

IMMUNOLOGICAL AND HISTOLOGICAL EFFECTS OF POMEGRANATE IN HEPATIC RATS

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ABSTRACT

Plants that contain medicinal properties are defined as everything of plant origin, containing one or more medicinal substances capable of treating or reducing a specific disease, or containing raw materials used in the preparation of medicinal substances. Plants that contain medicinal properties can be made more effective in medicine and therapy, so we worked on this investigation to evaluate the immunological and histological effects of pomegranate fruits (POF) and peel (POP) in hepatotoxicity in rats. The investigation was carried out on a caged animal. All rats were kept on a basal diet for one week prior to the study, and they were separated into seven groups, of six rats each. As control negative normal rats (C-ve), the first group sample was fed only the basal diet for 28 days. The remaining rats (n=36) were injected with carbon tetrachloride (Ccl4). Five groups were fed varying concentrations of (5%, 10%, 15% POF, 5%, and 10% pomegranate peel (POP)), whereas one group was diagnosed with the illness and disease, and didn't even feed the experimental diet. The results discovered non-significant variances in Phagocytosis between (C-ve) normal rats and Ccl4-intoxicated rats then fed on 5% and 10% POP Also, found non-significant differences in killing percent between rats injured by Ccl4 then fed on formulas 5%,10%, and 15% POF.

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Introduction

The body's immune network is a protective shield from an infection that may be harmed by distinguishing and responding to external antigens. The immune network is made up of a complex network of tissues, cells, and organs [1]. Hepatitis is an inflammatory disease of the liver and is considered one of the leading causes of death worldwide. Due to its scavenging activity, *Punica granatum* may be used for the treatment and prevention of liver diseases [2]. Pomegranate (*Punica granatum* L.) has an outstanding medicinal history and remarkable medicinal properties. Asia, especially India and Iran, was the place where pomegranates were originally cultivated. Since ancient times the fruit has been grown and naturalized in the entire Mediterranean area. pomegranate (POM, *Punica granatum*) is one of the edible fruits and is mainly grown in the middle east, India, southern Asia, and Mediterranean Europe, as well as in warm climate areas of the world including the Americas Pomegranate has been an attractive topic currently as a homeopathic agent with multiple therapeutic indications [3]. We also find that a lot of research has found that this plant may be used as a natural therapy for its ability to fight many pathogens. Some pomegranate parts, such as the bark, fruits, and peel, have been used to detect antimicrobial compounds. Pomegranate also has many plant compounds that have an antimicrobial effect. Among the antioxidants, ellagic acid and hydrolyzable tannins, such as Punicalagin, have the most anti-inflammatory agents. Since oxidative stress plays a critical role in liver disease pathogenesis and progression, antioxidants have been proposed as therapeutic agents and as drug adjuvants [4]. And the peels of the pomegranate protective activity of the liver. An examination was done on the in vivo effects of the extract of pomegranate in DENA-induced hepatocellular carcinogenesis, such as human hepatocellular carcinoma. The great chemopreventive capacity has been narrated because of the reduction in the incidence, size, and multiplicity of hepatic nodules. Pomegranate extract also reduces the oxidation of fats and proteins in the liver. [5] recommended and advocated for the dispensation of pomegranate-derived agents in the treatment and prevention of hepatocytes in humans [6]. Phytochemicals and antioxidants are abundant in pomegranate peel extracts due to the presence of ellagic acid, the primary polyphenol of

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pomegranate [7]. So, it is necessary to stimulate an increased focus on the production of new varieties of pomegranate and also the issuance of a law to protect the varieties (Brazilian *soc plant breeding*, 2021). The therapeutic abilities of pomegranate appear to be widespread. This has led to an increase in esteem as an innate compound and functional sustenance for centuries. Punicalagin pomegranate polyphenols are recognized to have strong anti-cancer activity in breast, cervical, and lung cells. All fruit parts have been reported to have healing activity including anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, anti-diabetic, liver-protective, antioxidant activity...etc [8].

Aim of the study: Evaluate the immunological and histological effects of pomegranate fruits (POF) and peel (POP) in hepatotoxicity rats.

Materials and Methods

Materials

Preparation of POF and POP

Pomegranate fruits and peel are properly cleaned, cut into small slices, and dried for 3-days at 50 °C in a drying oven, before crushing and grinding into a powder form.

Experiential Animals

In this investigation, 42 male albino rats of the Sprague Dawley breed weighing 150±10 g were expended.

Biological Experiment

Rats' Normal Diet

The basal diet contained 10% casein, 0.25% choline chloride, vitamin mixture (1%), 5% cellulose, 10% maize oil, 4% salt mixture, 0.35% methionine, and corn starch (69.5%).

The basal diet in the test contained CaCO₃ (600 mg), MgSO₄.2H₂O (204 mg), K₂HPO₄ (645 mg), CaHPO₄.2H₂O(150 mg), Fe(C₆H₅O₇) 26H₂O (55 mg), ZnCl₂ (0.5 mg), MnSO₄.4H₂O (10 mg), NaCl(334 mg), CuSO₄.5H₂O (0.06 mg) and K_i (1.6 mg), [9].

The basal diet in the test contained Vitamin A (200 Iu), Vitamin K (0.50 Iu), Vitamin E (10 Iu), Calcium panthothenic acid (0.40 mg), Thiamin (0.50 mg), Pyridoxine (1.00mg), Vitamin D (100 Iu), Folic acid (0.02 mg), Niacin (4.00 mg), Para-amino – benzoic acid (0.02 mg), Choline chloride (200 mg), Inositol (24 mg), Vitamin B12 (2.00 g) [10].

Diet Experiment

Table 1 shows the experimental diet, which is made up of the basic diet with powdered plants supplemented at a 10% rate.

Table 1. The basic and experimental diets' compositions

| Component (g) | Basal diet | 5% POF | 10% POF | 15% POF | 5% POP | 10% POP |
|-------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Test ingredients | --- | 5 | 10 | 15 | 5 | 10 |
| Casein | 20 | 20 | 20 | 20 | 20 | 20 |
| Corn oil | 4.7 | 4.7 | 4.7 | 4.7 | 4.7 | 4.7 |
| Mineral mix | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mix | 1 | 1 | 1 | 1 | 1 | 1 |
| Cellulose | 5 | 5 | 5 | 5 | 5 | 5 |
| Cholin chloride | 2 | 2 | 2 | 2 | 2 | 2 |
| Sucrose | 10 | 10 | 10 | 10 | 10 | 10 |
| Corn starch | Up to 100 | Up to 100 | Up to 100 | Up to 100 | Up to 100 | Up to 100 |

Carbon Tetrachloride (Ccl₄)

El-Gomhoria Company for Chemical Industries in Cairo, Egypt, provided the compound (Ccl₄) carbon tetrachloride in a 10% liquid solution. It was distributed in white plastic water bottles holding one liter as a deadly substance ingredient for liver disease, [11]. During the induction, it is diluted using paraffin oil obtained from the drugstore.

Rats

Mature, male, Sprague-Dawley, albino, rats weighing (B. Wt) 150-160 g. At the age of 14-16 weeks, the animals were transferred from the Animal Laboratory. The animals were kept in plastic cages with stainless steel covers and were kept in a very clean environment. For adaption, rats were given the basal diet for 7 days prior to the study. A smallmouth bottle

connected with a metallic tube and a piece of plastic tubing at the mouth provided Ad libitum water. As previously indicated, rats were acclimatized on a 12-hour light/12-hour night condition for seven days prior to the beginning of the research to allow for acclimation.

Induction of Liver Intoxication in Rats

[12] used intramuscular injections of (Ccl4) carbon tetrachloride into paraffin oil at 50% V/V (2ml/kg B.W.T.) twice per week for two weeks to stimulate chronic liver injury in male albino rats. Following Ccl4 injection, blood samples were extracted via the retro-orbital technique to confirm the presence of the injury to the liver and test its function.

Animal Groups and Experimental Design

- Each group of six rats was divided into seven groups. The following were the rat groups:
- G₁: Normal rats were fed a basic diet for 28 days without any treatment as the positive control (Control group).
- G₂: Rats with liver toxicity were kept as control negative and fed a basal diet for 28 days without any treatment.
- G₃: Rats with liver intoxication and fed on basal diet plus 5% of POF.
- G₄: Rats with liver intoxication and fed on basal diet plus 10% of POF.
- G₅: Rats with liver intoxication and fed on basal diet plus 15% of POF.
- G₆: Rats with liver intoxication and fed on a basal diet plus 5% POP.
- G₇: Rats with liver intoxication and fed on a basal diet plus 10% POP.

Biological Evaluation

Each day for the 28-day study, the amount of food consumed was noted, and body weight was recorded each week. The feeding efficiency ratio (F.E.R.), the body weight growth (B.W.G. %), and the organ weight were all calculated.

Blood Sampling

At the end of this study, blood samples were obtained following a 12-hour fast. The retro-orbital approach with highly specialized glass tubes was used to collect blood samples into a dry clean centrifuge tube and let them coagulate for half an hour in a water bath (37°C) at room temperature. Before testing for glucose, blood samples have been centrifuged for 10 minutes at 3000 rpm to extract the serum. The residue was carefully aspirated, put into clean, tight-fitting polypropylene tubes, and maintained frozen until analysis at (-20°C).

The liver, kidney, heart, and spleen were extracted and washed in salt solution before being weighed and preserved in 10% formalin, as specified by [13].

Immunologic Analysis

Lymphocyte Transformation Test. Lymphocytes: Boyum (1968) & Burrels and Wells (1977) [14, 15]. Using sterile Pasteur pipettes, the heparinized blood was layered carefully on the surfaces of the lymphocyte separation medium. Ficoll hypaque (1.1) in a 50ml sterile polystyrene centrifuge tube.

Phagocytosis and Killing Assay [16] Blood taken in heparin-coated universal containers was used to prepare leukocytes for bacterial phagocytosis and death. Heparinized blood samples were lysed with 0.83% ammonium chloride, washed thrice in cold phosphate buffer solution (PBS) at pH 7.2, and resuspended in MEM with 0.5 % inactivated fetal calf serum to produce a final concentration of 10 viable PMN cells per ml.

Determination of (CRP)

According to Kindmark *et al.*, (1972) the absorption used was 492 nm and, Concentration was expressed as mg/dl, in 370c [17].

Histopathological Testing

Liver specimens were taken immediately after the animals were sacrificed at the end of the experiment, set in 10% neutral formalin, dried in ethyl alcohol, cleared in xylene, and entrenched in paraffin wax. Hematoxylin and eosin staining were used to generate 4-6 thick slices [18].

Statistical Analysis

A one-way categorization was used to calculate the statistical analysis. According to the least significant difference (LSD) and analysis of variance (ANOVA) (LSD) [19].

Results and Discussion

The main goal of this study is to evaluate the immunological and histological effects of pomegranate fruits (POF) and peel (POP) in hepatotoxicity rats.

Immunologic Changes

Effect of POF and POP on GPX (ng/ml), CAT(mmol/l) and SOD (u/l) of hepatotoxicity rats:

Table 2 present the glutathione peroxidase (GPX) (ng/ml), catalase (CAT) (mmol/l), and superoxide dismutase (SOD) (u/l) for the negative control, positive control, and other different groups of hepatitis rats fed on POF and POP.

Table 2 shows the GPX (ng/ml), CAT(mmol/l), and SOD (u/l) for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP.

Table 2. The GPX (ng/ml), CAT(mmol/l), and SOD (u/l) for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP

| Groups | Parameters | | |
|-------------|------------------------|-------------------------|------------------------|
| | (GPX)(ng/ml) | (CAT)(mmol/l) | (SOD) (u/l) |
| Control (-) | 42+0.95 ^b | 78.2+0.321 ^a | 39.3+0.47 ^b |
| Control (+) | 17.1±0.32 ^c | 56.4+0.5 ^a | 18.3+0.43 ^f |
| 5% POF | 38.4+0.45 ^c | 64.4+0.45 ^c | 28+0.55 ^c |
| 10% POF | 32.2+0.65 ^d | 60.2+0.58 ^f | 29.1+0.32 ^d |
| 15% POF | 41.4±0.45 ^b | 69+0.3 ^c | 30.1+0.75 ^c |
| 5% POP | 43.5±0.5 ^a | 70.2+0.2 ^b | 40.5+0.0 ^a |
| 10%POP | 39.3±0.43 ^c | 65.4+0.45 ^b | 38.6+0.47 ^b |
| LSD | 1.000 | 0.735 | 0.914 |

Values denote arithmetic + Standard deviation of the mean. Means with different letters (a,b,c,d,e,f,g) in the same column differ significantly at P<0.05, while those with similar letters are non-significant by different.

Table 3 the rats inoculated with CCl₄ without treatment (C+ve group) mean values of GPX, CAT and SOD were (17.1+0.32 ng/ml, 56.4+0.5mmol/l and 18.3+0.43 u/l) respectively, but in (C-ve group) normal rats it was (42+0.95 ng/ml, 78.2+0.32mmol/l and 39.3+0.47u/l) respectively. These results indicate that there were substantial decreases in GPX, CAT, and SOD. Due to feeding on POF and POP means significantly increased compared to the C+ve group. The 5% POP group achieved high improvement of GPX, CAT and SOD, being (43.5+0.5 ng/ml, 70.2+0.2mmol/l and 40.5+0.55u/l) respectively.

These data revealed mostly significant differences 5%, 10%, 15% POF, and 10 %POP (7.5%) groups between them, but all values indicated significant differences as compared with C+ ve group.

Table 3 shows the lymphocytic transformation index for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP.

Table 3. The lymphocytic transformation index for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP

| Groups | Parameters | |
|-------------|--|-------|
| | Lymphocytic transformation Index (%) Mean ± S.E | IR• |
| Control (-) | 1.97 ± 0.12 ^a | 58.87 |
| Control (+) | 1.24 ± 0.12 ^c | - |
| 5% POF | 1.41 ± 0.03 ^d | 12.91 |
| 10% POF | 1.61 ± 0.09 ^c | 29.84 |
| 15% POF | 1.71 ± 0.13 ^b | 37.90 |
| 5% POP | 1.64 ± 0.05 ^c | 32.26 |
| 10%POP | 1.74 ± 0.08 ^b | 40.32 |

IR•: Means increase the ratio of the control positive group. Values denote arithmetic means ± Standard error of the mean.

Means with different letters (a, b,c,d) in the same column differ significantly at p ≤ 0.05 using one way ANOVA test, while those with similar letters are non-significant

Table 4 the rats inoculated with CCl₄ without treatment (C+ve), the percentage of lymphocytic transformation was 1.24±0.12% but in (C -ve) normal rats was 1.97±0.12%. These findings denote that there was a significant decrease in lymphocytic transformation index in the blood of rats poisoned by CCl₄ as compared to (C -ve) normal rats. Rats were imparted

Ccl4 then fed on all formulas, which revealed a significant increase in lymphocytic transformation index as compared to (C +ve). However, rats poisoned by Ccl4 before feeding on 15% POF and 5% POP showed the highest significant increase as compared to (C +ve), values were 1.71 ± 0.13 and $1.74 \pm 0.08\%$ respectively.

Table 4 shows the phagocytosis percent for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP.

Table 4. The phagocytosis percent for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP

| Groups | Parameters | |
|-------------|------------------------------------|-------|
| | Phagocytosis (%) Mean \pm S.E | IR• |
| Control (-) | 81.00 ± 1.15^a | 16.83 |
| Control (+) | 69.33 ± 2.19^d | - |
| 5% POF | 76.00 ± 0.58^b | 9.62 |
| 10% POF | 74.33 ± 1.85^c | 7.21 |
| 15% POF | 76.00 ± 0.58^b | 9.62 |
| 5% POP | 78.33 ± 0.33^{ab} | 12.98 |
| 10%POP | 78.76 ± 0.88^{ab} | 13.47 |

IR•: Means increase the ratio of the control positive group. Values denote arithmetic means \pm Standard error of the mean.

Means with different letters (a, b,c,d) in the same column differ significantly at $p \leq 0.05$ using one way ANOVA test, while those with similar letters are non-significant

Data in **Table 5** showed rats intoxicated with Ccl4, the phagocytosis percentage was 69.44 ± 2.195 compared to $81.00 \pm 1.15\%$ in (C -ve) normal rats. These findings show that there was a substantial reduction in phagocytosis percent of rats injected with Ccl4. All rats intoxicated with Ccl4 then fed on all tested formulas showed a considerable increase in the previously mentioned parameter as compared to the (C +ve) group. There were non-significant differences between (C -ve) normal rats and Ccl4-intoxicated rats then fed on 5% and 10% POP, values were 81.00 ± 1.15 , 78.33 ± 76 , and $78.76 \pm 0.88\%$ respectively.

Table 5 shows the killing percent of leucocytes upon Staphylococcus aureus bacteria in the blood for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP.

Table 5. The killing percent of leucocytes upon Staphylococcus aureus bacteria in the blood for the negative control, positive control, and other different groups of hepatotoxicity rats fed on POF and POP

| Groups | Parameters | |
|-------------|---------------------------------------|-------|
| | Killing percent (%) Mean \pm S.E | IR• |
| Control (-) | 77.67 ± 1.33^a | 19.49 |
| Control (+) | 65.00 ± 1.15^c | - |
| 5% POF | 73.67 ± 0.66^b | 13.34 |
| 10% POF | 72.67 ± 1.45^b | 11.80 |
| 15% POF | 73.33 ± 0.88^b | 12.81 |
| 5% POP | 76.33 ± 0.33^{ab} | 17.43 |
| 10%POP | 76.33 ± 1.20^{ab} | 17.43 |

IR•: Means increase the ratio of the control positive group. Values denote arithmetic means \pm Standard error of the mean.

Means with different letters (a, b,c,d) in the same column differ significantly at $p \leq 0.05$ using one way ANOVA test, while those with similar letters are non-significant

In rats injected with Ccl4, the killing percent of leucocytes was $65.00 \pm 1.15\%$ while in control -ve normal rats it was $77.67 \pm 1.33\%$. These results revealed that there was a sizable reduction in the (C +ve) group as compared to (C -ve) normal rats. In rats given Ccl4 then fed on all formulas, there was a major increase in the killing percent of leucocytes as compared to the control -ve group. There were trivial distinctions between the control -ve normal rats and Ccl4-poisoned rats then fed on 5% and 10% POP which were 77.67 ± 1.33 , 76.33 ± 0.33 and $76.33 \pm 1.20\%$ respectively. Moreover, there were insignificant variations between rats injured by Ccl4 then fed on formulas 5%, 10%, and 15% POF which were 73.67 ± 0.66 , 72.67 ± 1.45 , and $73.33 \pm 0.88\%$ respectively.

Histopathological Results

Microscopical liver examination of rat from control –ve normal group revealed the normal histological structure of hepatic lobule (**Figure 1**). On the other hand, the liver of a Ccl4-intoxicated rat without treatment (C +ve) group revealed kupffer cells activation, hepatocellular vacuolization (**Figure 2**), and local area of hepatic necrosis completely replaced by leucocytic cells infiltration (**Figure 3**). Examined liver of Ccl4-intoxicated rat and fed on 5% POF showed congestion of central vein and hepatic sinusoids (**Figure 4**). Meanwhile, examined liver sections of Ccl4-intoxicated rats and fed on 10% POF revealed necrosis of sporadic hepatocytes (**Figure 5**). However, the liver of a Ccl4-intoxicated rat fed on 15% POF showed no histopathological changes (**Figure 6**). Kupffer cells activation was the only histopathological finding observed in the liver of Ccl4-intoxicated rats fed 5% POP, (**Figure 7**). Meanwhile, examined liver of a Ccl4-intoxicated rat and fed on 10% POP revealed no histopathological changes.

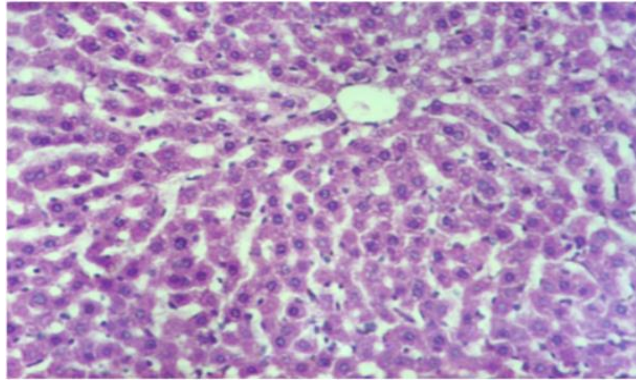


Figure 1. Liver of rat from control (+) group showing congestion of central vein and necrosis of sporadic hepatocytes (H and E $\times 200$)

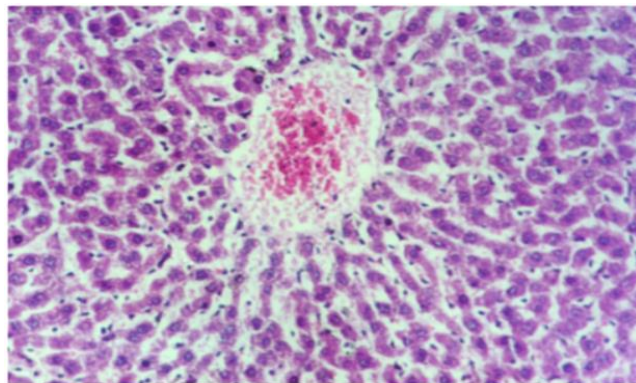


Figure 2. Liver of Ccl4-intoxicated rat without treatment (control +ve group) showing hepatocellular vacuolization (arrows). (H and E $\times 200$)

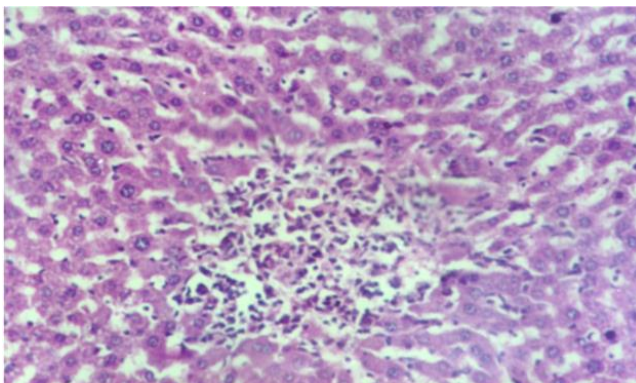


Figure 3. Liver of Ccl4-intoxicated rat fed on 5 % POF showing the local area of hepatic necrosis completely replaced by leucocytic cells infiltration (arrow). (H and E $\times 200$)

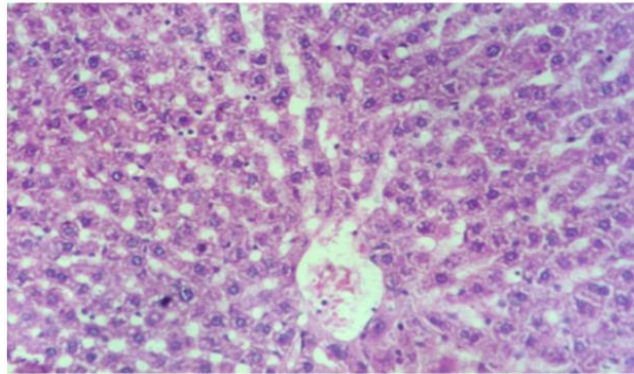


Figure 4. Liver of Ccl4-intoxicated rat then fed on 10% POF showed congestion of central vein and hepatic sinusoids (arrows). (H and E x 200)

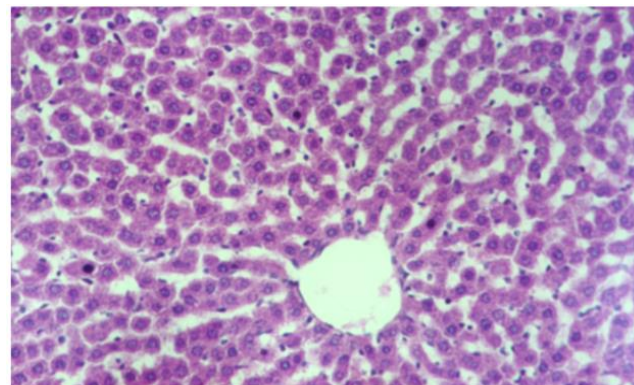


Figure 5. Liver of Ccl4-intoxicated rat then fed on f fed on 15% POF showing necrosis of sporadic hepatocytes (arrows). (H and E x 200)

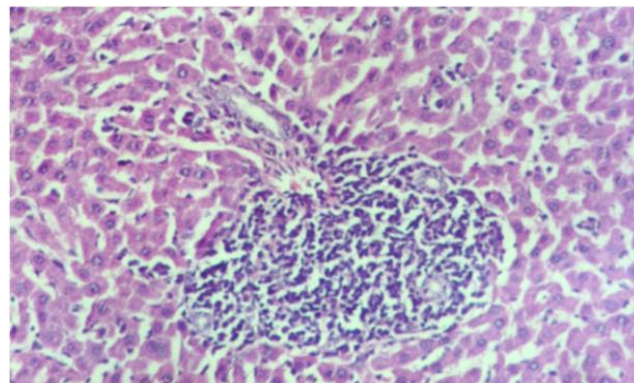


Figure 6. Liver of Ccl4-intoxicated rat then fed on fed on 5% POP showing no histopathological changes. (H and E x 200)

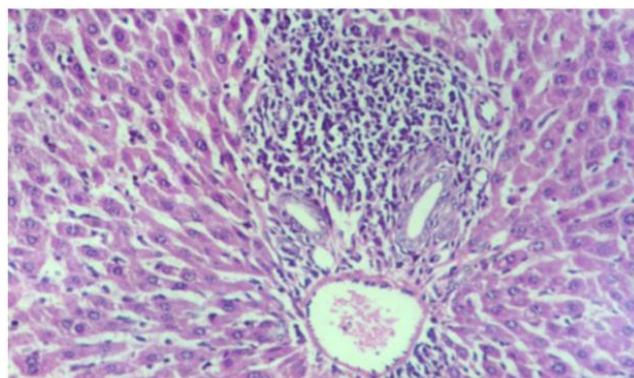


Figure 7. Liver of Ccl4-intoxicated rat then fed on fed on 10% POP showing kupffer cells activation (arrows). (H and E x 200)

The body's immune network is a protective shield from an infection that may harm by recognizing and responding to foreign antigens. The immune network consists of a complex network of cells, tissues, and organs [1]. The results (**Table 2**) supported that obtained by [20] and Pradeep *et al.*, (2007) [21], According to the researchers, the significant decreases in GSH, GPX, and CAT activity in NDEA-treated rats could be because of the reduced expression of these antioxidants after hepatocellular damage. Moreover, because GSH is a required co-factor for many enzymes, lower cellular GSH levels resulted in decreased activity. The findings were similar to those of [22], who discovered that NDEA induced hepatocellular damage by lowering hepatic GSH, GPX, and CAT activity, which was later restored by antioxidant treatment. In hepatocellular carcinoma, according to [23], there is a disruption in the oxidant and antioxidant equilibrium, with the bias being toward the oxidant side. Hepatocellular carcinoma, a familiar but deadly cancer, is a potent activator of oxidative stress. The peels of pomegranates have liver-protective effects. Pomegranate extract was tested in vivo on DENA-induced hepatocellular carcinogenesis, such as hepatocellular carcinoma in humans. Hepatic nodule decrease in size and multiplicity has been shown to have strong chemopreventive potential. Pomegranate extract also reduces both lipid and protein oxidation in the liver. Bishayee *et al.* proposed and supported the use of pomegranate-derived medications in people to treat and prevent hepatocyte injury [8]. Some studies also showed that pomegranate contains bioactive compounds that have a chemopreventive effect against DND-induced hepatocellular carcinogenesis in mice by inhibiting hepatic oxidation. The chemopreventive pomegranate possesses efficacy against hepatocellular carcinoma, probably through antioxidant signaling mechanisms without any toxic expression. The bioactive characteristics of pomegranate hinder the proliferation of the cell, govern the progression cell cycle, and induce cell death [5]. According to various studies, pomegranate has antioxidant, anti-proliferative, anti-inflammatory, and anti-tumor properties that can enhance the immune system by modifying various signaling pathways, making it a feasible chemotherapeutic and preventative diet [4]. The peel, juice, and oil of the pomegranate have been found to have anti-cancer characteristics, including impeding cancer cell proliferation, cell cycle, and angiogenesis. As a result, healthy persons can safely ingest pomegranate peels, juice, or extracts. There were no negative effects on liver or kidney function when people were given up to 1420 mg/day of pomegranate extract tablets. Antioxidants are also abundant in pomegranate extracts [6]. Developed as a natural supplement to address a suitable alternative form of bioactive polyphenol use [24].

Conclusion

The results showed that the pomegranate plant (the peel and fruits) had an effective effect on improving immunity and improving the functions of liver tissue in mice infected with hepatitis and the improvement rate increased in the group containing 10% POP and 5% POP, because it contains Phytochemicals and antioxidants are abundant especially in pomegranate peel

Recommendations

1. For hepatic patients, different POF and POP levels are recommended to increase immunity.
2. POF in different concentrations, especially 10% POP, can improve the histology of the liver.

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Conflict of interest: None

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Ethics statement: None

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