Trop J Nat Prod Res, February 2021; 5(2):233-237

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Short Communication

Hepatoprotective Effects of Soy Flour (*Glycine max* (L.) Merr.) Supplementation in Diazinon-Treated Wistar Rats

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ARTICLE INFO

Article history:
Received 28 November 2020
Revised 12 December 2021
Accepted 20 February 2021
Published online 01 March 2021

ABSTRACT

Diazinon is one of the most commonly used insecticides in agriculture that can cause hepatotoxicity through the induction of oxidative stress and apoptotic mechanisms. Isoflavones in soy flour act as antioxidants that prevent lipid peroxidation in liver tissue thus preventing damage to liver tissue. This study will evaluate the hepatoprotective effects of soy flour supplementation as a preventive therapy in diazinon-treated Wistar rats. A total of 30 rats were divided into 5 groups, 6 each (n = 6). Normal control (K_n) and negative control ($K_{(-)}$) groups were given normal saline, K_1, K_2, and K_3 groups were given soy flour with concentrations of 10% 15%, and 20% respectively for 28 days. All groups were induced by diazinon 40 mg/kg for 5 days, except K_n. Histopathological examinations of liver, liver function markers [(alanine aminotransferase (ALT), and aspartate aminotransferase (AST)], level of oxidative stress [(malondialdehyde (MDA), and gluthathione (GSH)] were measured at the end of the study. There was a significant decrease ($p < 0.05$) in the levels of liver biomarker enzymes (MDA AST, ALT) in K_2 and K_3 groups compared to $K_{(-)}$. The administration of soy flour in treatment groups (K_1, K_2 and K_3) caused a significant increase in GSH levels ($p < 0.05$) and a significant reduction in liver's histopathology score compared to $K_{(-)}$. The biochemical changes are consistent with histopathological observations suggesting significant hepatoprotective potentials. Further research is needed to find the most suitable form of soy flour for consumption that has a similar hepatoprotective effect.

Keywords: Antioxidant, Agromedicine, Pesticide, Toxicity, Functional food

Introduction

Insecticide has been used as an effort to optimize effectiveness in agriculture. Diazinon is one of the most commonly used insecticides, widely used by farmers to eradicate pests in their agricultural environment.[1] In general, it eradicates pests through inhibition of the Acetylcholinesterase (AChE) enzyme.[2,3] Diazinon, a type of organophosphate, can induce toxicity in the target (plant pests) and non-target organisms (humans).[2] Diazinon causes toxicity through the induction of oxidative stress and apoptotic mechanisms.[1] Diazoxon is a result of diazinon metabolism modulated by cytochrome P450 (CYP450) in liver tissue. These compounds are considered to be oxidative stress inducers because they increase the production of Reactive Oxygen Species (ROS), which bind to proteins and lipids to initiate lipid peroxidation and contribute to liver damage.[3,4] Several studies stated that the administration of diazinon at several doses has a hepatotoxic effect. It is indicated by changes in histopathological structure, increased levels of liver biochemistry; Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Malondialdehyde (MDA); and lowering the activity levels of Glutathione (GSH) and Superoxide Dismutase (SOD).[3,5]

Soybean is one of Indonesia's leading commodities that is known to have a lot of nutrition content, high-quality protein, essential oils, vegetable fats, vitamins A, B, E, K, and minerals K, Fe, Zn, P. Soybean also contains various chemical compounds which has high bioavailability, especially isoflavones as its main metabolites.[6] This compound has antioxidant and anti-apoptotic activity, which is mainly played by genistein and daidzein.[7,8] The higher isoflavone content was found in processed soy flour, with genistein at 98.77%, daidzein at 72.92%, and glycitin at 16.12%.[9] Previous study showed that adding 10% soy flour to bread made from wheat flour had a better effect on the liver of rats than other rats that were given bread without the addition of soy flour.[6] In that study, rats were not induced by substances that could damage the liver. Another study on the effect of soy flour on liver has been carried out in rats induced by CCl_4.[10] The results showed a significant decrease in the marker enzymes of Lipid Peroxidation (LPO), Xanthine Oxidase (XO), MDA, and hepatotoxicities such as AST, ALT, and Lactate Dehydrogenase (LDH). The study used a large dose range between treatment groups and a relatively short time of soy flour administration. Therefore, a longer administration period with a lower dose range needs to be evaluated. This may indicate a pattern of giving soy flour in terms of time and dose of different effectiveness as a hepatoprotective agent. Given the complex health-promoting potential of soybean and soy foods, it can be assumed that soy flour may inhibit diazinon-induced hepatotoxicity. The aim of this study was to evaluate the hepatoprotective effects of soy flour supplementation as a preventive therapy in diazinon-treated Wistar rats.

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Citation: Wisudanti DD, Normasari R, Hidayati T. Hepatoprotective Effects of Soy Flour (*Glycine max* (L.) Merr.) Supplementation in Diazinon-Treated Wistar Rats. Trop J Nat Prod Res. 2021; 5(2):233-237. doi.org/10.26538/tjnpr/v5i2.3

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Preparation of soy flour

The soybean used was a local variant, namely the Baluran variety, in which the Baluran soybean is one of the superior varieties produced by researchers from University of Jember. These soybeans have been determined at the Laboratory of Seed and Plant Production Technology, Faculty of Agriculture, University of Jember. Soy flour was made at the RPHP Laboratory of the Faculty of Agricultural Technology, University of Jember. A total of 200 grams of soybean seeds were soaked in 600 mL of clean water for 3 hours and renewed every 1-1.5 hours. The soybeans were drained and boiled for 5 minutes, followed by drying in the sun for 4 hours, then heating at 50°C for 24 hours. The dried soybeans were then ground and sieved twice.

Animal

Wistar male rats (*Rattus norvegicus)* (150 – 300 g) used for this experiment were obtained from the animal house of *Wistar Farm*, Malang, Indonesia. Wistar rats were divided into five groups, each group consisting of 6 rats. The animals were placed in standard cages, kept under constant conditions (temperature 25 ± 2°C and humidity 60 ± 5%), reared with pellet feed and water provided *ad libitum* on a 12-hour light-dark cycle. All experimental protocols have been approved by the Ethical Committee of Faculty of Medicine, University of Jember, No. 1295/H25.1.11/KE/2019.

Treatment of experimental animals

The animal was adapted for seven days at the Physiology and Pharmacology Laboratory of the Faculty of Medicine, University of Jember. A total of 30 rats were divided into five groups, K_n group as normal control was given normal saline, $K_{(-)}$ as negative control was given normal saline, followed by 40 mg/kg diazinon administration. The soy flour supplementation group consisted of K_1, K_2, and K_3, which were given soy flour concentrations of 10%, 15%, and 20%, respectively, followed by 40 mg/kg diazinon. Normal saline and soy flour were given for 28 days, and diazinon was given for five days. After 33 days of treatment, the rats were sacrificed under diethyl ether vapor. Furthermore, ventral surgery of the rat's body was performed to take samples of the liver and blood from the cardiac puncture, and these blood samples should be used immediately. The liver tissue was then fixed in 10% formalin solution.

Estimation of MDA

Measurement of MDA levels in rat liver used Thiobarbituric Acid (TBA) reagents and measured by the UV-Vis spectrophotometry method.[11,12] The liver was cut into small pieces and then washed with Phosphate-Buffered Saline (PBS) solution. One gram of liver is crushed in a mortar on an ice block then added to 0.9% cold NaCl. The mixture was then centrifuged at 8000 rpm for 20 minutes, and the supernatant part was transferred to a microtube. A total of 100 µL of liver supernatant were added with 550 µL of sterile distilled water, 100 µL of TCA (Tri Chloro Acetate), 250 µL of 1 M HCl, and 100 µL of Na-Thiobarbiturate. For every addition of 2 reagents, the solution was homogeneous using a vortex. The homogenates were heated in a water bath at 100°C for 20 minutes, removed, and maintained at room temperature. Furthermore, centrifugation was carried out at a speed of 500 rpm for 10 minutes. The supernatant portion of the centrifugation was transferred into a new micro-tube. The absorbance of the samples was measured at a maximum wavelength of 533 nm. Standard curves were prepared using MDA stock kits with concentrations of 0, 1, 2, 3, 4, 5, 6, 7 and 8 µg/mL plus 550 µL sterile distilled water, 100 µL TCA, 250 µL HCl 1 M, and 100 µL Na-Thiobarbiturat. For each addition of 2 reagents was homogeneous using vortex. The homogenates were heated in a water bath at 100°C for 20 minutes, removed, and maintained at room temperature. Furthermore, centrifugation was carried out at a speed of 500 rpm for 10 minutes, then the supernatant part of the centrifugation results was transferred to a new microtube. The absorbance of the samples was measured at a maximum wavelength of 533 nm. The absorbance value of the sample will be entered into the standard curve formula, and the final MDA value will be generated in units of nmol/dL.

Histopathology evaluation

Histopathological preparations were carried out at the Pathology Anatomy Laboratory, Faculty of Medicine, University of Jember used a light microscope. Observations were carried out based on the modified Roenigk classification to assess the histopathological changes of the liver (Table 1).[13] The histopathological preparations of the rat liver were observed under a 400x magnification light microscope with the zigzag method to observe the entire field of view in one preparation. Researchers used a double-blind system, and observations were made by at least two people. The assessment with the modified Roenigk classification has several conditions, including the preparations seen under a light microscope with a 400x magnification in 5 fields of view. Each visual counted 20 hepatocyte cells, and each cell was assessed for its damage, then the mean score of hepatocyte damage was calculated from the five visual fields.

Table 1: Assessment classification of liver histopathology

No	Damage rate	Score
1	Normal	1
2	Parenchymal degenerations	2
3	Degenerations of hydropic	3
4	Necrosis	4

GSH level

GSH levels were measured by the Ellman method based on its reaction with dTNB (5-5 'dithion bis-2-nitrobenzoic acid). GSH levels were measured by mixing 0.5 mL of 5% TCA solution and 0.5 mL of plasma to precipitate protein, and the mixture was centrifuged at 3000 rpm for 20 minutes. A total of 0.1 ml of the centrifuged supernatant was added with 1 ml of phosphate buffer (pH 8) and 0.5 dTNB (39.6 mg in 100 mL of 1% sodium citrate solution until a concentration of 1 mM was obtained), then left for 1 hour. The mixture was then examined using a spectrophotometer at a wavelength of 412 nm to determine the plasma GSH levels.[12]

AST and ALT level

AST and ALT levels were determined using blood specimens. Rat blood was left at room temperature and centrifuged for 15 minutes at 3500 rpm to obtain serum preparation. AST and ALT levels were measured using the *Diasys* reagent kit, the measurement method used was the International Federation of Clinical Chemistry (IFCC) method using *Biolyzer* 100. The reagent kit was maintained at 37°C, 1000 µL of reagent 1 was added to the test tube containing 100 µl of serum and homogenized. The mixture was incubated at 37°C for 5 minutes, and 250 µl of reagent 2 was added, and then homogenized. The mixture was then read using a biolyzer at a wavelength of 340 nm. The examination results were obtained in U/L units.

Statistical analysis

Data obtained from each examination were analyzed using the SPSS 16 application for Windows application using the One Way Anova different test. The analysis was continued with the Post Hoc Least Significant Difference (LSD) test to determine significant differences between groups. The p-value < 0.05 indicated the significance of the data.

Results and Discussion

Liver tissue was observed on gross anatomy and histopathology preparation. The gross anatomy result from the normal control group showed a normal structure (Figure 1A, 2A). The effects of diazinon on the treated rats' liver tissue are presented in Fig 1B-E, 2B-E. The diazinon-treated rats showed steatosis (in rough anatomy) and many histopathological changes, including parenchymal generation, hyponic

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

degeneration, and necrosis. Table 2 shows that diazinon induction causes an increase in the rate of liver cell damage histopathologically, an increase in AST and ALT levels, MDA level, and a decrease in GSH levels. Those results were significant compare to the group given only normal saline ($p < 0.05$).

The normality and homogeneity tests showed that the data were normally distributed, and the data variance was homogeneous ($p > 0.05$) at each examination. There was a significant decrease ($p < 0.05$) levels of liver biomarker enzymes (MDA, AST, ALT) in the experimental rats of 15% and 20% soy flour groups (K_2 and K_3) compared to the negative control group ($K_{(-)}$). The administration of soy flour to all treatment groups (K_1, K_2, and K_3) caused a significant increase in GSH levels ($p < 0.05$). The histopathology score of the rats liver treated with soy flour (K_1, K_2, K_3) showed a significant reduction in pathological features compared to the diazinon-induced group ($K_{(-)}$). The administration of soy flour in this study showed its potential to protect liver tissue at 15% and 20% concentrations. The biochemical changes are consistent with histopathological observations suggesting a significant hepatoprotective potential. Diazinon toxicokinetics includes the process of absorption, distribution, metabolism, and excretion from the body. Oral induced diazinon will rapidly be absorbed into the digestive tract. Diazinon that enters the body orally will then be metabolized in liver tissue mediated by CYP450. The metabolic process produces active metabolite products from diazinon, namely diazoxon, which is more toxic than diazinon itself.[3,14] This metabolite is a pro-oxidant that inhibits the AChE enzyme, resulting in the accumulation of the neurotransmitter acetylcholine (ACh). This resulted in the influx of intracellular Ca^{2+} resulting in increased formation of nitric oxide (NO), which is a radical compound. This ROS induces lipid peroxidation and oxidative stress.[14,15,16] Then will destroy the mitochondrial membrane and lead to the release of cytochrome c into the cytoplasm. Cytochrome c then interacts with Apoptotic Protease Activating Factor-1 (APAF-1), procaspase-9, and Adenosine Triphosphate (ATP) to form apoptosomes which cause activation of caspases-9 and caspases-3. Activated caspases-3 works by digesting structural proteins, deactivating enzymes for cell replication, and breaking the structures of chromosomal DNA into the nucleosome nuclei.[16,17,18]

Liver damage due to oral induction of diazinon can be evaluated through biochemical examination and tissue histopathology. The result of histopathological structure observation showed a significantly higher score in the $K_{(-)}$ group compared to the K_n and K_1, K_2, K_3 groups. The results showed the level of liver tissue damage in group $K_{(-)}$ due to diazinon induction characterized by sinusoidal dilatation, parenchymal degeneration, hydropic degeneration, and necrosis. This structural change is caused by its reactive metabolite, free radicals diazoxon, which causes structural damage and disruption of cell membrane function, even cell death.[1,3,19] The results of this study are in line with previous researches showing a change in liver histopathology, such as sinusoid dilatation, narrowed central veins, parenchymal degeneration, hydropic degeneration, fatty degeneration, and necrosis.[1,20]

Sinusoid dilatation in this study is in line with studies conducted by Al-Attar (2014) and Marzano *et al*. (2015), which occurs due to pressure on the cell walls due to containment of the veins by toxic substances.[20,21] Parenchymous degeneration that occurred in this study was characterized by swelling and cytoplasmic opacification. This damage results in the oxidation of the cells so that the damaged cells cannot eliminate water so that eventually, water is buried in the cells. Hydropic degeneration occurs due to the hydration of sodium ions due to impaired cell wall permeability due to the toxicity of reactive diazinon metabolites. This result is similar to previous studies, showed that the occurrence of hydropic degeneration is due to disruption of energy metabolism in cells, especially the active transport mechanism in Na^+/K^+-ATPase. Therefore hepatocytes are unable to pump out sodium ions of the cell, so most of the part cytoplasmic organelles have water-filled pockets.[6,17] Severe steatosis forms fatty vacuoles inside the cell, pushing the cell nucleus towards the edges. Steatosis can occur due to disruption of fat metabolisms, such as impaired mitochondria function, hypoxia which inhibits the oxidation of fatty acids that enter the cell. If fatty degeneration continues, hepatocytes may become necrotic.[1,6,20]

Aminotransferase enzymes (AST and ALT) catalyze the transamination reactions occurring in the mitochondria or cytoplasm. Damage to liver cells and tissue will cause these two enzymes to be released into the bloodstream. Therefore, AST and ALT are indicators to detect liver damage at the cellular level. The mean AST and ALT levels in the $K_{(-)}$ group showed the highest mean compared to all treatment groups, indicating massive liver tissue damage due to diazinon induction. Liver tissue damage due to apoptosis induced by diazoxon causes the enzymes in it (mitochondria or cytoplasm) such as AST and ALT to be released into the bloodstream. Our result is consistent with a previous study by Al-Attar et al. (2014), which stated that diazinon induction causes increased levels of ALP, AST, ALT, Gamma-Glutamyl Transferase (GGT), total bilirubin, creatinine, and MDA, as well as changes in the histopathological structure of the liver.[3,20] Other biochemical tests, including MDA and GSH, are biochemical indicators commonly used to identify tissue damage due to oxidative stress. Oxidative stress results from an imbalance of natural pro-oxidant and antioxidant agents in the body. In the event of production of pro-oxidant agents such as ROS and Reactive Nitrogen Species (RNS), the body's natural antioxidant system, such as GSH will try to stabilize these agents from causing damage. The accumulation of ROS compounds causes the depletion of the body's natural antioxidants, which is indicated by decreased levels of GSH.[3,5,](22) The results of the biochemical examination of GSH levels in rats showed the lowest mean GSH levels were in the $K_{(-)}$ group compared to the K_n and K_1, K_2, K_3 groups. The decrease in the average GSH level in the $K_{(-)}$ group indicates the depletion of natural antioxidants in the body due to an increase in ROS compounds.[1,5,22]

The natural depletion of antioxidants in the body causes an unstable buildup of ROS, which can potentially damage tissue. ROS is a compound that tends to oxidize other compounds that do not have ion pairs. ROS compounds will oxidize PUFA to form fat peroxidation and cause oxidative stress and tissue damage. The by-product of PUFA degradation by the ROS compound is produced in the form of MDA. If ROS accumulation occurs due to depletion of the body's natural antioxidants, MDA levels will increase.[1,23,24] The results of examining MDA levels in this study showed that there was an increase in the mean MDA levels in the $K_{(-)}$ group compared to the K_n and K_1, K_2, K_3 groups. This shows that the massive accumulation of ROS will increase PUFA degradation and then increase the by-products (MDA). Physiologically, the body's natural antioxidant can stabilize the formation of ROS compounds.[24] Massive increase in ROS compounds in the body can cause depletion of antioxidants (such as GSH), leading to ROS accumulation, then oxidizing PUFAs and forming LPO, which causes oxidative stress. The by-product of PUFA degeneration is MDA, which is a mutagenic compound in the body. Increased levels of MDA can cause fragmentation and lead to tissue fragility. Increased ROS then induces apoptosis by increasing Bax protein activity, increasing the Bax/Bcl-2 ratio. Apoptosis is caused by degeneration of DNA structure due to caspase-3 activation. Tissue fragmentation and fragility due to increased MDA and cell death due to apoptosis can cause hepatocellular damage, which then manifests as increased AST and ALT levels and histopathological changes.[16,23,25] The results of the histopathological structure observations in the K_1, K_2, and K_3 groups showed a lower average score than in the $K_{(-)}$ group. These results indicate the hepatocellular mechanism of protecting soy flour content. This is supported by the results of liver biochemical examinations in this study, including AST, ALT, MDA, and GSH, which showed a decrease in the average level in the soy flour treatment groups. The average of AST and ALT levels in the K_1, K_2, and K_3 groups showed a lower number than the $K_{(-)}$ group with the lowest mean in the K_3 group, which was given the highest concentration of soy flour (20%). The examination of GSH levels in the soy flour group (K_1, K_2, and K_3) showed an increase in the average GSH levels. It shows that giving soy flour before diazinon induction can maintain GSH levels in the body so that antioxidant depletion does not occur and oxidative stress can be prevented. It is supported by the MDA biochemical test results,

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

which showed a decrease in the average MDA levels in the K_1, K_2, and K_3 groups.

The results of histopathological and biochemical examinations of this study showed that soy flour had a protective effect. The difference in the mean increase or decrease in observations indicates synergy between the large doses given, with the resulting protective effect. Soy flour which contains isoflavones, especially genistein and daidzein, can act as a scavenger against free radical agents.[25,26] Daidzein and genistein act as a hydrogen atom transfer (HAT) through hydrogen atom donors, and as a single electron transfer (SET) through electron transfer to reduce metal ions, radicals, and carbonyl. The role of these compounds in counteracting free radicals is through preventing the initiation of fat peroxidation through its reaction with radical lipids. In addition, these compounds work by converting hydroxyl and peroxyl radical compounds into flavonoid radicals that are more stable.[7,27] Isoflavones in soy flour also work as anti-apoptosis by inhibiting Bax protein activation and reducing caspase-3 activity, which functions to destroy DNA in the apoptotic mechanism.[17,26,28]

The observations in this study are consistent with previous studies, which reported that soy flour has effectiveness as a hepatoprotective agent. It is indicated by increased levels of GSH, decreased levels of MDA, AST, ALT, ALP, and decreased SOD and catalase activity.[10,25,27,29,] Current suggests that soy flour may play an important role in cell protection from increased peroxidative injuries by diazinon. Further research is needed to find the most suitable form of soy flour for consumption, which has a similar hepatoprotective effect.

Table 2: Effect of soy flour administration on rats-diazinon induces histopathologic and biochemical alteration

Treatment groups	Histopathology score	GSH (µmol/L)	MDA (µg/mL)	AST (U/L)	ALT (U/L)
K_n	1.11 ± 0.05^{a}	5.48 ± 0.32^{a}	6.35 ± 0.76^{a}	81.76 ± 4.99^{a}	41.59 ± 1.52^{a}
$K_{(-)}$	3.13 ± 0.11^{b}	2.41 ± 0.19^{b}	9.23 ± 1.35^{b}	126.27 ± 9.16^{b}	55.40 ± 2.01^{b}
K_1	2.75 ± 0.13^{c}	3.23 ± 0.21^{c}	$8.85 \pm 0.33^{c,b}$	106.11 ± 5.13^{c}	50.52 ± 2.25^{c}
K_2	2.34 ± 0.09^{d}	3.72 ± 0.21^{d}	$7.88 \pm 0.61^{d,c}$	92.28 ± 4.56^{d}	45.72 ± 1.83^{d}
K_3	2.07 ± 0.03^{e}	4.10 ± 0.33^{e}	$7.04 \pm 0.59^{a,d}$	82.85 ± 2.96^{a}	$43.86 \pm 2.05^{d,a}$

Data presented as mean ± SD, n = 6, K_n: normal control; $K_{(-)}$: negative control; K_1: 10% soy flour; K_2: 15% soy flour; K_3: 20% soy flour, significant value at $p < 0.05$ compared to $K_{(-)}$.

The values with the same superscript in the same column are not significantly different. Histopathology scores and GSH levels were significantly different in all groups. MDA levels of K_n, K_2, K_3 decreased significantly, AST and ALT levels of K_n, K_1, K_2, K_3 decreased significantly, compared to the $K_{(-)}$.

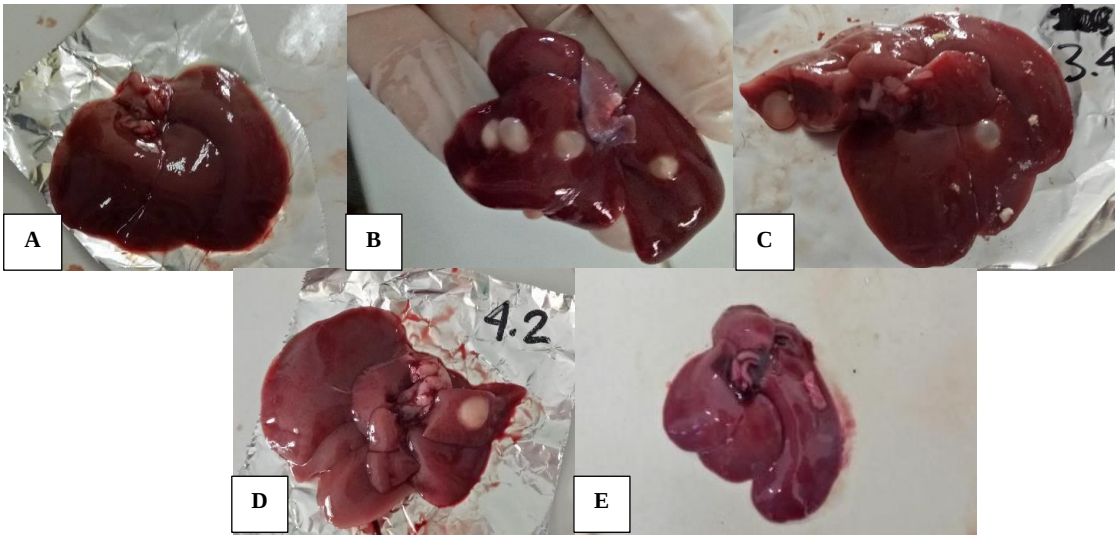

Figure 1: Macroanatomy structure of liver in each experimental group

(A) showing the normal structure of liver tissue, rats treated with normal saline only (K_n); **(B-E)** showing various steatocytes macroanatomically, which Fig. 1B showing more steatocytes than the other groups, rats treated with normal saline followed by diazinon ($K_{(-)}$). **(C)**, **(D)**, **(E)**: rats treated with 10% , 15%, 20% concentrations of soy flour, respectively, followed by diazinon (K_1, K_2 and K_3).

Conclusion

The results of this study suggest that soy flour may play an essential role in hepatocellular protection from increased peroxidative injuries by diazinon. Therefore, this study concludes that soy flour has a beneficial effect in reducing the histopathological and biochemical changes induced by diazinon in male Wistar rats.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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