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Research Paper

Raphanus sativus extract prevents and ameliorates zearalenone-induced peroxidative hepatic damage in Balb/c mice

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Abstract

Objectives *Raphanus sativus* (radish) is a species of crucifer, which includes widely consumed vegetables, distributed in Asia, Africa and Europe. It is a rich source of bioactive molecules including anthocyanins, glucosinolates, isothiocyanates and other flavonoids, and miscellaneous phenolic substances. We have evaluated the hepatoprotection of *R. sativus* extract against zearalenone, an estrogenic mycotoxin initiating hepatotoxicity in male Balb/c mice.

Methods Animals were divided into seven treatment groups and treated orally each day for twenty eight days as follows: a control, an olive oil group, group I, group II, and group III treated with radish extract alone (5, 10 and 15 mg/kg, respectively), group IV treated with zearalenone (40 mg/kg), and group V treated with zearalenone plus the lowest dose of radish extract.

Key findings Administration of zearalenone alone resulted in significant decreases in the levels of alkaline phosphatase, lactate dehydrogenase, alanine and aspartate aminotransferases in the liver, suggesting hepatic damage. Moreover, a marked increase in the level of lipid peroxide and concomitant decrease of glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, glutathione-S-transferase, RNA and DNA concentrations were also observed in the liver tissue of zearalenone-treated mice. Co-treatment with *R. sativus* extract plus zearalenone succeeded in reversing the condition back to normal levels for all studied parameters.

Conclusions By itself *R. sativus* extract did not show any toxic effects and could be considered as a potent hepatoprotectant.

Keywords antioxidants; hepatotoxicity; hepatoprotectant; *Raphanus sativus*; zearalenone

Introduction

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin biosynthesized through a polyketide pathway by *Fusarium* fungi, including *F. graminearum* (*Gibberella zeae*) and *F. culmorum*, which are common soil fungi in temperate and warm countries. They are regular contaminants of cereal crops worldwide.[1] Fungi-producing zearalenone contaminates corn and also colonizes, to a lesser extent, barley, oats, wheat, sorghum, millet and rice. The toxin has been detected in cereal products such as flour, malt, soybeans and beer. There is now overwhelming evidence of global contamination of cereals and animals with *Fusarium* mycotoxins, particularly zearalenone.[2] In addition, *Fusarium* species have been implicated in several human outbreaks of mycotoxicosis.[3] Zearalenone from toxic *Fusaria* has been linked to scabby grain toxicosis in the USA, China, Japan, and Australia. Symptoms included nausea, vomiting and diarrhoea.[4] Moreover, zearalenone has been shown to be hepatotoxic, haematotoxic, immunotoxic and genotoxic.[5–7] It induces adverse liver lesions with subsequent development of hepatocarcinoma.[8] Recently, Ben Salah-Abbès *et al.*[9] demonstrated that zearalenone caused alterations in the reproductive tract of laboratory animals. Abbès *et al.*[5] have reported haematological, biochemical and liver toxicity of zearalenone after a single oral dose in mice. Livers from mice treated with zearalenone (40 or 500 mg/kg) showed focal necrosis, vascular dilatation and lymphoid infiltration indicating portal tract inflammation. Moreover,

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in-vivo investigations by Tiemann *et al.*[10] have shown that feeding pre-pubertal gilts with 0.358 mg/kg zearalenone-contaminated wheat caused dysfunction of liver and spleen cells. They described abnormal mitochondria in the cytoplasm of some hepatocytes from foetuses whose mothers consumed a zearalenone-contaminated diet (swollen mitochondria and disappearance of mitochondrial cristae).

Dietary intakes of zearalenone have been reported for a few countries from around the world. The mean dietary intakes for zearalenone have been estimated at 20 ng/kg body weight per day for Canada, Denmark and Norway and at 30 ng/kg per day for the USA.[11–13] The Joint FAO/WHO Expert Committee on Food Additives established a provisional maximum tolerable daily intake (TDI) for zearalenone of 0.5 μg/kg of body weight, but this TDI is not respected in several countries.[14] Due to economic losses engendered by zearalenone and its impact on human and animal health, several strategies for detoxifying contaminated food and feed have been described in the literature, including physical, chemical and biological process.[7,15–17] Several studies sustain the beneficial role of *R. sativus* in the human diet because these vegetables contain a wide variety of antioxidant compounds and provide protection against oxidative damage.[18,19] Several reports attribute the beneficial effect of a *R. sativus*-based-diet to the presence of a characteristic class of compounds named glucosinolates, which are hydrolysed into isothiocyanates by vegetal myrosinase or by thioglucosidase activity of the intestinal microflora.[20] Those compounds are supposed to prevent cancer and degenerative diseases by increasing cellular intrinsic mechanisms that deactivate potential carcinogens/toxins and reactive oxygen species (ROS), in the so-called 'electrophile counterattack'.[21] Glucosinolate is, however, just one compound among the multitude of molecules existing in the *R. sativus* matrix, some of which have exhibited an antioxidant activity such as glycosinolate, peroxidases, L-tryptophan and flavonoids.[22,23] Botanical derivatives obtained from medicinal plants usually contain several classes of compounds endowed with a polyhedric mechanism of action, which often act synergistically on the same target. Recent research has shown that the complex mixture of phytochemicals in fruits and vegetables provides a better protective effect on health than single phytochemicals.

Our previous studies demonstrated that some of the undesirable effects of zearalenone treatment in Balb/c mice could be ameliorated by the antioxidant nature of the *R. sativus* extract.[9,17,24] These findings prompted us to evaluate the antihepatotoxic efficacy of *R. sativus* extract against zearalenone-induced hepatotoxicity in Balb/c mice.

Materials and Methods

Chemicals

Zearalenone was purchased as pure crystals from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and dissolved in pure olive oil obtained from a local market. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT) and quercetin were purchased from Sigma (St Louis, MO, USA). Riboflavine was purchased from Merck (Darmstadt, Germany). All other chemicals were of the highest purity commercially available.

Plant material and preparation of extract

Tunisian radish plants (*Raphanus sativus*) were harvested from Eletha region (centre of Tunisia) in April 2007. The plants were botanically identified by Professor El Ouni (Department of Botany, Faculty of Sciences, Tunisia), according to the flora of Tunisia.[25] A voucher specimen has been kept for future reference (R.S.19-89). Fresh roots and aerial parts of radish (500 g) were crushed, and the mixture juice of roots and aerial parts was extracted through fine mesh. The extracts were purified and characterized according to the method described previously.[26] The filtrate was concentrated in a freezing dryer to yield a thick powder (28 g). From the yield 10 g was mixed with 100 ml 80% aqueous methanol to extract the phenolic compounds. The mixture was shaken at room temperature for 12 h and then centrifuged at 2000*g* for 20 min. After centrifugation, the methanol supernatant was crystallized and yielded 6.23 g.

Preliminary phytochemical analysis

Plant materials were screened for the presence of tannins, flavonoids, coumarins and phenols by using the methods described by Tona *et al.*[27] Reverse phase C18 high-performance liquid chromatography (HPLC; Shimadzu LC-10AT system, USA) was used to identify antioxidant phenolic compounds in the radish extract. The HPLC comprised a column (C18, 250 × 4.6 mm, 5 μm; Waters, MA, USA) and photodiode array detector (SPD-M10V). The column was equilibrated with HPLC water containing 0.05% trifluoroacetic acid. A sample of the methanolic extract (100 μl) was injected and eluted with HPLC water containing 0.05% trifluoroacetic acid and acetonitrile at a flow rate of 1 ml/min. The absorbance of the eluant was scanned from 200 to 500 nm by the photodiode array.

In-vitro study

Free radical scavenging activity by DPPH assay

Free radical scavenging activity of radish extract was determined using the DPPH method.[28] A sample (0.5 ml) of methanol solution containing different amounts of radish extract was added to 3 ml of daily prepared ethanol DPPH solution (0.1 mM); the maximum concentration of the extract employed was 100 μg/ml. The optical density change at 517 nm was measured 30 min later using a spectrophotometer. Results were expressed as 'percentage inhibition' of the DPPH. The IC50 parameter is defined as the concentration (μg/ml) of substrate that causes 50% loss of DPPH activity and was calculated using the Litchfield and Wilcoxon[29] method.

Determination of superoxide radical scavenging

The inhibition of NBT reduction by photochemically generated O_2^- was used to determine the superoxide anion scavenging activity of the extracts by using the methods described by Siddhurrajir *et al.*[30] Quercetin was used

as a positive control. The degree of the scavenging was calculated by the following equation:

$$\text{Scavenging } (\%) = ((\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}}) \times 100 \quad (1)$$

The reference substance and the sample were assayed at concentrations 10, 3, 1, 0.3 and 0.1 mg/ml with three repetitions each.

In-vivo study

Animals and treatments

Forty-two male Balb/c mice (Sexuel, St Doulchard, France) were used (25 ± 0.3 g, 6-weeks-old). Animals were looked after under the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes. Experimental protocols were approved with the guidelines of the Ethical Committee of Medicine Faculty of Monastir, Tunisia.

The mice were given a standard granulated chow and drinking water was freely available. Mice were divided into seven treatment groups, of six mice each as follows: control group (first negative control group), mice given water; olive oil group (second negative control group), mice given olive oil; group I, mice given 5 mg/kg extract in distilled water; group II, mice given 10 mg/kg extract in distilled water; group III, mice given 15 mg/kg extract; group IV, mice given 40 mg/kg zearalenone (corresponding to 8% of the LD50 (the dose required, when administered all at once, to cause the death of 50% of the test group)) in olive oil; group V, mice simultaneously given 40 mg/kg zearalenone + 5 mg/kg extract.

Mice were treated each day for twenty-eight days and the experiments were conducted according to the guidelines of animal laboratory use in our university. The test was performed in compliance with the Commission Directive 2000/32/EC and the OECD Guideline 474.[31] Dosing was by the oral route using ten successive daily treatments followed by one 24-h sampling time. All samples of zearalenone and/or *R. sativus* extract were orally administered by a tube once, in 200 μl for each mouse, during the 28 days.

Blood and liver collections

At the end of the 28-day experimental period (72 h after zearalenone administration), blood samples were collected from the retro-orbital sinus. Animals were then killed by cervical dislocation. Serum was separated from all blood samples for biochemical assays. The liver was excised immediately, rinsed in ice-cold physiological saline and homogenated in Tris-HCl buffer (0.1 M, pH 7.4) to give a 10% homogenate. A section of liver was set aside for histological processing. Samples of the tissue homogenate were suitably kept for enzyme assays.

Biochemical parameters

The blood samples were left to clot and the sera were separated using cooling centrifugation for the determination of the biochemical assays. Serum albumin (Alb), total proteins (TP), cholesterol (Chl), uric acid (UA) and the activity of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were measured using commercial kits on an auto-biochemical parameter analyser (C × 9 PRO, Beckman).

Lipid peroxidation

Hepatic lipid peroxidation (LPO) was estimated by the measurement of malondialdehyde (MDA) by measuring thiobarbituric acid-reactive substances (TBARS) as follows: approximately 1 g liver was cut into small pieces and immersed into 2 ml ice-cold Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4), sonicated (for 10 s) and centrifuged (5000*g*, 30 min, 4°C). The resulting supernatants were collected and stored at −20°C until use. For the assay, 175 ml thawed supernatant was mixed with 175 ml 20% trichloroacetic acid containing 1% butyl-hydroxytoluene and centrifuged for 10 min. A 200 ml sample of the resulting supernatant was mixed with 40 ml 0.6 M HCl and 160 ml 26 mM Tris (pH 7.4) buffer containing 0.72 mM thiobarbituric acid, then allowed to stand for 10 min at 80°C. After cooling down, optical density was measured at 530 nm.

Liver antioxidant enzymes status

Catalase (CAT) was assayed by the method of Sinha.[32] In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. Chromic acetate thus produced was measured colorimetrically at 610 nm. Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund.[33] The unit of enzyme activity is defined as the enzyme required giving 50% inhibition of pyrogallol autooxidation. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.*[34] based on the reaction between glutathione remaining after the action of GPx and 5,5′-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. Glutathione reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Staal *et al.*[35] Glutathione-S-transferase (GST) was assayed by the method of Habig *et al.*[36] Total reduced glutathione (GSH) was determined by the method of Moron *et al.*[37] Glucose-6-phosphate dehydrogenase (G6PD) was assayed by the method of Beutler[38] wherein the increase in absorbance was measured when the reaction was started by the addition of glucose-6-phosphate.

DNA and RNA content

The extraction of liver DNA and RNA was carried out according to the method of Sambrook *et al.*[39] The determination of DNA content in the liver was carried out using the colorimetric diphenylamine method as described by Durton[40]; the determination of RNA content in the liver was carried out by the orcinol reaction method of Sambrook *et al.*[39] The liver content of DNA and RNA was expressed as mg/g wet liver.

Statistical analysis

Results of the in-vivo data are expressed as mean ± standard deviation (SD). Differences between groups were assessed by one-way analysis of variance using the SPSS software

package for Windows. Post-hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at P-values < 0.001, < 0.01 and < 0.05 have been given respective symbols in the tables. The data of the in-vitro test were evaluated using the nonparametric Mann–Whitney U-test. The a-priori P level for statistical significance was $\alpha = 0.05$, and was used in all cases.

Results

Preliminary phytochemical screening

One major peak and more than six small peaks were separated from the methanolic extract of the radish by C18 HPLC (Figure 1). Among these peaks, three peaks labelled 5, 6 and 8 accounted for 60, 11 and 17% of the total area of peaks, respectively. Further studies revealed that these three peaks contained six phenolic compounds (i.e. gallic acid, ferulic acid, isoferulic acid, sinapic acid, methyl ferulate and methylsinapate) that were identified by gas chromatography/mass spectrometry after acidic hydrolysis and chemical derivatization.

Radical scavenging activity on DPPH

The antioxidant activity of *R. sativus* extract was evaluated by the ability to scavenge DPPH free radicals (Figure 2). The radical scavenging activity of the extract can be measured as a decolourizing effect following the trapping of the unpaired electron of DPPH. *R. sativus* extract showed scavenging activity with a percentage decrease, vs the absorbance of DPPH standard solution, of 57.1, 61.9 and 68%, at a concentration 10, 30 and 100 μg/assay, respectively. The IC50 value was 6.5 μg/ml. The control without *R. sativus* extract showed a weak antioxidant activity compared with α-tocopherol.

Determination of superoxide radical scavenging

Figure 3 shows the superoxide radical scavenging effects of *R. sativus* extract. The assay was based on the capacity of the extract to enhance the formation of formazan in comparison with the NBT/riboflavine reference signal. The increase of the purple colour, typical to formazan, was followed spectrophotometrically at 560 nm. *R. sativus* extract was a very potent radical scavenger. This extract decreased respectively by 73, 87 and 98% NBT photoreduction at a concentration of 10, 30 and 100 mg/ml, and had an IC50 value of 6.5 μg/ml. The *R. sativus* extract was more active than the positive control, quercetin, in the assay.

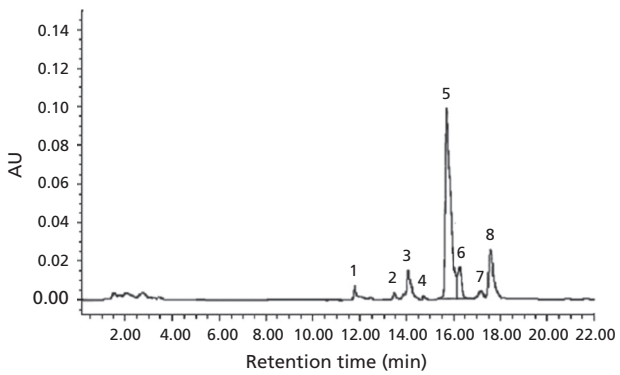

Figure 1 HPLC profile of the phenolic compounds of the methanolic extract of radish. One major peak and seven small peaks were separated from the methanolic extract of the radish by C18 HPLC. The peaks labelled as 5, 6 and 8 accounted for 60, 11 and 17% of the total area of peaks, respectively.

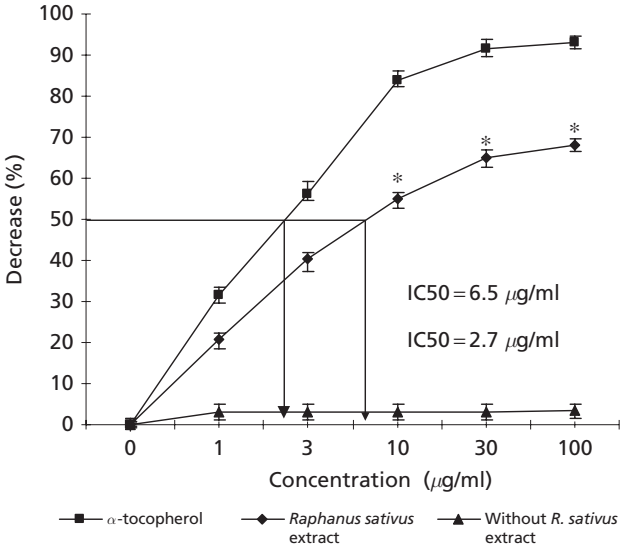

Figure 2 Scavenging effects of *Raphanus sativus* extract against the radical 2,2-diphenyl-1-picrylhydrazyl. Values represent the average of three measurements ± SEM. $^*P < 0.05$, comparison between groups by Mann–Whitney U-test.

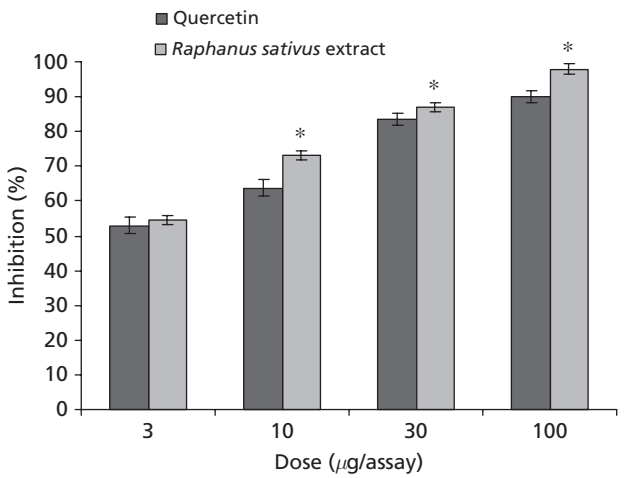

Figure 3 Antioxidant activity of *Raphanus sativus* extract on superoxide free radical (O_2^-) scavenging. Values represent the average of three measurements ± SEM. $^*P < 0.05$, comparison between groups by Mann–Whitney U-test.

Lipid peroxidation

The strong increase in the level of TBARS in liver of zearalenone-treated mice is shown in Figure 4. In the liver of the mice co-treated with zearalenone + *R. sativus* extract, the

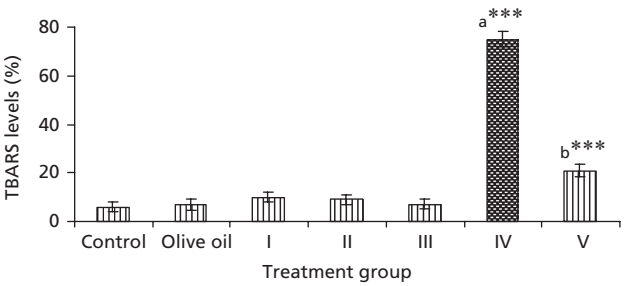

Figure 4 Level of TBARS in liver tissue of mice treated with zearalenone and *Raphanus sativus* extract alone or in combination. Treatment: group I, extract 5 mg/kg; group II, extract 10 mg/kg; group III, extract 15 mg/kg; group IV, zearalenone 40 mg/kg; group V, extract (5 mg/kg) + zearalenone (40 mg/kg). Comparisons were made between: [a]group control and group olive oil, group I, II, III, IV, V; [b]group olive oil and IV, V. Data represent mean ± SEM of six experiments. $^{***}P < 0.001$.

level of TBARS was found to be much lower compared with that seen with zearalenone alone, and the values were towards the normal level. Treatment with *R. sativus* alone at an oral dose from 5 to 15 mg/kg significantly decreased the level of TBARS as compared with zearalenone tested at an oral dose of 40 mg/kg. These results indicated the efficacy of *R. sativus* extract to counteract the LPO in the liver, since the latter was the mean zearalenone-target organ.

Biochemical parameters

Table 1 reveals the abnormal activity of biochemical parameters and serum enzymes in mice, clearly indicating cellular damage caused by zearalenone treatment (group IV). It produced a significant decrease in the levels of serum albumin, total proteins, cholesterol and uric acid compared with the control group. Moreover, the activity of the serum enzymes LDH, AST, ALT and ALP decreased by more than 2-fold in the zearalenone-treated group when compared with control. The *R. sativus* extract-treated mice showed that these enzymes and the biochemical parameter levels were similar to control values. The activity of these marker enzymes and the biochemical parameters were significantly restored in the sera of zearalenone + *R. sativus* extract-administered mice, indicating a hepatoprotective role of this extract.

Liver antioxidant enzyme status

Table 2 shows the effects of *R. sativus* extract on zearalenone-induced enzyme antioxidant status (group IV).

Table 1 Alterations in serum biochemical and enzyme activity in zearalenone-induced mice and the effect of co-treatment with *Raphanus sativus* extract

	Control	Olive oil	Group I	Group II	Group III	Group IV	Group V
Biochemical parameters							
Serum albumin (g/l)	3.1 ± 0.2	2.9 ± 0.3	3.0 ± 0.3	2.9 ± 0.3	3.1 ± 0.3	1.2 ± 0.2^{a***}	3.0 ± 0.3^{b***}
Total proteins (g/l)	14.1 ± 0.6	13.9 ± 0.5	12.9 ± 0.7	13.0 ± 0.2	12.7 ± 0.3	5.3 ± 0.3^{a***}	12.2 ± 0.4^{b***}
Cholesterol (g/l)	101.0 ± 4.9	103.1 ± 4.3	102.3 ± 5.9	104.3 ± 4.4	104.2 ± 5.9	49.2 ± 3.2^{a***}	100.3 ± 4^{b***}
Uric acid (mmol/l)	4.7 ± 0.7	4.5 ± 0.6^{a}	4.4 ± 0.5	4.5 ± 0.7	4.4 ± 5.7^{a}	8.3 ± 0.5^{a***}	4.6 ± 0.3^{b***}
Enzymes (U/ml)							
Alanine aminotransferase	39.5 ± 2.14	37.9 ± 2.03	41.5 ± 1.22	41.5 ± 1.22	41.5 ± 1.22	89.1 ± 4.33^{a***}	41.5 ± 1.22^{b***}
Lactate dehydrogenase	53.1 ± 4.3	52.9 ± 4.5	51.3 ± 4.1	53.2 ± 4.1	51.5 ± 4.5	118.3 ± 6.5^{a***}	56.1 ± 3.5^{b***}
Aspartate aminotransferase	50.4 ± 2.2	49.5 ± 3.4	48.9 ± 3.5	50.1 ± 4.1	49.9 ± 3.2	187.3 ± 8.3^{a***}	53.8 ± 6.2^{b***}
Alkaline phosphatase	70.1 ± 6.2	71.5 ± 5.1	72.2 ± 7.1	70.3 ± 5.1	70.9 ± 4.2	149.1 ± 9.2^{a***}	69.1 ± 7.2^{b***}

Treatment: group I, extract 5 mg/kg; group II, extract 10 mg/kg; group III, extract 15 mg/kg; group IV, zearalenone 40 mg/kg; group V, extract (5 mg/kg) + zearalenone (40 mg/kg). Comparisons were made between: [a]group control and group olive oil, group I, II, III, IV, V; [b]group olive oil and IV, V. Values are expressed as mean ± SD for six animals in each group. $^{***}P < 0.001$.

Table 2 Alterations in antioxidant enzymes in the liver of zearalenone-induced mice and the effect of co-treatment with *Raphanus sativus* extract

	Control	Olive oil	Group I	Group II	Group III	Group IV	Group V
Glutathione peroxidase	6.1 ± 0.6	6.5 ± 0.5	6.8 ± 0.2	6.4 ± 0.8	6.8 ± 0.4	3.1 ± 0.5^{a***}	5.9 ± 0.6^{b***}
Superoxide dismutase	8.1 ± 0.9	7.9 ± 0.8	7.9 ± 0.9	8.0 ± 0.6	7.9 ± 0.6	3.2 ± 0.8^{a***}	7.6 ± 0.6^{b***}
Catalase	301.0 ± 14.9	303.1 ± 11.3	302.3 ± 15.1	299.3 ± 12.4	304.2 ± 10.9	149.2 ± 13.1^{a***}	285.3 ± 14.1^{b***}
Total reduced glutathione	4.7 ± 0.5	4.5 ± 0.6	4.2 ± 0.6	4.6 ± 0.6	4.4 ± 0.7	0.9 ± 0.1^{a***}	3.8 ± 0.2^{b***}
Glutathione reductase	0.25 ± 0.04	0.26 ± 0.03	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.09 ± 0.01^{a***}	0.19 ± 0.02^{b***}
Glucose-6-phosphate dehydrogenase	2.1 ± 0.3	2.2 ± 0.4	2.3 ± 0.2	2.1 ± 0.3	2.3 ± 0.5	1.1 ± 0.2^{a***}	2.0 ± 0.3^{b***}

Units: glutathione peroxidase, μg total reduced glutathione utilized/min/mg protein; superoxide dismutase, U/mg protein (1 U = amount of enzyme that inhibits the autooxidation of pyrogallol by 50%); catalase, μmol H_2O_2 consumed/min/mg protein; glutathione-S-transferase, μg/mg protein; glutathione reductase, nmol NADPH oxidized/min/mg protein; glucose-6-phosphate dehydrogenase, nmol NADPH formed/min/mg protein. Treatments: group I, extract 5 mg/kg; group II, extract 10 mg/kg; group III, extract 15 mg/kg; group IV, zearalenone 40 mg/kg; group V, extract (5 mg/kg) + zearalenone (40 mg/kg). Comparisons were made between: [a]group control and group olive oil, group I, II, III, IV, V; [b]group olive oil and IV, V. Values are expressed as mean ± SD for six animals in each group. $^{***}P < 0.001$.

A significant decrease ($P \leq 0.05$) in the activity of enzymic antioxidants (GPx, SOD, CAT, GSH, GR and G6PD) was seen in the zearalenone-treated mice (group IV) compared with the negative control. *R. sativus* extract-treated mice did not show any decrease in the activity of antioxidant enzymes at any of the tested doses (5, 10 or 15 mg/kg).

R. sativus + zearalenone co-treatment restored the levels of these enzymic antioxidants towards the control level, thereby indicating that *R. sativus* co-treatment protected the liver against oxidative stress-induced depletion of antioxidants.

Effects on DNA and RNA content

The effects of the extract and zearalenone on DNA and RNA contents in the liver are presented in Table 3. Zearalenone caused a significant decrease ($P \leq 0.05$) in the content of both nucleic acids, while the treatment with extract alone at oral doses from 5 to 15 mg/kg produced a nonappreciable change in the content of DNA and RNA in the liver. On the other hand, the combined treatments with extract plus zearalenone significantly increased the liver content of both nucleic acids towards the values of the controls, but not entirely.

Discussion

The hepatotoxic effect of zearalenone has been documented well in a variety of animals and associated with a high incidence of primary human liver cancer worldwide, especially in Africa and Europe.[41,42] Humans are exposed to zearalenone by consumption of food which has been directly contaminated through growth of fungi, and by food products derived from exposed animals.[43] Zearalenone has been shown to be immunotoxic, hepatonephrotoxic, apoptotic and an enhancer of LPO.[6,17,24,44] Epidemiological studies also show that zearalenone intake is associated with the aetiology of human and animal cancer.[45] To eliminate the damage caused by zearalenone, different techniques have been investigated, such as using bioactive compounds from plant extracts, and adsorbents have been evaluated and found to be effective as post-harvest methods for the prevention of zearalenone toxicity.[5,9]

Table 3 Effect of *Raphanus sativus* extract on the nucleic acid contents of liver in zearalenone-treated Balb/c mice

Treatment	Nucleic acid contents of liver (mg/g wet liver)	
	DNA	RNA
Control	3.57 ± 0.19	4.11 ± 0.22[a]
Olive oil	3.63 ± 0.23	4.16 ± 0.25[a]
Group I	3.86 ± 0.25	4.23 ± 0.26[a]
Group II	3.97 ± 0.29	4.29 ± 0.26[a]
Group III	4.01 ± 0.29	4.44 ± 0.29[a]
Group IV	2.07 ± 0.14[a***]	2.16 ± 0.12[a***]
Group V	3.27 ± 0.21[b***]	4.02 ± 0.21[b***]

Treatments: group I, extract 5 mg/kg; group II, extract 10 mg/kg; group III, extract 15 mg/kg; group IV, zearalenone 40 mg/kg; group V, extract (5 mg/kg) + zearalenone (40 mg/kg). Comparisons were made between: [a]group control and group olive oil, group I, II, III, IV, V; [b]group olive oil and IV, V. Values are expressed as mean ± SD for six mice in each group. [***]$P < 0.001$.

R. sativus is an important vegetable in Asia, Europe and Africa. Its intrinsic health benefits are due to bioactive compounds such as defensins, glucosinolates, carotenoids, isothiocyanates and other flavonoids, and miscellaneous phenolic compounds.[46,47] Our study indicated that *R. sativus* extract exerted an important antioxidant activity towards the free radical DPPH. Antioxidant reacts with DPPH, which is a stable free radical, and converts it to α,α-diphenyl-β-picryl hydrazine. The degree of discolouration indicated the scavenging potential of the antioxidant compounds of *R. sativus* extract. It showed scavenging activity with a decreasing percentage vs the absorbance of DPPH standard solution of 55.1, 64.9 and 68% at a concentration of 10, 30 and 100 μg/ml, respectively. These results were correlated with the chemical composition of the extracts. In fact, the chemical study of *R. sativus* extract by HPLC revealed the presence of important quantities and kinds of phenolic compounds such as gallic acid, ferulic acid, isoferulic acid, sinapic acid, methyl ferulate and methylsinapate. Similar results were found by Kim *et al.*[47] and indicated that the level of these phenolic compounds increased significantly when *R. sativus* was infected by methyl jasmonate. Otherwise, phenols are known to inhibit LPO and exert these effects as antioxidants, free radical scavengers and chelators of divalent cations.[48,49] In a previous study, we demonstrated the effectiveness of *R. sativus* extract as an antioxidant and radical scavenger against zearalenone toxicity in Balb/c mice.[17]

In this study, we have observed that the addition of zearalenone to mice conferred health toxicity and a negative promotional growth response. To some extent, this could be a result of the decrease of total protein, total cholesterol and albumin. It seemed that zearalenone treatment could damage the availability of protein either by a protein catabolism process by making the critical amino acids less available, or by liver and kidney dysfunction as more commonly occurs.[6] Concerning the decrease of total cholesterol, there is considerable evidence to suggest that the influence of zearalenone dose on plasma triacylglyceride levels is caused by the suppression of very low density lipoprotein secretion by the liver.[50,51] Evidence for the inhibition of serum albumin was seen by the inhibition of protein synthesis and the augmentation of uric acid and urea.[52]

Metabolism of zearalenone takes place in the liver with production of metabolites, essentially α and β-zearalenol. According to data of the National Toxicology Program USA[8], zearalenone was found to produce hepatocellular adenoma, as well as pituitary tumour. In general, its toxicity is manifested in reproductive tracts, livers and kidneys. The degree of damage in the tissues or in the whole body can be assessed by specific enzyme tests.[53] In clinical diagnosis, determination of transaminases is of great importance.[54] For example, the intracellular enzyme AST is released into the blood in proportion to the level of damaged cells. In liver-function disorders, ALT is also specific. In this study, zearalenone alone was found to modify AST and ALT levels; this reflected initial hepatocellular damage as described by Conkova *et al.*[55] Similar results were observed by Abbès

et al.[5] 48 h after a single oral administration of zearalenone to mice. The high level of blood serum ALP reached indicated the hepatotoxicity, the augmentation of this enzyme activity level resulting in the degeneration of the hepatic tissues and the perturbation of the biliary system. Moreover, zearalenone-induced free radical production has been referred to as a possible contributor to the hepatotoxicity.[17] LPO is one of the main manifestations of oxidative damage initiated by ROS and it has been linked with altered membrane structure and enzyme inactivation. It is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane.[56] The data from this study revealed that zearalenone administration produced a marked oxidative impact, as evidenced from the significant increase in LPO. The increase in lipid peroxides may have resulted from an increased production of free radicals and a decrease in antioxidant status. The oxidative stress observed in our study was in accordance with other reports in which zearalenone-induced hepatotoxicity was implicated.[57] In this study, co-treatment with *R. sativus* extract significantly reduced the zearalenone-induced LPO by its ability to scavenge the free radicals.

GSH plays a critical role in the protection of tissues from the deleterious effects of activated zearalenone.[17] GSH is a tripeptide containing cysteine that has a reactive –SH group with reductive potency. It can act as a nonenzymic antioxidant by direct interaction of the –SH group with ROS, or it can be involved in the enzymatic detoxification of ROS, as a cofactor or a coenzyme.[58] In zearalenone-induced mice, we observed a significant decline in the levels of GSH. This finding was in accordance with results of Raney *et al.*[59] They indicated that GSH conjugated with mycotoxin and/or their metabolites thereby decreased the intracellular glutathione content.

Antioxidant enzymes such as SOD, CAT and GPx form the first line of defence against ROS and a decrease in their activity was observed with zearalenone administration.[17,57] The above finding corroborated our results, where we observed a decline in SOD, CAT and GPx activity. SOD is a family of metallo-enzymes known to accelerate the dismutation to H_2O_2 of endogenous cytotoxic superoxide radicals, which are deleterious to polyunsaturated fatty acids and structural proteins of plasma membrane.[60] The H_2O_2 produced by SOD is further removed by CAT. Decline in the activity of these enzymes after zearalenone administration may have been due to the inactivation of these enzymes by ROS. The activity of GPx, which is a constituent of the GSH redox cycle, decreased during zearalenone administration. The reduction in the activity of GPx with zearalenone treatment may have been due to a decrease in the availability of substrate (GSH) and also because of alterations in their protein structure by ROS.[58]

The decrease in the levels of glutathione metabolizing enzymes (G6PD and GR) in zearalenone-treated mice occurred as a result of the impaired flux of glucose-6-phosphate through the hexose monophosphate shunt and a decreased supply of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for the conversion of GSSG to GSH in the presence of GR. Under conditions of oxidative assault, the $NADP^+$/NADPH ratio will switch in favour of $NADP^+$, indicating decreased G6PD activity. This study showed a similar finding in the levels of these enzymes, indicating increased onslaught of oxidative radicals. In this study, zearalenone ingestion resulted in a significant decrease in the DNA and RNA contents in the liver. Roebuck and Maxuitenko[61] stated that the covalent binding of mycotoxin with the nucleic acids occurs within minutes of mycotoxin administration. This binding results in a precipitous decrease in both DNA and RNA synthesis in the liver.

Treatment with *R. sativus* extract prevented the formation of zearalenone-induced liver injury as indicated by the significant improvement in biochemical parameters, enzymatic antioxidant status and LPO level. Previous reports indicated that flavonoids and glycosides had a protective effect against oxidative stress of tissues resulting from zearalenone exposure.[17,57] In this study, animals treated with one of three different doses of *R. sativus* extract alone were comparable with the controls regarding all tested parameters. Tissue damage mediated by oxygen free radicals and LPO occurs as a result of imbalance between the oxygen free radical-producing system and the oxygen free radical-scavenging system i.e. the antioxidant defence system.[62] Also, it was found to eliminate oxygen free radicals such as hydroxyl radical, singlet oxygen, H_2O_2, peroxyl radical and hypochlorous acid through its direct scavenging activity.[63] *R. sativus* extract increased the GSH status resulting in the increase in SOD activity, thereby preventing the deleterious effect of superoxide radicals. Thus *R. sativus* extract indirectly influenced the activity of SOD and CAT. The main role of GPx is to remove H_2O_2 and lipid peroxides by catalysing the conversion of lipid hydroperoxide to hydroxy acids in the presence of GSH. The increased intracellular GSH content following *R. sativus* co-treatment may have activated GPx, thereby preventing the accumulation of H_2O_2.

Treatment with *R. sativus* extract significantly improved the activity of GR and G6PD. This finding was supported by Sunitha *et al.*,[64] who previously reported that oral administration of antioxidant plant extracts changed the tissue redox system by scavenging the free radicals and improving the antioxidant status of the liver during cadmium-induced hepatotoxicity.

Similar to our observations, Kouadio *et al.*[65] reported that zearalenone treatment resulted in an increase in radical oxygen species, increased the rupture of cellular membrane, and high damage to genomic DNA, thus confirming the involvement of oxidative stress. Addition of flavonoid glucosides reduced free radical species production and prevented genomic DNA damage. Barillari *et al.*[66] reported that 4-methylthio-3-butenyl glucosinolate had a protective role and good redox properties against oxidative stress. These compounds have the ability to reduce the production of ROS, the inhibition of protein and DNA synthesis and the apoptosis caused by zearalenone mycotoxin.[17]

The protective role of *R. sativus* extract against the oxidative stress resulting from zearalenone treatment supported the antioxidant properties, which may have been due to its high content of isothiocyanate, kaempherol glycosides and L-tryptophan compounds and its ability to scavenge free radical intermediates of LPO. In a previous study, we

reported that *R. sativus* extract was able to eliminate, or at least overcome the reproductive toxicity and the immunosuppressive effects of zearalenone in mice such as effects on lymphocyte phenotypes, cytokines and immunoglobulin profile.[9,24] The cruciferous compounds in the *R. sativus* extract activated the antioxidant defence system in liver processes, which was mediated by several enzymes functioning in a concerted manner by removing peroxide and superoxide anions generated within the cell after zearalenone damage.[67] Besides, the peroxidase enzymes in *R. sativus* such as GPx and catalase were able to react with hypochlorous acid and H_2O_2 generated by superoxide dismutase in cytosol and mitochondria by oxidizing the GSH to GSSG.[26,68,69]

Regarding the genotoxicity, this study has indicated that zearalenone induced alteration in DNA and RNA, as demonstrated by a decrease of DNA and RNA liver contents in zearalenone-treated mice. This diminution resulted from genetic alteration in DNA and RNA after treatment with zearalenone. Liver samples from mice treated with zearalenone plus *R. sativus* extract did not show any modification in DNA and RNA contents.

The protection offered by *R. sativus* extract against zearalenone toxicity was offered by neutralizing the ROS. In addition, it could not be excluded that *R. sativus* extract acted as an antigenotoxic complex enhancing the DNA repair system or DNA synthesis, which was proved by the disappearance of DNA and RNA content modification caused by zearalenone treatment.

Conclusions

R. sativus extract, rich in bioactive phytochemical compounds, induced a meaningful reduction of zearalenone-toxicity in mouse liver. This extract could be commercially exploited and applied as a hepatoprotectant. However, further studies are needed to elucidate the antioxidant mechanisms and the exact compounds which offer this chemoprevention in liver against zearalenone toxicity.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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