RESEARCH ARTICLE

PROTECTIVE EFFECT OF AQUEOUS EXTRACT POMEGRANATE PEEL AGAINST STERIGMATOCYSTIN TOXICITY IN RAT

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ABSTRACT:

Introduction and Aim: Sterigmatocystin (Stg) a mycotoxin with mutagenic and carcinogenic properties is commonly found as the contaminant in grains and animal feeds. Pomegranate peel is a rich source of antioxidants, flavonoids and other phenolic compounds. So the aim of the current study was to evaluate the protective effects of aqueous extract of red Pomegranate peel against Stg toxicity in liver, kidney, intestine and lung as well as final body weight using male rats.

Methods: Forty eight Sprague-Dawley male rats were divided into six groups (8 rats/group) including the control group that fed on a standard diet and water without any treatment, group 2 fed on standard diet plus aqueous extract of RPP (250 mg/rat/day), group 3 fed on standard diet plus aqueous extract of RPP (500 mg/ rat/day), group 4 fed on a standard diet and orally Stg dissolved at a dose (18μg/rat/day), group 5 fed on a standard diet and Stg plus aqueous extract of RPP (250mg/day) and group 6 fed on a standard diet and Stg plus aqueous extract of RPP (500mg/day). At the end of the experimental period, blood samples were collected for serum biochemical analyses. After collecting the blood samples all animals were sacrificed and dissected samples of liver, kidney, intestine and lung were collected for histological examination.

Results: The total phenols and total flavonoids, compounds in aqueous extract of RPP were 1.38 mg/ml and 680.28 mg/ml, respectively. However, the antioxidant activity amounted to 68.0% in the determination of radical DPPH scavenging activity. On the other hand, results indicated that rat orally Stg plus aqueous extract of RPP with low dose and high doses showed a significant improvement in final body weight compared with group administrated of Stg alone. While, the effect of aqueous extract of RPP on kidney and liver function of rats, the results indicated that the rat orally Stg alone caused significant increased in urea, creatinine and uric acid compared with the control group. The aqueous extract of RPP alone at the two tested doses did not induce any significant changes in the biochemical parameters or the histological picture. The combined treatment showed significant improvements in all tested parameters and histological pictures in the liver tissues. Moreover, this improvement was more pronounced in the group received the high dose of aqueous extract of RPP.

Conclusion: From results it can be concluded that the aqueous extract of RPP has a potent antioxidant activity and a protective effect against Stg toxicity and this protection was dose dependent.

Keywords: Sterigmatocystin, Red pomegranate peels (RPP), aqueous extract, liver and kidney.

INTRODUCTION:

Sterigmatocystin (Stg) is a mycotoxin produced by Aspergillus versicolor and Aspergillus nidulans. The stg has been shown to be carcinogenic, cause cancer in a number of animal species and classified as class 2B carcinogen (as possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC). Stg is a biogenic precursor of aflatoxin B1. The stg is one of the predominant contaminating mycotoxins in food and grains in high-incidence areas of malignant tumors in China. Stg is closely correlated to the incidence areas of malignant tumors in China. Stg is closely correlated to the occurrence of gastric carcinoma, hepatocellular carcinoma and esophage carcinoma. Stg can potentially induce hepatocellular carcinoma, lung cancer and gastric cancer. Stg is commonly detected in human food, animal feed and in the indoor environment, such as carpets and building materials. Several in vivo studies have shown that Stg may induce lung adenocarcinoma in mice. In addition, our previous in vitro studies have demonstrated that Stg could induce malignant transformations in human fetal lung tissue. Some countries have set already relatively low maximum levels for Stg (e.g., Czech Republic and Slovakia at the level 5 µg/kg of rice, vegetables, potatoes, flour, poultry, meat, milk, and 20 µg/kg for other foods) and soon after Stg was recognized as a highly toxic compound, the California Department of Health Services used TD50 values from the Cancer Potency Database to produce "no significant risk” intake levels for humans. The level, resulting was 8μg/kg body weight/day for a 70 kg adult. The pomegranate (Punica granatum L) is one of the oldest edible fruits, which has a long history as a medicinal fruit and has been used extensively in the folk of many cultures. The pomegranate cultivated around the world in subtropical and tropical regions. The world pomegranate production amounts to approximately 1,500,000 tons. Pomegranate...
peels which constitute up to 40% of the whole fruit remain as a byproduct after pomegranate juice production. The pomegranate fruit peel is an inedible part obtained during processing of pomegranate juice. Pomegranate peel is a rich source of tannins, flavonoids and other phenolic compounds. Pomegranate peel extracts exhibited marked antioxidant capacity in several studies using unsafe solvents such as methanol and a mixture of methanol, acetone, ethyl acetate and water. The pomegranate peel is a rich source of polyphenolic compounds, which possess antioxidant properties that can play an important role against cancer cells. Pomegranate peel is rich in polyphenols including ellagitannins, gallotannins, ellagic acids, gallic acids, catechins, anthocyanins, ferulic acids, and queretins. These polyphenols exhibit various biological activities, such as eliminating free radicals, inhibiting oxidation and microbial growth, and decreasing the risk of cardio- and cerebrovascular diseases and some cancers. Researchers have shown that preparations containing the pomegranate rind extract can be used to prevent and/or cure atherosclerosis, diarrhea, gastric ulcer, venereal disease, and estrogen-related diseases. Accordingly, the aim of the present study was to investigate the ability of aqueous extract of pomegranate peel as a natural source for phenolics, flavonoids and antioxidant possibly will protect against sterigmatocystin toxicity in male rat.

MATERIAL AND METHODS:

Material

Red pomegranate peels (RPP):

Red pomegranate peels (RPP) were obtained from fruit purchased from a local market. The peels were separated manually from the fruit, sun-dried and powdered, and then kept at room temperature for further study.

Sterigmatocystin

Sterigmatocystin was purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.).

Kits:

Urea, creation, uric acid (UA), ALT, AST, ALP and bill kits were purchased from Biodiagnostic Company, Cairo, Egypt.

Experimental animals

Two months old, mature male rats were purchased from the Animal House Colony, National Research Centre, Giza, Egypt.

Methods:

Preparation of aqueous extract of RPP:

The thirteen gram of the powder RPP was extracted with 200 mL distilled water at room temperature for 24 h in a shaker, and then filtered under vacuum. Finally the extract was concentrated under vacuum with a rotary evaporator.

Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu procedure. Briefly, the extract (100 µl) was transferred into a test tube and the volume adjusted to 500 µl with distilled water and oxidized with the addition of 250 µl of Folin-Ciocalteu reagent. After 5 min, the mixture was neutralized with 1.25 ml of 20% aqueous Na₂CO₃ solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, expressed as µg of gallic acid equivalent (GAE) per g of sample.

Analysis of total flavonoid content

The total flavonoid content was determined according to Zilic et al. (2012). Briefly, 50 µl of 5% NaNO₂ was mixed with 100 µl of extract. After 6 min, 500 µl of a 10% AlCl₃ solution was added. After 7 min, 250 µl of 1 M NaOH was added, and the mixture was centrifuged at 5000 g for 10 min. Absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was expressed as µg of catechin equivalent (CE) per g of sample.

Determination of antioxidant activity:

Determination of radical DPPH scavenging activity

Free radical scavenging capacity of the Moringa peel extract was determined using the stable 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) according to . The final concentration was 200 µM for DPPH and the final reaction volume was 3.0 mL. The absorbance at 517 nm was measured against a blank of pure methanol at 60 min. Percent inhibition of the DPPH free radical was calculated by the following equation:

\[
\text{Inhibition} \% = 100 \times \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}
\]

Where: \( A_{\text{control}} \) is the absorbance of the control reaction (containing all reagents except the test compound). \( A_{\text{sample}} \) is the absorbance with the test compound.

Determination of Phenolic acids profile by High Performance Liquid Chromatography (HPLC):

Extraction of phenolic compounds

The sample was alkaline hydrolyzed according to . Briefly, 1mL sample was placed in quick fit conical flask and 100 ml hexane was added and shacked for 4 h at room temperature 2M NaOH was added. The flask was flushed with N₂ and the stopper was replaced. The samples were shacked for 4 h at room temperature. The pH was adjusted to 2 with 6 M HCl. The samples were centrifuged at 5000 rpm for 10 min and the supernatant was collected. Phenolic compounds were extracted twice with 50mL ethyl ether and ethyl acetate (1:1). The organic phase was separated and evaporated at 45°C and the samples re-dissolved in 3mL methanol.

Experimental animals: Two-month old Sprague-Dawley male rats (100-120 g) were maintained on a standard diet (protein: 16.04; fat: 3.63 a; fiber 4.1g/kg and metabolic energy: 2887Kcal/Kg). After an acclimation period of one week, animals were divided into six groups (8rats/group) and housed in filter –top polycarbonate cage housed in a temperature controlled
and artificially illuminated room free from any source of chemical contamination.

Experimental design:

Animals within different treatment groups were treated daily for four weeks as follows:

Group (1): normal control animals which fed on a standard diet and water without any treatment.

Group 2 (PIX1): Fed on standard diet+ aqueous extract of RPP (250 mg/rat/day)

Group 3 (PIX2): Fed on standard diet+ aqueous extract of RPP (500 mg/rat/day)

Group 4 (Stg): Fed on a standard diet and Stg dissolved in corn oil (18µg/rat/day)

Group 5 (PIX1+Stg): Fed on standard diet and Stg+ aqueous extract of RPP (250mg/day)

Group 6 (PX2+Stg): Fed on standard diet and Stg+ aqueous extract of RPP (500mg/day)

At the end the experimentation period (4 weeks), blood samples were collected from all animals from the retro-orbital venous plexus after they had fasted for 12h for different biochemical analyses. After the collection of blood samples, all animals were sacrificed and the blood samples were collected in a dry clean centrifuge tube. The tubes were kept for 30 min, to allow blood to clot before centrifugation at 3000 rpm for 10 min using cooling centrifuge. Serum was separated and stored at -20ºC for analysis. Other samples from the liver, kidneys, intestine and lung were excised and fixed in 10% neutral formalin for histopathological studies. The tissue samples were dehydrated in ascending grades of ethanol, cleaned with xylene and embedded in paraffin. Section (8µm)

Biochemical assay: The kidney function including creatinine, uric acid and urea are determined in serum according to 19, 20, respectively. On the other hand the liver function (AST, ALT and ALP) are determined according to21, 22, respectively. Total Bilirubin (T.Bil) was determined according to 23

Statistical analysis:

All data were statistically analyzed using the General Linear Model procedure of the SPSS var.18. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio. All statements of significance were based on probability of P<0.05.

RESULTS:

Total phenolics and flavonoids contents in aqueous extract of RPP:

The results in (Table 1) shows the content of total phenols and total flavonoids, compounds in aqueous extract of RPP. The total phenol contents were 1.38 mg/ml, while the total flavonoids were 680.28 mg/ml. On the other hand the antioxidant activity amounted to 68.0% in the determination of radical DPPH scavenging activity. The phenolic acids were determined by HPLC in (Table 2). That showed the aqueous extract of RPP contained the garlic, sinapic and Ferulic at a level of 272.73, 112.71 and 61.29 µg/ml, respectively.

Effect of aqueous extract of RPP on final body weight of male rats:

The results in (Figure 1) showed that rat oral administration of Stg (18µg/rat/day) for 4 weeks showed a significant decrease in final body weight compared to the control group and/or the groups received an aqueous extract of RPP with tested two doses. Results indicated that rat received Stg plus aqueous extract of RPP at both low and high doses showed a significant improvement of final body weight when compared with the group received Stg alone.

Effect of aqueous extract of RPP on kidney and liver function of rats

The results in (Table 3) shown levels of urea, creatinine and uric acid in blood serum of rats received stg alone, RPP at two tested doses and/or combined with stg.

| Red pomegranate peel prepared as aqueous extract | Total phenols (mg/ml) | Total flavonoids (mg/ml) | Antioxidant activity (%) |
|-----------------------------------------------|-----------------------|--------------------------|-------------------------|
|                                               | 1.38                  | 680.28                   | 68.0                    |

| phenolic acids | Concentration (µg/ml) |
|----------------|-----------------------|
| Gallic         | 272.73                |
| Chlorogenic    | 34.0                  |
| Vanillin       | 14.03                 |
| Ferulic        | 61.29                 |
| Sinapic        | 112.71                |
| Coumarin       | 72.8                  |
| Cinnamic       | 16.78                 |
Stg caused significant increased in kidney function compared with control group and PX1, PX2 groups. On the other hand, group administrated Stg at dose 250 mg of RPP were significant increased in urea that represented (35.5±2.08 mg/dl), while the group treated with Stg and 500 mg of RPP (PX2+Stg) had a significant decrease in urea compared with Stg group. Oral administration of rats with Stg caused significant increase in creatinin and uric acid compared with a control group and other groups that had oral RPP aqueous extract alone or with Stg.

The results in (Table 4) indicated that rats orally received of Stg at a dose (18µg/rat/day) showed a significant (p≤0.05) increase in serum biochemical parameters i.e ALT, AST and ALP. Moreover the aqueous extract of RPP alone at two tested doses did not effect on ALT and AST, while caused a significant increase of ALP. It is worthy to report that administration and combination of RPP aqueous extract with the rats received stg succeeded to restore or eliminate or moderate the tested biochemical parameters towards the control level. A marked elevation in the concentration of T.Bil, was observed in the rats orally administration of Stg for 4 weeks. The aqueous extract of RPP showed a significant protection the T.Bil.

![Figure 1: Effect of Stg and an aqueous extract of RPP at doses 250 and 500 mg/rat/day on the final body weight of rats.](image)

**Table 3: Effect of Stg and aqueous extract of RPP on the kidney function of rats**

| Test     | Control     | PX1         | PX2         | Stg         | PX1+Stg     | PX2+Stg     |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|
| Urea (mg/dL) | 14.75±1.71<sup>a</sup> | 19.0±2.16<sup>a</sup> | 17.0±2.94<sup>a</sup> | 58.0±5.35<sup>d</sup> | 35.5±2.08<sup>c</sup> | 27.75±2.22<sup>b</sup> |
| Creatinin (mg/dL) | 0.85±0.13<sup>a</sup> | 0.91±0.04<sup>a</sup> | 0.75±0.14<sup>a</sup> | 1.45±0.26<sup>b</sup> | 0.79±0.11<sup>a</sup> | 0.72±0.06<sup>a</sup> |
| Uric acid (mg/dL) | 4.15±0.66<sup>a</sup> | 4.8±0.65<sup>ab</sup> | 4.77±0.79<sup>ab</sup> | 6.35±0.64<sup>c</sup> | 5.4±0.45<sup>b</sup> | 4.97±0.52<sup>ab</sup> |

<sup>*mean±SD</sup>  **Mean values in each raw having different letter (a, b, c, d) are significant**

**Table 4: Effect of Stg and aqueous extract of RPP on the liver function of rats**

| Test     | Control     | PX1         | PX2         | Stg         | PX1+Stg     | PX2+Stg     |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|
| ALT (IU/L) | 21.25±2.5<sup>a</sup> | 21.5±2.38<sup>a</sup> | 21.0±2.16<sup>a</sup> | 37.25±1.71<sup>c</sup> | 27.25±1.7<sup>b</sup> | 21.5±3.11<sup>a</sup> |
| AST (IU/L) | 23.5±3.69<sup>a</sup> | 21.75±3.86<sup>a</sup> | 19.75±3.3<sup>a</sup> | 41.25±2.21<sup>b</sup> | 21.0±3.91<sup>a</sup> | 19.5±2.64<sup>a</sup> |
| ALP (IU/L) | 79.5±2.64<sup>a</sup> | 85±2.16<sup>b</sup> | 93.25±5.37<sup>b</sup> | 117.75±6.84<sup>d</sup> | 88.25±6.24<sup>bc</sup> | 95.75±7.27<sup>i</sup> |
| T.Bil (mg/dL) | 0.44±0.06<sup>a</sup> | 0.53±0.05<sup>a</sup> | 0.51±0.09<sup>a</sup> | 1.42±0.21<sup>c</sup> | 0.71±0.06<sup>b</sup> | 0.56±0.09<sup>ab</sup> |

<sup>*mean±SD</sup>  **Mean values in each raw having different letter (a, b, c, d) are significant**
The histopathological study:

The histological examination of the kidney and liver tissues:

The biochemical results were confirmed by the histological examination in kidney, liver, intestine and lung tissues. The histological examination of the kidney in the control group showed normal histological structure, the kidney of the control rat showing the normal histological structure of renal parenchyma (Figure 2a). Kidney of rat treated with Stg showed thickening of the parietal layer of bowman’s capsule and vacuolation of renal tubular epithelium (Figure 2b). On the other hand the kidney of rats treated with Stg showed focal tubular necrosis associated with inflammatory cell infiltration (Figure 2c). It is worthy to report that the kidney of rat treated with aqueous extract of RPP showing no histopathological changes at two doses (Figure 2d). The obtained results of the histological examination of rats, kidney administrated with Stg plus a low dose of aqueous extract of RPP (250 mg/rat/day) showing congestion of the glomerular tuft as shown in (Figure 2e), while the kidney section of rats received orally Stg plus a high dose of aqueous extract of RPP (500 mg/rat/day) showing significant improvements in kidney tissues or showing no histopathological changes relationship with control group (Figure 2f).
The histological examination of the control group showed normal histological structure of the liver lobule and the central vein (Figure 3a). While, the liver sections of the rats administrated with Stg for 4 weeks (18µg/ rat/day) showed focal hepatic necrosis associated with inflammatory cell infiltration and addition fibrosis of portal tract (Figure 3b & Figure 3c). On the other hand the rats treated with the aqueous extract of RPP at the low or the high doses showed normal hepatocytes and portal tract (Figure 3d). The liver sections of the rats orally administrated of Stg plus low dose of aqueous extract of RPP showed focal hepatic necrosis associated with inflammatory cells infiltration and apoptosis of hepatocytes (Figure 3e). Moreover the liver of rat orally administration of Stg plus high dose of aqueous extract of RPP showed significant improvement of the liver tissues and normal hepatocytes compared with control group (Figure 3f).
The histological examination of the intestine and lung tissue:

The current study focused on the investigation of the histopathological changes in intestine and lung of rat orally received Stg alone and/or aqueous extract of RPP at doses 250 and 500mg/rat/day. Figure 4a shows the histologically normal appearance of the intestine layers of the control group. On the other hand the intestine of rat orally received Stg showing edema in lamina propria associated with few inflammatory cell infiltrations (Figure 4b). The rat orally received of aqueous extract of RPP at two doses showing no histopathological changes (Figure 4c). Furthermore, the intestine of rat orally received Stg plus a high dose of aqueous extract of RPP showing slight activation of mucous secreting glands and inflammatory cell infiltration of the lamina propria (Figure 4d).
The microscopic examination of the lung section of control rat showed the normal structure of the lung (Figure 5a). The lung of rat orally received Stg showed focal interstitial pneumonia. Furthermore, notice focal aggregation of mononuclear inflammatory cells (Figure 5b). The rat orally received an aqueous extract of RPP showed congestion of pulmonary blood vessel and focal interstitial pneumonia (Figure 5c). Lung of rat treated with Stg plus a high dose of aqueous extract of RPP showing slight thickening of interalveolar tissue (Figure 5d).

**DISCUSSION:**

Pomegranate peels are characterized by an interior network of membranes comprising almost 26–30% of the total fruit weight and are characterized by substantial amounts of phenolic compounds, including flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, Gallic and ellagic acid). These compounds are concentrated in pomegranate peel and juice. Pomegranate peel extracts are suitable for applications in the food industry due to its important source of phenolics, flavonoids and tannins as a natural ingredient. The pomegranate age, the mean annual precipitation and agro-climatic and environmental conditions could explain the change in the polyphenolic composition between different cultivars. The light and sun exposure of selected fruits seems to be another factor influencing the phenol and the tannin synthesis. The RPP waste can provide a cheap source of flavonoids, compounds with strong antioxidant properties.

The reduction of body weight due to toxin ingestion that could be alters various digestive enzymatic activities that give rise to a mal absorption syndrome, characterized by steatorrhea, hypocarotenoidemy, and to
lowering of bile, pancreatic lipase, trypsin, and amylase. A significant decrease in the final body weight in groups received toxin due to affect the liver capacity for protein synthesis. The pharmaceutical, pharmacological and medicinal bioactivities of compounds (including tannins, flavonoids, alkaloids, organic acids, triterpenes and steroids, etc.) from different parts of the pomegranate plant impart hypolipidemic, antioxidant, antiviral, anti-neoplastic, anticancer, antibacterial, anti-diabetic, anti-diarrheal, helminthic, vascular and digestive protection, and immunomodulation effects. Polyphenols are well known plant antioxidant principles responsible for several important pharmacological properties namely anticarcinogenic (antimutagenic and chemopreventive), antimicrobial, anti-diabetic, hepatoprotective, cardioprotective etc. Certain phenolic compounds such as ellagic acid found in strawberries, raspberries, grapes, walnuts, etc. have been found to be anticarcinogenic due to their excellent antioxidant potential. The ellagic acid and punicalagin arrest cancer cell growth by inducing apoptosis-a multistep cell death program. Antioxidants, either from synthetic or plant sources, are thought to be a preventive approach against cancerogenesis. Natural antioxidants, despite the presence of synthetic ones in the market, have gained a wide acceptance due to their high safe edible limits. Pomegranate peel is a high natural source of phenolic compounds: anthocyanins, ellagic acid glycosides, free ellagic acid, ellagitannins, punicalagin, punicalin and gallotannins. Pomegranate peel exhibited strong antimicrobial, preservative and high antioxidant activity, and could enhance kidney and liver functions.

The histopathological study:
The toxicity of Stg is primarily confined to the liver and kidney and closely correlated to the occurrence of hepatocellular carcinoma, gastric carcinoma and esophageus carcinoma. The Stg also has been demonstrated to inhibit RNA synthesis in rat liver. Early experiments are emphasizing interactions with DNA, because chemical fixation of damage in this macromolecule could be a prelude to genetic alterations resulting in mutation, cancer, and other diseases resulting from functional alterations of the genetic apparatus. Stg can potentially induce hepatocellular carcinoma, lung cancer and gastric cancer. Phenolics are linked to a negative association with stomach cancer incidences and tumor growth. Stg can potentially induce hepatocellular carcinoma, lung cancer and gastric cancer. Several studies have substantiated the chemo protective role of the pomegranate peel extract antioxidant fraction against chemically induced injuries. These studies suggest an inhibitory and protective role against oxidative damage in carcinogenesis and its progression. In vivo studies have validated the attenuation of oxidative stress by the pomegranate ellagitannins in the treatment of placental dysfunction. Prenatal ingestion of the metabolic fractions of ellagitannins in juice form reduced oxidative stress and apoptosis in placental tissues, confirming the in vitro results of sub-culturing placental tissues of pomegranate administered to expectant mothers suffering from hypoxia.

CONCLUSION
Based on the aforementioned results, it could be concluded that pomegranate peel powder, prepared as aqueous extract is a rich and cheap source of antioxidant, total phenolics and flavonoids. Furthermore, a protective effect against Stg toxicity and this protection was dose dependent. So, the aqueous extract of pomegranate peel powder could be used as natural antioxidants to enhance the antioxidant properties of functional food.

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