighing 70 kg (Anonymous, 2006). Non-mutagenicity of rice bran oil was demonstrated using the bacterial reverse mutation Ames mutagenicity assay (Polasa and Rukmini, 1987). Other *in vivo* long-term toxicity studies evidenced the non-carcinogenic properties of the bran oil active constituents as γ-oryzanol and ferulic acid derivatives in rats and mice (Tamagawa et al., 1992; Yasukawa et al., 1998). Lack of toxicity testing of heat stabilized rice bran as well as its alcohol extract amplifies the need for further toxicological studies in order to determine its safety. Moreover the majority of the relevant reported toxicity studies focused solely on either the oil or on an individual active component of rice bran rather than that of the whole bran or its alcohol extract. Stating these facts encouraged us to conduct our experiments on bran oily extract following stabilization process. In this study we used the ethanol extracted oil (RBE) from heat stabilization rice bran grain *O. sativa* variety of Sakha 101 (short grain). The phytochemical study and nutritional values evaluation of the RBE was verified (Al-OKbi et al., 2013). In addition to recently reported studies on its pharmacological potential activities, such as its anti diabetic effect as well a promising role in Alzheimer's disease management (Kaup et al., 2013; Hagl et al., 2013, 2015) effects against hypertension and inflammation is under investigation (59th International congress and annual meeting of the society for medical plants and Natural Product Research, 4th to 9th September, 2011, Antalya, Turkey). Very limited numbers of toxicological studies provide insufficient data for setting the accurate safety limits for daily intake level of the rice bran. Furthermore no reported literature on the safety of stabilized rice bran extracts has been published so far, therefore we conducted a combined study of acute and chronic toxicity. In the present study RBE was fed to experimental rats at various selected doses for the assessment levels of dietary consumption. This was done by examining its effect on body weight gain, hematological, biochemical and histological parameters. The gentoxicity assays are essential to evaluate the chronic toxicity. Accordingly we performed a number of *in vitro* mutagenic, cytogenetic assays for the RBE in an attempt for estimation of any possible toxicity from continuous dietary exposure.

MATERIALS AND METHODS

Test substance and chemicals

RBE was obtained from International Trade and Marketing Company S.A.E Giza, Egypt (IT&M). First the stabilized rice bran was prepared by applying high temperature short time technique (HTST) to rice bran after milling to inactivate lipase enzyme. Ethanol extraction was carried out using 95% alcohol by maceration overnight (1:3 w/v) at 50°C for three successive times. The combined filtrates were concentrated under vacuum. The produced oily extract was referred to as rice bran extract (RBE). The oily extract was stored in refrigerator and was warmed in water bath at 37°C with sanitation just before use. *Salmonella typhimurium* TA100, TA2638, TA97a, TA98 and TA102 bacterial strains as well as the positive control mutagenic agents such as Sodium azid, 2-Aminoanthracene (2-AA) were purchased from Bonnie Kuenstler, Discovery Partners International Inc., San Diego, CA, USA, and Trinova Biochem GmbH – Gießen, Germany. Methotrexate and Benzo[a] pyrene were purchased from Sigma Aldrich, Germany. Blood samples were collected under sterile conditions from a healthy volunteer in vacutainer tubes containing heparin to avoid blood coagulation, and then further processed for human peripheral lymphocytes (HPL) separation. SK-N-SH cell line were supplied from Viva cell laboratories (Biotechnology GmbH (VC)-Freiburg - Germany).

Animals

White albino rats (aged 6 to 8 weeks) were housed in cages. Each cage contained 5 rats of the same sex. Food and water were given *ad lib* and 12 h light/dark cycles were provided. Environmental conditions were maintained at a temperature of 25 ± 2°C and a relative humidity of 60 ± 10%. All animal experiments were conducted according to NIH guidelines for treatment and care of laboratory animals (NIH publication 85-23 revised 1985) and approved by the animal and human ethics committee at the

German University in Cairo (GUC).

HPLC fingerprint of Egyptian RBE extract

Separation was carried out on a Lichrospher C18 column (250 mm × 4 mm, 5 µm), preceded by a guard column (10 mm × 4 mm id, 5 µm) using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) adapted with a quaternary pump and degasser G1322A, series1200. Mobile phase was composed of methanol (100%). Linear isocratic program at a flow rate of 1.9 ml/min was performed; temperature and UV wavelength were set at 25°C and 295 nm, respectively. The γ-oryzanol reference standard (40 mg) was accurately weighted and dissolved in methylene chloride then diluted to give different concentrations and the peak area detected by high performance liquid chromatography-ultra violet (HPLC-UV).

Acute toxicity

The RBE was directly administered (without dilution). The limit test was used according to acute oral toxicity procedure, (OECD guidelines test N^0: 423, 2002). Test was carried out on a group of 5 female rats. Animals were orally administered the oil at a dose of 2000 mg/kg/day once and observations for toxic signs were performed at 10, 30, 60, and 120 min and at 4 and 6 h after dosing during the first day and on daily basis at least once for 14 days. The control group of 5 female rats received 1 ml saline/rat. The weight of each rat was recorded on day 0 and at weekly intervals throughout the course of the study. At the end of the treatment period, animals were fasted overnight. The following day, each animal was anaesthetized and blood samples were collected from the orbital sinus with and without ethylenediaminetetraacetic acid (EDTA) for hematological and biochemical parameters measurements.

Repeated dose (28-day) study

The RBE was orally administrated according to the repeated 28 days oral toxicity study in rodents (OECD guidelines test N^0: 407, 2008). A total of 40 healthy rats were assigned to the 28-day study. Animals were divided into four groups (gpI-gpIV) of 10 animals each. Each group contains 5 males and 5 females. Group I (gpI_M and gpI_F) served as control and the other three groups ($gpII_M$ and $gpII_F$, $gpIII_M$ and $gpIII_F$ and $gpIV_M$ and $gpIV_F$) orally administered the oily extract at doses of 1000, 500, 100 mg kg^{-1} daily throughout the experiment period. The weight of each rat was recorded on day 0 and at weekly intervals. At the end of the treatment period, animals were fasted overnight. The following day, each animal was anaesthetized and blood samples were collected from the orbital sinus with and without EDTA. Hematological parameters were measured only in the group administered RBE at a dose of 1000 mg kg^{-1}. Biochemical analysis for all 28-day toxicity animal groups was performed on serum obtained after centrifugation of total blood without EDTA at 6000 rpm for 10 min. Biochemical analysis was determined via diagnostic kits test (Quimican Clinica Alpicada S.A, Spain) using spectrophotometric determination (Shimadzu UVPC 2401 v 3.9 Shimadzu, Kyoto, Japan). Liver, kidney, brain and cardiac muscle specimens were removed from the sacrificed animals and fixed in 10% formol saline for 24 h. Paraffin blocks were prepared and sections were subjected to the histological study using hematoxylin and eosin (H&E) (Kiernan, 2001). All morphometric analysis were performed on H&E stained tissue sections using Leica Qwin 500 LTD computer assisted image analysis system (Cambridge, UK) at the Histology Department, Faculty of Medicine, Cairo University.

Cytotoxicity testing

Cell viability was estimated by using Alamar Blue method according to manufacture instruction. The assay was used to measure quantitatively the proliferation of SK-N-SH cell line in presence of different concentrations of RBE ranging from 0.1 to 100 µg ml^{-1}. The RBE was prepared in 20 mg ml^{-1} stock solution in dimethyl sulphoxide (DMSO) and serial dilutions were prepared. The fluorescence intensity was measured at 1 h intervals for 4 h using spectrofluoremetry at excitation wavelength of 550 nm and Emission of 590 nm.

Ames mutagenicity test (Bacterial reverse mutation assay)

Salmonella mutagenicity assay was performed using *Salmonella typhimurium* strains TA100, TA2638, TA97a, TA98 and TA102 (Maron and Ames, 1983; Gomaa et al., 2013). The RBE concentrations of 100, 150 and 200 µg ml^{-1} were dissolved in 1% DMSO, which was used as a reference negative control. The study was conducted in accordance with the OECD guidelines (Part 471), European commission (2000).

***In vitro* comet assay (single cell gel electrophoresis assay) of HPL cells**

Human peripheral blood lymphocytes (HPL) from blood samples were used. The comet assay was conducted according to Singh et al. (1988). A suspension of 10^6 cells were incubated at 37°C for 1 h with either 30 µg ml^{-1} Methotrexate (MTX) as a positive control, or the different dilutions of RBE (100, 150 and 200 µg ml^{-1} in 1% DMSO) in RPMI serum depleted medium to minimize DNA repairing mechanisms. Viability of treated cells were determined using trypan blue exclusion method and monitored at 75% with reference to PBS treated cells in order to exclude cytotoxic effects at the interpretation of the assay results (Henderson et al., 1998). For scoring the comet results, duplicate slides from each dose and positive control groups were counted. A total number of 100 cells in each slide were examined using a fluorescent microscope (Carl Zeiss, Axiostar plus, 37081) connected to an image analysis system (Comet Imager, metasystems GmbH). Quantification of DNA damage was assessed as percentage of damaged DNA migrating to the tail. Results were expressed as percentages of comet tail intensity (fraction of DNA in the tail divided by the amount of DNA in the cell, multiplied by 100%). The comet tail moment (product of the tail and the mean distance of migration in the tail [arbitrary units]) was selected as the parameter to quantify basal levels of DNA damage.

***In vitro* Karyotyping for HPL cells**

Duplicates for whole blood cultures of 0.5 ml peripheral blood sample from a healthy female donor were set up for preparation of metaphase chromosomes according to Gomaa et al. (2012). Treatment with different RBE concentrations was given for 3 or 24 h, before harvesting the metaphase chromosomes. Replicate slides of metaphases was prepared from each culture and stained with Giemsa banding for 10 min. One hundred metaphase cells for each treatment group were analyzed for chromosomal damage. All slides were coded and screened in a random order. Aberrations were identified according to Savage (1976) as chromosome and

chromatid type damage. Displaced and un-displaced fragments separated by a non-staining region equal to or greater than the width of the chromatid were scored as deletions. Unstained regions of less than the chromatid width were scored as gaps. Prior to mutational analysis and karyotyping, the mitotic index (MI) defined as the number of metaphases per total number of 1000 blast cells for each treated sample with either MTX or SRB in comparison to the untreated cells was also assessed.

Statistical analysis

In vitro results were expressed as mean ± SD. Data was subjected to statistical analysis byTukey Multiple comparison ANOVA test using Graphpad Prism (San Diego, CA, USA). Treatment groups were considered significantly different from the control group at value of $p < 0.05$. For morphometric analysis, quantitative data were summarized as mean ± SD and compared using one-way analysis-of-variance (ANOVA). Any significant ANOVA was followed by Bonferroni post-hoc test to detect which pairs of groups caused the significant difference. *p*-Values < 0.05 were considered statistically significant. Calculations were made on SPSS software (Emsley,et al., 2010)

RESULTS

HPLC finger-print of SRB oil extract

γ–Oryzanol content in the RBE sample determined by HPLC was 2.42%. Chromatogram for RBE in comparison to chromatogram of γ-oryzanol reference standard is shown in Figure 1.

Acute toxicity

Weight gain for female rats orally administered the extract at a dose of 2000 mg kg^{-1} is shown in Table 1. Animals showed no signs of intoxication during the period of treatment. No differences were found in growth between treated animals and control ones ($p > 0.05$). Hematological examination presented in Table 2, showed no significant differences for all measured parameters between treated groups and control group ($p > 0.05$). All values were found to be within the normal range.

Repeated dose toxicity

Animals orally administered the RBE at three doses did not show unusual changes in behaviour or in loco-motor activity. Moreover, no signs of intoxication were observed during the 28 days period. Significant differences were observed in weight gain between male and female only in group administered RBE at a dose of 1000 mg Kg^{-1} compared to control. No significant difference was observed in other treated groups compared to the control group ($p > 0.05$) (Table 1).

Hematological analysis

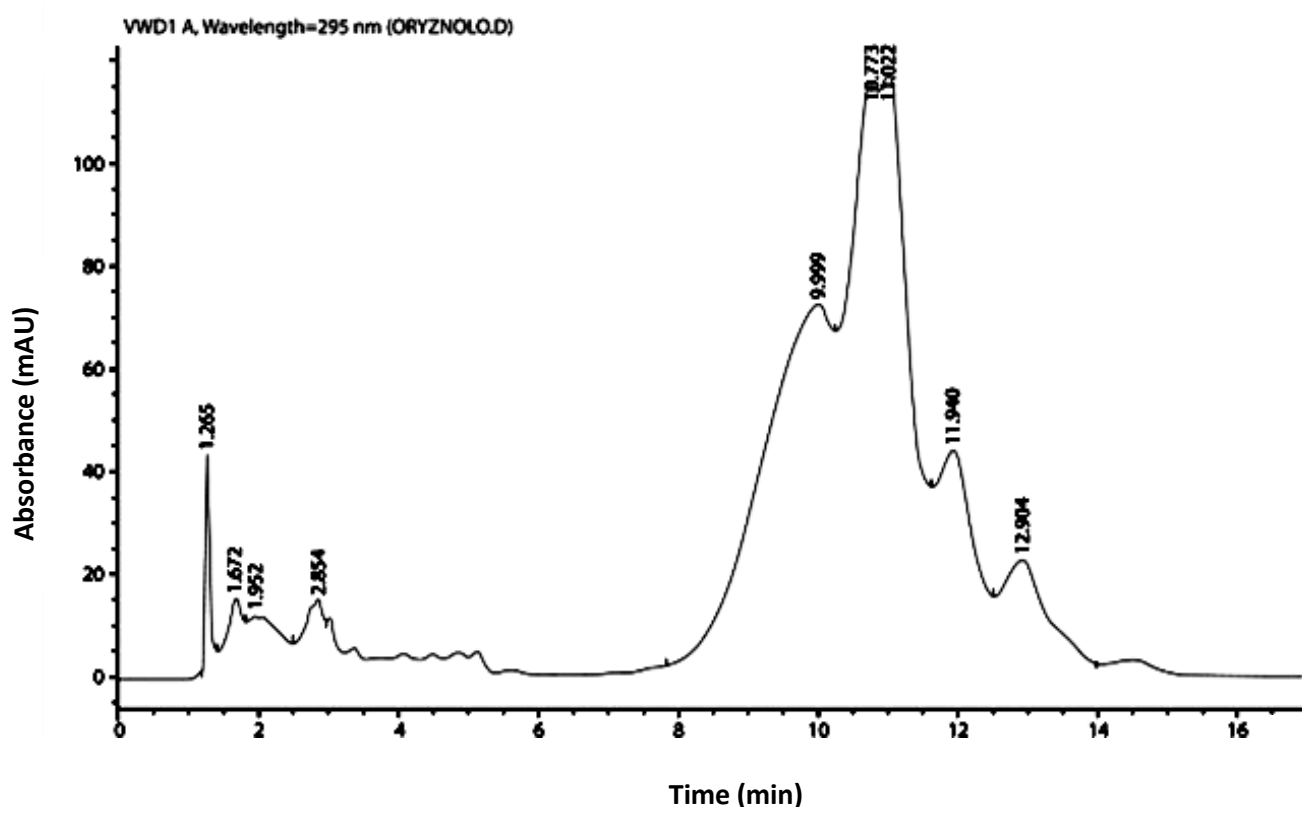
Results were recorded in animals ($gpII_M$, $gpII_F$) administering RBE at a dose of 1000 mg kg^{-1} day^{-1}. Results presented in Table 2 showed insignificant difference between the two sexes and between control and experimental groups ($p > 0.05$).

Biochemical analysis

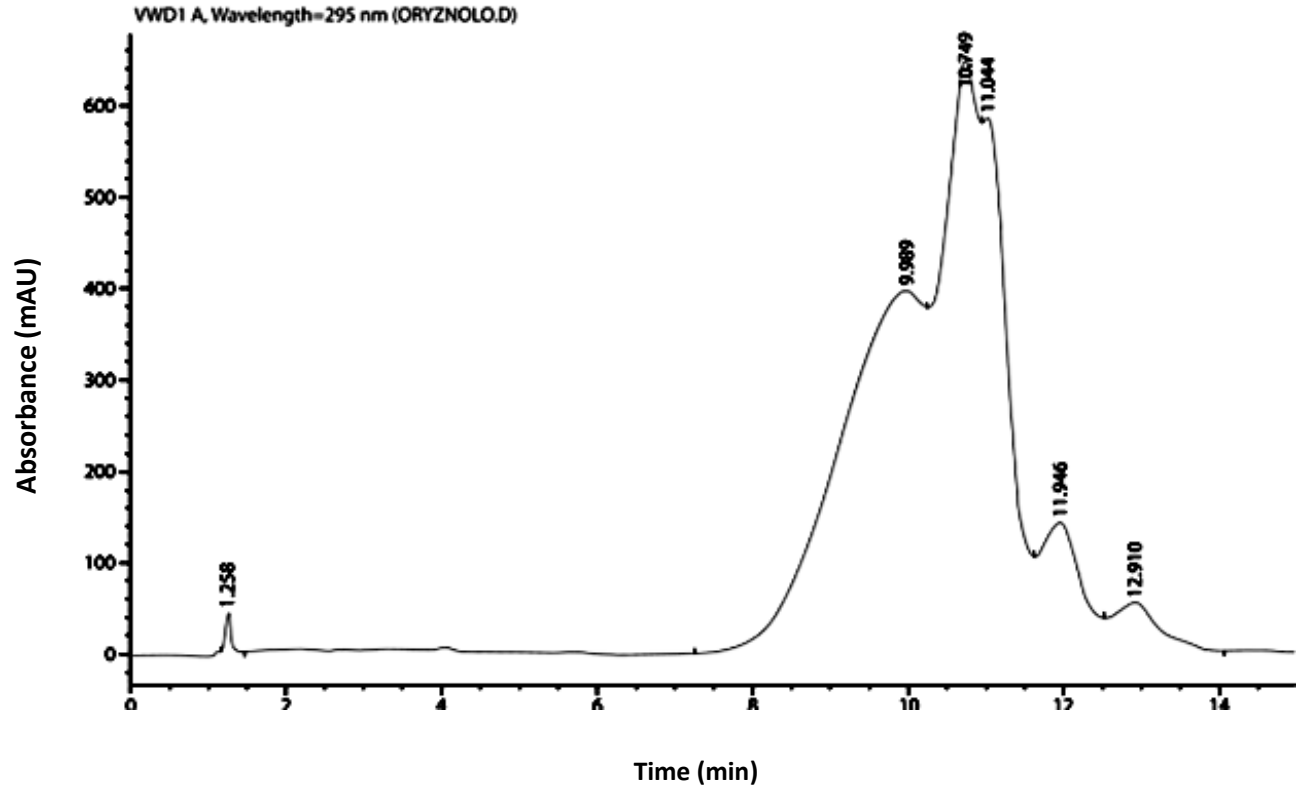
Animals treated at the highest dose level of 1000 mg kg^{-1} showed significant increase in blood sugar , bilirubin and SGOT levels in treated male compared to control male group ($p<0.05$). Total protein, creatinine, and bilirubin levels as well as alkaline phosphates enzyme levels were significantly lower in female group ($gpII_F$) compared to their corresponding values in the female control group ($p< 0.05$) (Table 3). Other activities of enzymes analyzed in plasma showed neither significant differences between the two sexes nor between control and experimental groups. Biochemical parameters of animals treated at 500 mg kg^{-1} showed significant increase in blood urea level of treated female groups compared to their corresponding values in the control ($p < 0.05$). All other biochemical parameters at 500 and 100 mg kg^{-1} showed neither significant differences between the two sexes nor between the groups of treated animals ($gpIII_F$,$gpIII_M$, $gpIV_M$, $gpIV_F$) compared to the control (gpI_M, gpI_F) (Tables 4 and 5).

Histological and morphometric analysis

Photomicrograph of rat liver sections administrating low dose (100 mg kg^{-1}) showed few hepatocytes with vacuolated cytoplasm and dark nuclei around congested central vein compared to some hepatocytes with vacuolated cytoplasm and dark nuclei around dilated congested central vein in rats administered the 500 mg kg^{-1} dose. In addition multiple hepatocytes with vacuolated cytoplasm and dark nuclei around obviously distended congested central vein were observed in rats administered high dose (1000 mg kg^{-1}) (Figure 2A). Histological results of kidney sections of low dosed rats showed very few malpighian renal corpuscle (MRC) with distended glomerulus obliterating the Bowman's space compared to some and multiple MRC with distended glomeruli obliterating the Bowman's space in addition to vacuolated cytoplasm of cells lining some tubules (Figure 2B) in sections of rats administered medium and high doses, respectively. Photomicrograph of sections in the cerebrum of rat group administrating low doses showed an accidental apoptotic neuron exhibiting dark nucleus among the neurons compared to sections of rat groups administrating medium and large doses which showed fewer apoptotic neurons exhibiting dark nuclei among the neurons (Figure 2C). Photomicrograph of cardiac muscle sections of animals administrating medium dose showed moderately congested vessels among the muscle fibres while those administering large dose showed markedly



A. Rice bran oil extract



B. γ -oryzanol reference standard

Figure 1. HPLC chromatogram of rice bran ethanol extract.

Table 1. Body weight of female rats orally administered RBE after acute toxicity study and male and female rats after repeated oral (28 day) toxicity study.

Sex	Dose (mg kg^{-1})	Week 0	Week 1	Week2	Week3	Week4
Female	Control	155±18.0	173.8±11.0	180.6±12.5	-----	-----
	2000	144.4±20.6	155.2± 20.3	171.2±9.7	-----	-----
Male	Control	153±9.7	174±17.4	199.0±21.9	217.4±23.4	238.0±28.3
	1000	211.2±8.5*	223.2±11.0*	251.7±5.6*	264.0±10.4*	267±11.8*
	500	151.6±8.4	168.6±8.1	199.6±12.8	234.3±31.9	256.7±25.4
	100	155.4±11.1	172.4±8.9	200.4±11.5	217.4±16.4	249.4±23.8
Female	Control	140.0±10.7	143.2±10.1	152.4±8.6	161.4±10.8	169.6±12.0
	1000	152.7±8.5	151.5±4.5	164.2±5.7*	169.25±19.9*	188.5±12.9*
	500	137.8±4.4	150.4± 2.8	159.2±4.8	165±6.0	176.2±5.8
	100	134.6±13.5	143.2±15.7	156±14.1	161.2±14.1	176.6±16.2

Values represents mean ± S.D, n= 5.* P <0.05 = Significance difference vs control.

Table 2. Hematological data of female rats orally administrated RBE in acute study and of male and female rats orally administered RBE in repeated dose toxicity study (28 day).

Group	Hb (g%)	RBC (10^6/mm^3)	Rt (%)	HCT (%)	MCV (µm^3)	MCH (pg)	MCHC (%)	Platelets (10^5/mm^3)	Total leukocytes (10^3/mm^3)	N %	L %	E %	M%
2 g/kg													
Fc	13.22±0.68	6.39±0.45	1.47±0.23	37.98±3.6	59.56±3.45	20.7±1.03	34.84±2.31	3.18±0.52	9.94±3.41	17.52±2.87	72.9±4.2	1.55±0.47	2.77±0.54
Ft	13.96±0.12	6.02±0.13	1.7±0.105	38.5±0.65	53.05±1.84	19.34±0.51	35.09±0.29	3.4±0.66	10.7±1.77	17.025±1.3	71.9±1.2	1.902±0.3	2.98±0.11
1 g/kg													
Mc	14.08±0.8	7.10±0.76	1.15±0.12	37.5±3.6	57.32±10.2	19.9±1.4	33.4±2.4	3.18±0.5	9.94±3.41	17.5±2.8	74.7±2.6	1.55±0.47	3.02±0.34
Mt	113.97±1.5	7.8±0.4	1.19±0.16	37.7±5.4	57.3±10.2	19.9±3.5	37.2±3.5	3.5±0.4	10.5±1.9	18.02±1.00	74.1±1.3	1.76±0.23	3.02±0.5
Fc	13.22±1.0	6.39±0.45	1.47±0.2	37.9±3.6	59.56±3.4	20.7±1.03	34.84±2.3	3.74±0.5	9.7±2.4	17.8±0.8	72.9±4.2	1.44±0.2	2.77±0.5
Ft	12.9±0.12	7.02±0.13	1.3±0.1	36.5±0.65	52.05±1.84	18.4±0.51	35.4±0.29	3.74±0.5	11.7±2.45	18.4±0.5	72.9±1.9	1.57±0.29	2.98±0.11

Mc (male control) , Mt (male test) Fc (Female control) , Ft (Female test). Values represents mean ± S.D, n= 5.* p<0.05 = Significance difference vs control. Hemoglobin (Hb), mean corpuscular volume (MCV), total erythrocyte count (RBC), reticulocyte concentration (Rt), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets and total and differential leukocyte count; neutrophils (N) , lymphocytes (L) , eosinophils (E) and monocytes (M).

Table 3. Biochemical parameters of male and female rats orally administered RBE after repeated toxicity study (28 days).

Sex	Blood sugar (mg%)	Tot. protein (g%)	Albumin (g%)	Creatinine (mg%)	Bilirubin (mg%)	Blood urea (mg%)	SGOT (Iu/ml)	SGPT (Iuml^{-1})	Alkaline phosphatase (u/L)
Male									
Control	71±19.7	5.4±0.62	2.6±0.44	0.57±0.1	0.19±0.169	46.7±5.9	105.5±30.6	62.7±11.2	228±27.7
1000 mg/kg	114.7±34.1*	4.8±0.29	2.7±0.38	0.57±0.05	0.35±0.12*	40.4±5 1.7*	154.7±30.6*	69.7±14.3	230.0±7 47
Female									
Control	87.5±22.7	7.5±1.1	4.08±0.21	0.655±0.07	0.22±0.043	40.75±0.21	94.75±18.2	72.75±9.3	288.25±115.7
1000 mg/kg	88.7±25.1	5.82±0 .49*	3.23±0.37	0.53±0.05*	0.145±0.03*	38.5±4.94	94.75±18.9	61.5±17.7	162.5±69.1*

Values represents mean ± S.D, n= 5.* p< 0.05 = Significance difference vs control.

Table 4. Biochemical parameters of male and female rats orally administrated after repeated dose toxicity study (28 days).

Sex	Dose (mg kg^{-1})	Blood glucose (mg %)	Total protein (g%)	Albumin (g%)	Creatinine (mg%)	Blood urea (mg%)	Billirubin (mg%)	Cholesterol (mg%)	Serum triglycerides (mg%)	Sodium mmol/L	Potassium mmol/L
Male	Control	44.6±8.96	8.02±0.33	3.68±0.14	0.88±0.09	38.4±2.07	0.096±0.01	132.2±18.86	104.6 ±22.5	187.7±22.0	7.6±2.69
	500	58.6±15.7	8.2±0.5	3.7±0.26	0.84±0.14	38.5±4.07	0.09±0.02	126±8.71	149.7±58.4*	163.2±2.16	11.07±3.3
	100	36.6±1.98	8.06±0.7	3.9±0.55	0.96±0.1	45.33±9.2	0.10±0.03	136±18.5	120.8±40.2	163.2±14.7	10.32±2.1
Female	Control	53.5±9.46	9.3±0.90	4.67±0.29	0.78±0.03	34±2.16	0.145±0.03	127±11.86	194.3±43.0	170±17.4	8.6±1.2
	500	37.5±14.4	8.87±0.4	4.2±0.5	1.03±0.3	44.5±5.6*	0.19±0.05	141±39.7	83±22.3	167.8±11.9	8.1±1.2
	100	54.6±8.11	8.6±0.62	4.0±0.5	0.82±0.13	35±7.39	0.09±0.008	142±40.4	118±52.8	153.8±4.7	7.2±0.17

Values represents mean ± S.D, n= 5.* p< 0.05 = Significance difference vs control.

congested vessels among the muscle fibres with an accidental degenerated muscle fibre. Sections of the cardiac muscle of low dose administered rat groups showed normal cardiac muscle fibres with very few congested vessels (Figure 2D).

In morphometric analysis, the mean area (μ^2) of vacuolated cytoplasm and dark nuclei were measured in H&E stained liver sections. Results indicated significant increase ($p < 0.05$) in medium and high doses compared to low doses (Table 6). Kidney sections results of the mean area (μ^2) of vacuolated cytoplasm and mean glomerular area (μ^2) indicated significant increase ($p < 0.05$) in medium and high doses compared to the lowdose (Table 7). In the cerebral stained sections, results presented in Table 8 showed the mean area (μ^2) of apoptotic nerve cells, which indicated non-significant difference between the three studied doses.

Cytotoxicity

Results from viability testing (Almar blue) revealed that the maximum concentration of rice bran oil extract not affecting viability is 100 µg ml^{-1} (Figure 3). Higher concentrations used in the present study showed cytotoxicity not exceeding 75% of the treated cells as estimated using trypan blue exclusion method.

Ames mutagenicity test (bacterial reverse mutation assay)

Results showed insignificant mutagenic activity ($p > 0.05$) for all concentrations when incubated with TA100, TA97a and TA98 in presence and absence of metabolic activation. Only the 200 µg ml^{-1} RBE concentration showed mutagenic activity with TA2638 strain both in absence and presence of S9 liver extract ($p < 0.001$ and $p < 0.001$), respectively compared to values of negative control. However, evidence of mutagenicity was observed for RBE with TA 102 strain at low dose of 100 µg ml $^{-1}$ (Figure 4).

In vitro comet assay (single cell gel electrophoresis assay) of HPL cells

Genotoxic effect of RBE concentrations was studied using HPL cells in comparison to untreated cells. Results revealed insignificant ($p > 0.05$) DNA damage of RBE extract at any concentration levels compared to a standard genotoxic agent (MTX) used as a positive control (Figure 5).

In vitro karyotyping for HPL

Results revealed the absence of chromosome and chromatid type mutations upon treating blood lymphocytes (HPL) with any of the RBE concentrations. However, treatment of blood lymphocytes with MTX for 24 h resulted into hypoploidy for several chromosome numbers in all examined metaphases (Figure 6). Subsequently, evaluation of the RBE influence on mitotic index proved that the mean percentages of mitotic indices with any of the RBE concentrations (100 to 200 µg ml^{-1}) has no significant influence ($p > 0.05$) on mitotic indices of the metaphase chromosomes seen as (92 to 74%) in comparison to the non treated control cells (Figure 6). Positive control MTX responded as expected for both treatments with each of RBE concentrations at 3 or 24 h, incubation periods ($p < 0.001$). To our knowledge, this is the first study to investigate the chromosomal aberrant effect of SRB on blood lymphocytes.

DISCUSSION

The purpose of the present study is to investigate the toxicity of the RBE extracted from rice bran after stabilization process. As reported by Al-Okbi et al. (2013), no changes in the active constitutes of the stabilized rice bran extract compared to those cited in the literature, suggesting the preservation of the active principles. In the present study RBE was standardized in relation to the bioactiveγ-oryzanol not less than 2.42% from total extract.

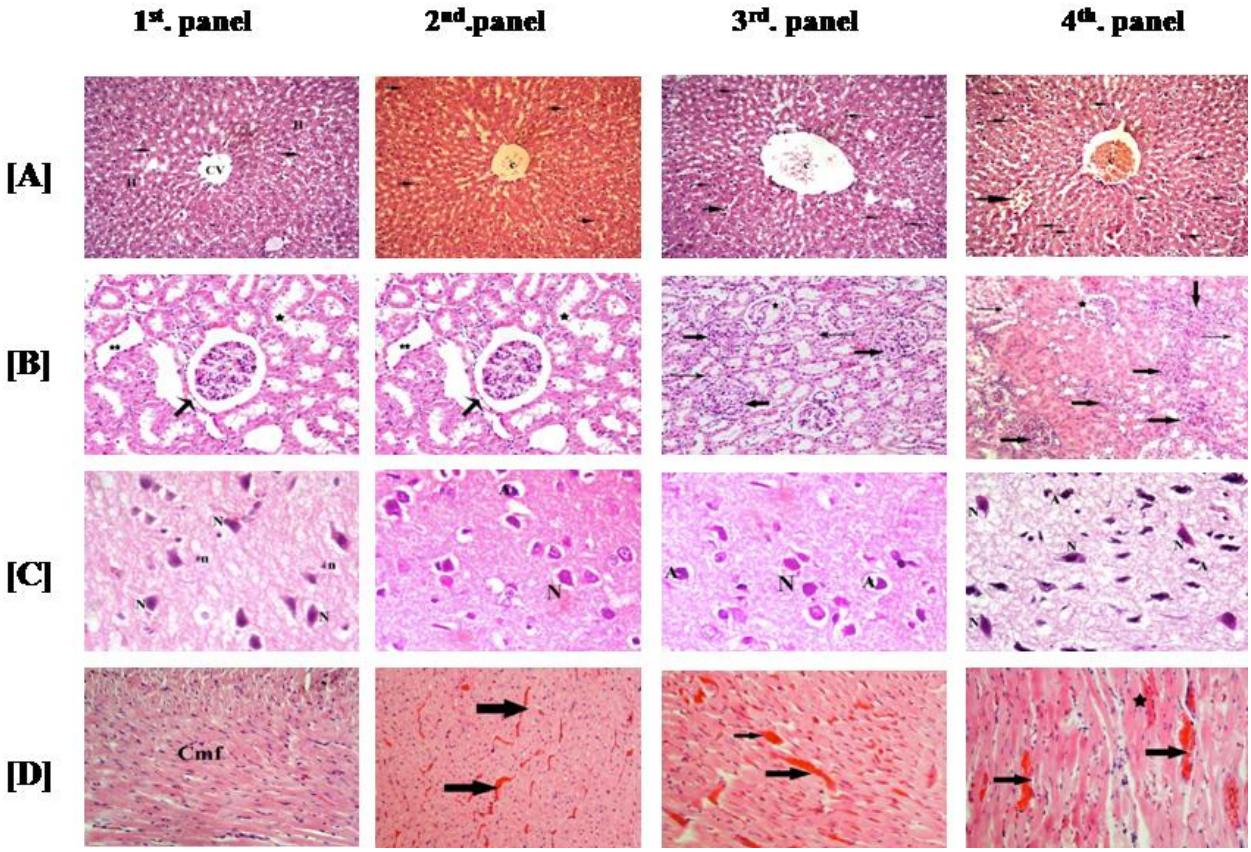


Figure 2. Photomicrograph of tissue sections**.** [A] Rat liver biopsy showing; Control tissue (1st panel) hepatocytes cords (H), acidophilic cytoplasm and pale nuclei around a central vein (CV), separated by blood sinusoids (arrows). Treated tissue with low dose (2nd. panel) showing few hepatocytes with vacuolated cytoplasm and dark nuclei (arrows) around a congested central vein (c).Treated tissue with medium dose (3rd. panel) showing some hepatocytes with vacuolated cytoplasm and dark nuclei (arrows) around a dilated congested central vein (c) and separated by few dilated congested blood sinusoids (thick arrow).Treated tissue with high dose (4th panel) showing multiple hepatocytes with vacuolated cytoplasm and dark nuclei (arrows) around a distended obviously congested central vein (c) and separated by few obviously distended congested blood sinusoids (thick arrow) (H&E, ×200). [B] Rat kidney biopsy showing; Control tissue (1st panel) with Malpighian renal corpuscle (MRC) (arrow), proximal convoluted tubule (*) and distal convoluted tubule (**). Treated tissue with low dose (2nd panel) showing a MRC with distended glomerulus obliterating the Bowman's space (thick arrow), another MRC with shrunken separated glomerulus (*) and vacuolated cytoplasm of the cells lining few tubules (thin arrow). Treated tissue with medium dose (3rd panel) showing some MRC with distended glomeruli obliterating the Bowman's space (thick arrows), another MRC with separated glomerulus (*) and vacuolated cytoplasm of the cells lining some tubules (thin arrows).Treated tissue withhigh dose (4th panel) showing multiple MRC with distended glomeruli obliterating the Bowman's space (thick arrows), another MRC with separated glomerulus (*) and vacuolated cytoplasm of the cells lining some tubules (thin arrows) (H&E, ×200). [C] Rat cerebrum biopsy showing; control tissue (1st panel) showing neurons (N) with pale nuclei and basophilic cytoplasm and dark nuclei of neuroglial cells (n). Treated tissue with low dose (2nd panel) showing an accidental apoptotic neuron (A) exhibiting dark nucleus among the neurons (N). Treated tissue with medium dose (3rd panel) showing few apoptotic neurons (A) exhibiting dark nuclei among the neurons (N).Treated tissue with high dose (4th panel), showing few apoptotic neurons (A) exhibiting dark nuclei among the neurons (N) (H&E, ×400). [D] Rat cardiac musclebiopsy showing;control tissue (1st panel) at which cardiac muscle fibers (Cmf) arranged in different directions, treated tissue with low dose (2nd panel) showing congested vessels (arrows) among the muscle fibers, treated tissue with medium dose (3rd panel) showing moderately congested vessels (arrows) among the muscle fibers, treated tissue with high dose (4th panel) showing markedly congested vessels (arrows) among the muscle fibers. An accidental degenerated muscle fiber is recognized (*) (H&E, ×200).

Safety of Egyptian stabilized rice bran hexane extract according to Al-Okbi et al. (2013) was declared to be up to 12 g kg^{-1} mice body after acute oral administration. To further support those findings the present study examined the acute and chronic toxicity of the alcohol extract taking into consideration that the hexane extract is not recommended to be used as food supplement, but the alcohol extract is accepted (alcohol is categorized as food). Here we conducted additional hematological, biochemical and histological measurements in addition to

Table 5. Enzyme activities in plasma of male and female rats orally administered of RBE after repeated dose toxicity study (28 day).

Sex	Dose (mg kg^{-1})	SGPT ($IU\ L^{-1}$)	SGOT ($IU\ L^{-1}$)	Alkaline phosphates ($IU\ L^{-1}$)
Male	Control	51±10.17	186.6±27.7	151±31.8
	500	47.7±4.03	129.7±7.03	191±32.3
	100	36.8±7.5	174±38.7	188±60.3
Female	Control	46.8±7.5	141±7.5	95.3±11.8
	500	42±7.3	146.2±29.9	109±56.3
	100	32.6±7.0	118±6.4	142±44.3

Values represents mean ± S.D, n= 5.* $p< 0.05$ = Significance difference vs control.

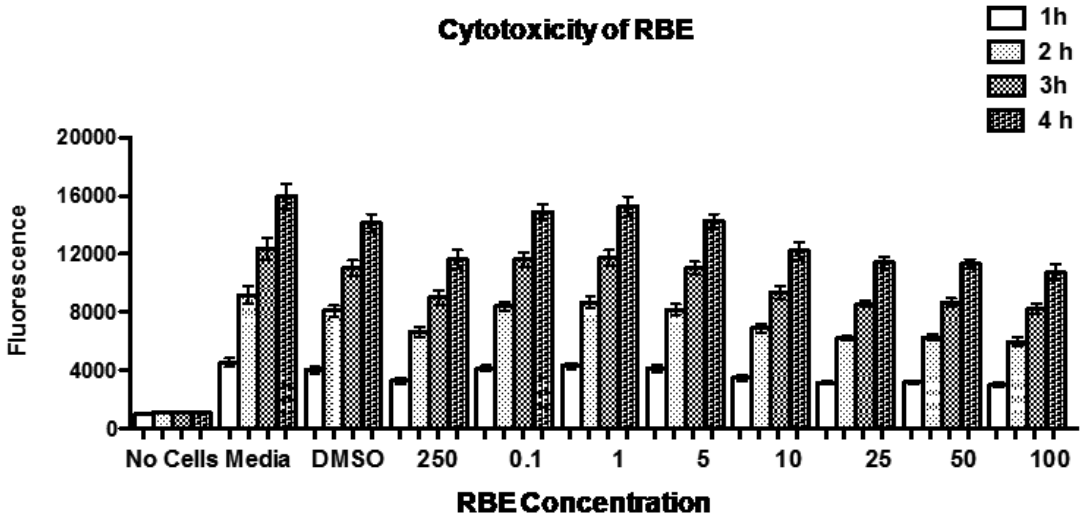


Figure 3. Cytotoxicity of RBE extract.

genotoxic analysis using standard *in vivo* and *in vitro* toxicological testing.

The study revealed that the limit dose test of acute toxicity indicates that oral administration of RBE is likelyto be non-toxic. Safety was evidenced by normal weight gain and hematological findings in female rat group. Results of 28 days oral treatment in rat group, at dose level 1000 mg kg^{-1}, indicated the lack of alteration in the haematopioetic system. Some of the measured biochemical parameters were not altered. However, significant changes in other biochemical values in the female ($gpII_F$) and male ($gpII_M$) treated groups indicated possible damage from long term administration at the high dose (equivalent to 10 g oil daily 70 kg^{-1} individualhuman consumption) (Paget and Barners, 1946). In relation to the present study, even though the rice bran and/or its active constituents in experimental animals and humans was reported to have anti-hypercholesterolemic and hypolipidemic effects on oral administration (Rukimini and Raghuram, 1991; Edward and Radcliffe, 1994), we suggest that these effects are apparent only under the aforementioned metabolic disorders. Despite significant changes in the blood urea level found in the female rat group ($gpIII_F$) administering medium dose (500 mg kg^{-1}), and serum triglycerides in male group $gpIII_{m,}$ the majority of biochemical parameters in both sexes administering medium and low doses (500 to 100 mg kg^{-1}) showed no significant changes. Reason for the aforementioned finding needs further investigation.

In accordance with the results of the present study, Ahmed et al. (2006) also investigated the effect of heat stabilized process on different variety of rice bran for dietary safety. Experimental animals were fed heat stabilized rice bran at 5 and 10% levels of their daily food intake for 6 weeks. No hematological, biochemical and body weight gain changes were detected, suggesting the safe use of the heat stabilized rice bran up to 10% of the

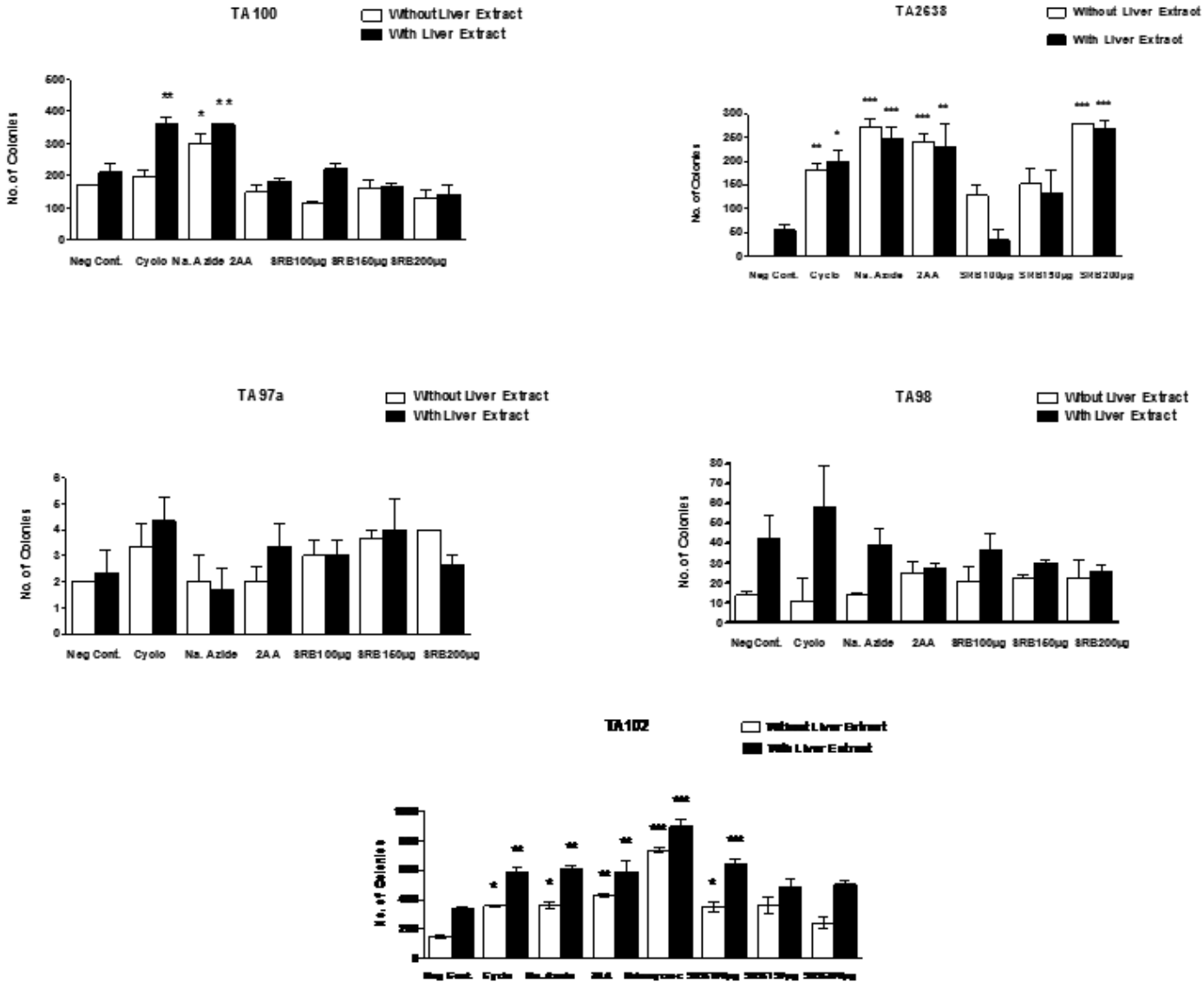


Figure 4. Mutagenic effect of RBE in presence and absence of S9 liver extract. Results are reported as No. of reverting colonies with strains TA100 (A), TA2638(B), TA97a (C), and TA98 (D) and TA 102 . Cyclophosphamide, sodium azide and 2-AA represent the positive controls. Results show the mean value ± SE of three independent experiments. Significant difference from the control group at value of $p< 0.05$ (*), $p< 0.01$ (**), and $p < 0.001$ (***).

diet which is translated into 0.5 to 1 g rice bran 200 g animal body weight per day. The average oil content of rice bran equals 10 to 20% of the total bran which in return correlates Ahmed et al. (2006), given doses with those administered in the present study, thus providing further support to the safety of our selected doses under the stabilization conditions.

On examining the present histological features of different tissue sections, results revealed the presence of damage in the tissues at the high dose compared to the medium and lower ones (Figure 2A to C). Morphometric analysis further confirmed the findings. Histological findings correlated to changes in the measured biochemical parameters of the liver and kidney revealed that RBE extract might have toxic potential to the vital organs proportional to dose increments after long-term administration. Moreover, minor changes were observedin histological findings (Tables 6 and 7) of treated groups administering medium and low doses of RBE, although their respective biochemical parameters were within normal values (Tables 4 and 5). This may conclude that the detected histological alterations may not be of toxicological significance at medium and low doses of RBE. The detected changes may be attributed to extra load on the liver and the kidney in both metabolism and reabsorption of RBE constituents. To

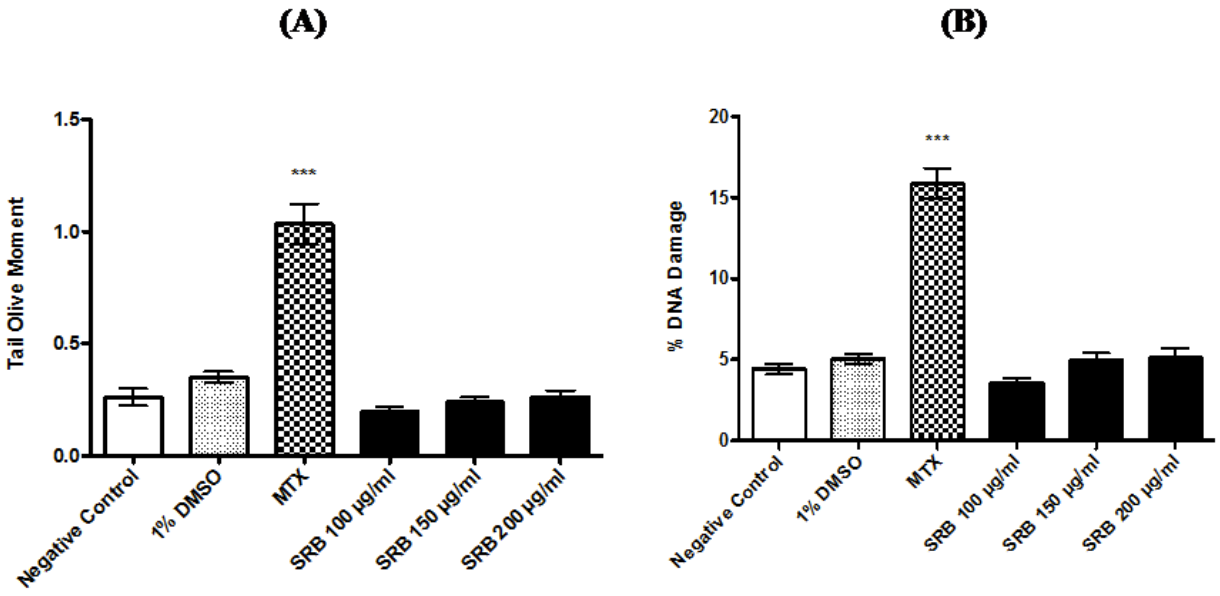


Figure 5. Genotoxic effect of RBE after 60 min on HPL (A, B). Results are reported as tail moment and %DNA damage, respectively. Methotrexate represents the positive control. Results show the mean value ± SE of three independent experiments. Significant difference from the control group at value of $p< 0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***).

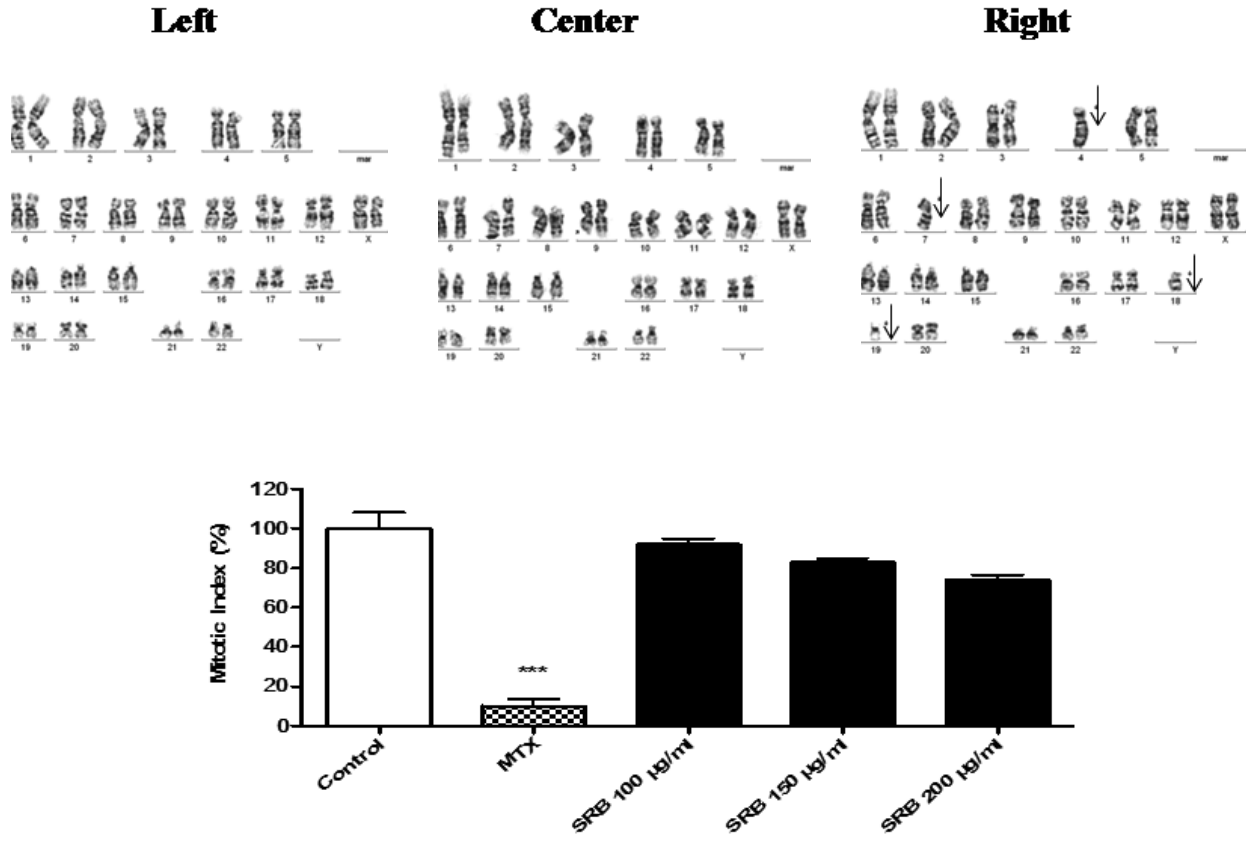


Figure 6. G-banded human Karyotypes of normal control cells (left), SRB treated cells at either 100, 150 or 200 µg/ml (center), and methotrexate (showing chromosomal hypoploidy (42, XX, - 4, - 7, - 18 & 19) (right). Mitotic index of human peripheral blood lymphocytes treated with different concentrations of SRB at 24 h. Results represented as % of the normal control. Methotrexate represents the positive control. Results show the mean value ± SE of three independent experiments. Significant difference from the control group at value of $p<0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***).

Table 6. Area of cytoplasmic vacuolations and area of dark nuclei in liver sections.

Groups (mg kg^{-1})	Cytoplasmic vacuolations in liver of male rats	Cytoplasmic vacuolations in liver of female rats	Area of dark nuclei in liver of male rats	Area dark nuclei in liver of female rat
Control	_	_	_	_
100	7.77±1.43	7.82±1.65	11.07±2.77	11.11±2.02
500	20.98±3.01*	23.18±3.65*	20.98±3.013*	23.18±3.65*
1000	30.11±4.13*	27.82±3.59*	30.11±4.13*	27.82±3.59*

Table 7. Area of cytoplasmic vacuolation sand glomerular area in kidney sections.

Groups (mg kg^{-1})	Cytoplasmic vacuolations in kidney of male rats	Cytoplasmic vacuolations in kidney of female rats	Glomerular area in kidney of male rats	Glomerular area in kidney of female rats
Control	_	_	204.62±55.13	205.77±8.81
100	4.70±1.23	6.27±0.65	243.04±28.41	216.55±37.47
500	14.28±2.93*	15.78±4.05*	620.98±83.01*	663.08±98.65*
1000	19.21±3.13*	18.96±4.20*	770.11±94.13*	727.82±89.59*

Table 8. Area of apoptotic nerve cells in cerebral sections.

Groups (mgkg^{-1})	Area in cerebrum of male rats	Area in cerebrum of female rats
Control	_	_
100	3.52±0.47	4.82±0.09
500	5.08±1.01	4.78±1.65
1000	6.01±1.13	6.12±1.59

Values represents mean ± S.D, n= 5.* $p < 0.05$ = Significance difference vs control.

further support this conclusion, morphometric analysis of other tissues such as cerebrum revealed insignificant changes between the three administered doses (Figure 6C, Table 8), in addition to normal cardiac muscle fibres with very few congested vessels at the low dose level (Figure 6D). However the need of histological examination of other tissues could furnish more information regarding RBE toxicity.

Results showed the absence of mutagenic activity of the RBE in the selected strains at the experiment concentrations, with the exception of 200 µg ml^{-1} with TA2638 strain. It has been reported that *S. typhimurium* TA102 could detect mutagenic activity of certain oxidizing mutagens, cross-linking agents and hydrazines not detected by other *S. typhimurium* strains (OECD guideline for the Testing of Chemicals test N°. 471). Therefore, the present study attempted to detect thepresence of such substances and the obtained results showed the presence of mutagenic activity of RBE on TA 102 strain at the low dose of 100 µg ml-^{1}. To explain the later results, it is worth to mention that some *in vitro* methods for testing the genotoxic potency of chemicals such as the Ames/Salmonella tests showed some false-positive rate for predicting *in vivo* genotoxicity and carcinogenicity (Mathijs et al., 2010). In this respect Raisfeld-Danse and Chen (1983) reported that drugs containing the nitrate moiety sometimes show positive results for Ames when they are indeed safe and nitroglycerin is an example that gives a positive Ames test, yet is still used in treatment today.

The genotoxic activity of the RBE has also been investigated by Comet assay. As far as we know, no data has been previously reported on the effect of whole rice bran extract on the DNA damage. Rice bran individual active constituents such as tocotrienol and the phenolic phenylpropanoid ferulic acid, were previously investigated for their effect on DNA damage. Results revealed that ferulic acid showed a dose-dependent decrease in DNA strands breaks *in vivo* on the bone marrow cells of mice experiment after exposure to γ-radiation. In another study, ferulic acid was non genotoxic in comet assay tested on hepatoma cell line (HTC) (Maistro et al., 2011). Ferulic acid (FA) with its phenolic nucleus and an extended side chain conjugation forms a resonance stabilized phenoxy radical, which accounts for its potent antioxidant potential. Ultraviolet (UV) absorption

by FA catalyzes stable phenoxy radical formation, thereby giving it the ability to terminate free radical chain reactions. Moreover tocotrienol, another active constituent present in the rice bran oil extract, was reported to prevent DNA damage (Budin et al., 2009). The previous reported results provides an evidence to the possible protective effect of RBE on DNA damage which expand our present results on the safety of RBE on genetic material.

The chromosome aberration test is also crucial in the evaluation of genotoxic effects. The comet assay detects primary DNA damage, while the formation of DNA adducts or strand breakage is often the first step in mutation induction. This damage can also be repaired (Schwaab et al., 2005). On the other hand, the loss of chromosomal fragments during the division (clastogenic effects) (Andrade et al., 2008) or whole chromosomes (aneugenic effects), is not detected by the comet assay. In this respect we performed cytogenetic test to better understand the genotoxic behaviour of rice bran extract. Structural chromosomal aberrations may be of two types, chromosome or chromatid type breaks. The majority of chemical mutagens, induced aberrations are of the chromatid type without excluding the probability of chromosome type aberrations occurrence. An increase in polyploidy may indicate that a chemical has the potential to inhibit mitosis. In the present study *in vitro* cytogenetic results revealed the absence of chromosome and chromatid type of mutations upon treating blood lymphocytes (HPL) with any of the RBE concentrations. Additionally, influence of RBE on the mitotic index, was not significant as ($p > 0.05$) in the metaphase stage (Figure 6).

Thus, the *in vivo* results suggest that the RBE at medium and low dose levels are likely to have non-toxic effect on various metabolic processes of vital organs as the liver and kidney. Moreover the studied RBE might have hepato and nephrotoxicity at high dose of 1000 mg kg^{-1} which is equivalent to a daily consumption of 10 g of the oily extract. The latter value is 10 times higher than the possible daily human intake and is regarded as an unlikely quantity to be reached. In order to clarify the disparity between histological and biochemical results, we suggest carrying out more histological examination on other body organs especially at medium and low doses. The presented results show that RBE is not mutagenic, not genotoxic in the HLP cells *in vitro* and in the chromosomal aberration test.

Conclusion

The stabilization process used for bran rice preserved the integrity of its active constituents and does not change the acute toxicity profile of the ethanol extract. Lower dose at 100 mg kg^{-1} (equivalent to 1 g oil daily consumption) is safe to be administered for long term use. Higher doses (equivalent to 10 g oil daily consumption 70 kg^{-1} human) needs to be further investigated. It is in the view of the present study that the aforementioned high dose is 10 times higher than the possible daily human intake which is impossible to be reached. Recently several pharmacological studies were reported on Egyptian stabilized rice bran extract. The objective of these studies aimed at investigating the potential therapeutic health benefits of RBE as a dietary supplement for human consumption. This study was the extension to the latter investigations with the aim to explore some of its toxicological profiles. Results of this study are needed to assess RBE impacts on human health in form of dietary supplement as primary step to introduce the RBE as food supplement in the Egyptian market and abroad.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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