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HEPATOPROTECTIVE EFFECTS OF ETHANOLIC EXTRACT OF *TRIDAX PROCUMBENS* LINN. AGAINST PARACETAMOL-INDUCED HEPATOTOXIC RATS BY INHIBITING INFLAMMATORY RESPONSE

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ABSTRACT

Paracetamol (PCM) overdose has been associated with hepatotoxicity. Medicinal plants such as T*ridax procumbens L.* (TP) may be used to relief PCM hepatotoxicity. This study evaluated the hepatoprotective effects of TP against PCM-induced hepatotoxic rats by inhibiting inflammatory response. Twenty-four rats were used for this study, randomly distributed into six groups with four rats per group: Group A received no treatment; Group B received 300 mg/kg PCM; Group C received 50 mg/kg Silymarin; Groups D, E and F received 100, 200 and 400 mg/kg of TP respectively for 8 days. Groups C to F were treated with 300 mg/kg PCM on 9th and 10th day. The live body weight of all the rats were taken at 11th days. Blood samples were collected into plain sample bottles from all rats. Rats were sacrificed and livers were harvested, weighed and later soaked in formalin for histopathological and immunohistochemical analysis. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analyzed using Randox kits. Immunohistochemical techniques were used to determine Cytochrome P450 (CYT P450) and Tumour necrotic factor-α (TNF-α) antigen–antibody reactions in the hepatocytes of rat. Data were statistically analyzed with graph pad prism 5.0 using Tukey post doc test. There were decreased live body and live body-liver weight ratio, AST, ALT and ALP of rats treated with 200 and 400 mg/kg TP compared with rats treated with PCM. Reduced necrosis and inflammatory infiltrate were observed in liver of TP-treated groups. Immunohistochemical analysis showed reduced expression of inflammatory markers with CYT P450 and TNF-α in liver tissues of TP-treated rats, thus establishing the fact that TP may have hepatoprotective effects. It can therefore be inferred that TP can be incorporated into herbal preparation as a hepatoprotective medicinal plant for livestock production.

Keywords: Hepatotoxicity, Inflammation, Paracetamol-induced, Rats, *Tridax procumbens.*

INTRODUCTION

Paracetamol (PCM) also known as acetaminophen, is a common drug sold without physician and or veterinary prescription across the counter in most drug stores in the developing countries (Afful *et al.,* 2022). It is a non-opioid analgesic and antipyretic agent used to treat fever, as well as mild to moder-

ate pain (Saragiotto *et al.*, 2019). There are adverse effects in the misuse of paracetamol or overdose which is associated with liver function abnormalities. Paracetamol toxicity can cause abdominal pain and nausea which have adverse effects on the gastrointestinal tract (Conaghan *et al.*, 2019). Paracetamol overdose has adverse effects on liver and kidney in which it develops acute liver failure (Moore *et al.,* 2016). Paracetamol is metabolized in the liver and excreted as reactive intermediates in the blood as APAP- glucoronides, in bile as APAP-sulphate and in urine as APAP- cysteine. These reactive metabolites can be neutralized by conjugation with glutathione (Saragiotto *et al.*, 2019). The covalent binding of N-acetyl-P-benzoquinoneimine as an oxidative product of paracetamol to sulphydryl groups of protein results in lipid peroxidation with degradation of glutathione and causing cell necrosis in the liver (Gulati *et al.*, 2018). The abuse of PCM is a common practice in livestock in most developing countries which invariably can cause liver damage in animals. This act of PCM abuse in livestock is done with a good fate of improving efficiency of livestock production in developing nations like Kenya, Gambia, and Nigeria (Afful, *et al.,* 2022). Abuse of PCM can lead to residual effects in animals and cause public health danger to man when such livestock are consumed. Mistreatment with PCM is a regular practice in animals (livestock and small animals), thereby causing unpleasant side effects at high doses in animals. This invariably causes severe liver toxicity and eventually death, with or without veterinary intervention, depending on the time of presentation (Faryal *et al.,* 2022).

Liver damage can be confirmed by evaluation of liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (Schmidt *et al.,* 2002). The increased levels of AST in blood, indicating liver damage and also ALT which catalyzes the conversion of alanine to pyruvate and glutamate are more specific for detecting liver cell function and liver damage. Hepatotoxicity can arise in liver due to abnormality in the drug metabolism and excretion mechanism (Gulati *et al.*, 2018). Hepatotoxicity can be caused by administration of paracetamol which produces necrosis of centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm followed by excessive hepatic lesions (Gulati *et al.*, 2018). Cytochrome P450 enzymes are members of family of heme-containing proteins found in all organisms. In mammals, about one-third is found in the liver. Cytochrome P450 enzymes play crucial role in the metabolism of drugs and xenobiotics in the liver (Guengerich, 2005). In the case of paracetamol, the CYP2E1 isoform is primarily responsible for its metabolism, leading to the generation of toxic N-acetyl-p-benzoquinone (NAPQI) imine (Rotundo and Pyrsopoulos, 2020). The NAPQI can overwhelm the antioxidant defense mechanisms of the liver, contributing to inflammation and hepatotoxicity (Rotundo and Pyrsopoulos, 2020). Cytochrome P450 reactions include hydroxylation, dealkylation, epoxidation, heteroatom oxygenation and dealkylation, group migration, oxidation of olefins and acetylenes, and heme inactivation (Montellano *et al.,* 2019). Cytochrome P450s may deactivate a drug to increase its clearance from the body, as is the case with heteroatom dealkylation reaction with caffeine. Summarily, Cytochrome P450 is always released for xenobiotic reactions in the body, especially when there is need to establish hemostasis with reductions of reactive oxygen

species (ROS) in the body. The activities of Cytochrome P450 enzymes can be measured by immunohistochemistry (Reiger *et al.*, 2015). Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine in disease pathogenesis such as liver injury, which governs development of the immune system, cell survival signaling pathways, proliferation, and regulates metabolic processes (Lai *et al.,* 2019). TNF-α is important for liver regeneration and tissue repair following acetaminophen (APAP)-induced hepatotoxicity (Chiu *et al.,* 2003). It has been established that TNF-α could be either protective, as in host defense, or deleterious, as in autoimmunity or toxic shock. Thus TNF-α has dual function in liver injury, either aggravating or alleviating injury, which presents a challenge for designing treatments to prevent liver injury (Grivennikov *et al.,* 2005).

Studies have shown that liver injury induced by different causes, such as viruses, bacteria, etc., has relatively specific inflammatory microenvironment characteristics, which could induce rapid immune response, infiltration of inflammatory cells, and production of inflammatory factors in the liver, thus destroying the immune balance in the liver and inducing a series of liver pathological processes. However, some drugs and some chemicals (APAP, CCl_4, etc.) can induce hepatic injury, which starts with hepatocytes (Krenkel *et al.,* 2014). Tumour Necrotic Factor-α can lead to multiple pathways of inflammation, proliferation and apoptosis (Aly *et al.*, 2020). As a result of liver damage and liver inflammation, the inflammatory cells and the hepatocyte itself release cytokines, such as TNF-α and reactive oxygen species (ROS), which can lead to peroxidation of plasma and mitochondrial membranes, causing cell death due to necrosis or apoptosis (Del campo *et al.*, 2018).

Coat buttons or Tridax daisy (*Tridax procumbens)*, is a species of flowering plant in the family *Asteraceae.* It is a native of tropical America and naturalized in tropical Africa, Asia, and Australia (Bhagat and Kondawar, 2019). *Tridax procumbens* has been strongly proved for its medicinal and analgesic activities in animal studies with the protective actions due to the presence of flavonoids and sterols (Vinod *et al.,* 2013*). Tridax procumbens* has been commonly used for pharmacological purposes, due to the presence of myriad of phytochemical properties. These include analgesic, anticoagulant, antileishmanial, antioxidants, anticancer, immunomodulatory agent, insecticidal, anthelmintic cardiovascular, antiseptic, antimicrobial, and insecticidal properties (Varsharani *et al.*, 2022). This study evaluated the hepatoprotective effects of ethanolic extract of *T. procumbens* on paracetamol-induced hepatotoxic male Wistar rats using biochemical, histopathology and immunohistochemistry to analyze the inflammatory progression. The objectives of this study were to determine the anti-inflammatory effects of *Tridax procumbens* by analyzing with Cytochrome p450 and Tumour Necrotic factor- α (TNF-α) antibodies using PCM-induced hepatotoxic male Wistar rats that were treated and not treated with ethanolic extract of *Tridax procumbens.*

MATERIALS AND METHODS

Ethical Approval

The animal procedures were performed in accordance with the recommendations of the guide for the care and use of Laboratory Animals as approved by the Ethical Committee of College of Veterinary Medicine (COLVET), Federal University of Agriculture, Abeokuta (FUNAAB). The ethical approval for this study was secured with the ethical number FUNAAB/ COLVET/ CREC/2024/05/03.

Collection and Authentication of Plant

Tridax procumbens was harvested in its natural habitat in Abeokuta, Ogun State, Nigeria and was deposited at Herbarium Unit, Department of Botany, University of Ibadan, Ibadan where it was authenticated with the voucher number UIH 22443.

Plant Extraction

The harvested plant was rinsed with water, air-dried leaves were separated and grinded to increase surface area and improve efficiency. The grinded sample (10.46 g) was soaked in 100% ethanol concentration for 72 hours. The solution was separated with filter paper and stored in amber bottle. A rotary evaporator was used to separate the ethanol from the extract during concentration process. The resulting extract had a yield of 75.72% which weighed 7.92g. The ethanolic extract of *T. procumbens* was stored in refrigerator at 4^{o}C until needed for use, as described by Abubakar and Haque (2020).

Experimental Animals Management

A total of twenty-four (24) apparently healthy male Wistar rats were purchased from the Department of Veterinary Pharmacology and Toxicology, COLVET, FUNAAB. The rats were housed in individual metal cages in animal house of Department of Veterinary Physiology and Biochemistry, COLVET, FUNAAB. The rats were acclimatized for two weeks to ensure uniform weight. The rats were fed with rats' feed (Grower mash) and served with water *ad libitum*. The experimental rats were thereafter randomly distributed into six (6) groups with each group having four rats (n=4) with the following treatment regimen:

Group A were given 1.0 ml/kg of normal saline *per os* for 10 days

Group B were given 300 mg/kg/bw of PCM, intramuscularly on 9th – 10th day of the study.

Group C were given 50 mg/kg/bw Silymarin orally for 8 days and later treated with PCM at 300 mg/kg/bw on 9th – 10th days.

Group D were given I00 mg/kg/bw *T. procumbens* (TP) extract for 8 days and received 300 mg/kg/bw PCM on 9th – 10th days.

Group E were given 200 mg/kg/bw TP for 8 days and later treated with 300 mg/kg/bw PCM at the 9th – 10th day.

Group F were given 400 mg/kg/bw TP for 7 days and later treated with 300 mg/kg/bw PCM at the 9th – 10th day. This was done according to the study of Sucheta *et al.* (2023) modified.

Sample Collection

The rats were weighed before collecting blood samples at the end of the experiment on the 11th day through retro-orbital puncture into plain sample bottle for biochemical analysis to evaluate liver function enzymes (AST, ALT and ALP). The rats were humanely sacrificed, dissected and livers were harvested from all the rats. The harvested livers were rinsed with normal saline, weighed and soaked in 10% buffered formalin for histopathological analysis.

Laboratory Analyses

Assessment of biochemical parameters: AST and ALT were done by colorimetric method of Reitman and Frankel (1957); estimation of blood ALP was also carried out using the method of Reitman and Frankel (1957). These were done according to Randox manufacturer kits guide. These procedures were done in the Laboratory of Department of Veterinary Physiology and Biochemistry, COLVET, FUNAAB. The liver histopathology and immunohistochemistry analyses were done in the Laboratory of Department of Veterinary Pathology, COL-

VET, FUNAAB. The immunohistochemistry assays were done using MACH 43, Rabbit AP polymer detection and Warp Red chromogen kits according to the manufacturer (Thermo Fischer^{R}) procedure. Cytochrome p450 was done with the dilution rate 1:200 while TNF-α was done with the dilution rate of 1:100.

Data Analysis

The data generated were presented in tabular form and expressed as mean ± Standard Error of Mean (SEM). The results obtained were statistically analyzed by one way analysis of variance (ANOVA) with Graph pad Instat software version 5.0 California USA. The differences in mean were considered statistically different at $P<0.05$ using Tukey multiple comparison postdoc test.

RESULTS

Comparison of live body weight, liver weight and ratio of live body-liver weight of PCM- induced hepatotoxic male Wistar rats treated and not treated with ethanolic extract of Tridax procumbens

The live body weight of Wistar rats treated with 200 mg/kg of TP was significantly higher compared to body weight of Wistar rats treated with normal saline. The body weight of Wistar rats treated with 400 mg/kg of TP was significantly lowered when compared with body weight of rats treated with PCM (Table 1).

The liver weight of rats treated with 200 mg/kg and 400 mg/kg TP were significantly lowered when compared with the liver weight of rats treated with PCM (Table 1). It was observed that there were significant increases in the live body weight - liver weight ratio of rats treated with PCM compared with 200 mg/kg and 400 mg/kg TP- treated rats (Table 1).

Table 1: Comparison of body weight, liver weight, body weight and liver ratio weights of Paracetmol - induced hepatotoxic male Wistar rats treated and not treated with ethanolic extract of *Tridax procumbens*.

GROUP	Live Body weight (g)	Liver weight (g)	Ratio of Body/ liver weight
A: NS	145.5±14.50	6.34±0.79	22.87±0.58
B: PCM	186.3±13.48 [a]	6.60±1.04	28.78±1.99[a]
C: SLY	140.5±28.50 [b]	6.68±0.76	21.02±1.90
D: 100 mg/kg of TP	131.5±7.86 [b]	4.77±0.41 [a]	27.79±1.11 [a]
E: 200 mg/kg of TP	153.5±3.97 [a b]	5.18±0.31[a b]	29.84±1.10 [a]
F: 400 mg/kg of TP	144.8±8.11 [a]	4.91±0.33 [a]	29.33±2.48[a]

Result of serum chemistry of Paracetamol - induced hepatotoxic male Wistar rats treated and not treated with ethanolic extract of TP

The AST values of Wistar rats treated with 100, 200 and 400 mg/kg TP (15.75±1.31; 17.50±1.56; 20.00±2.65iu/L respectively) were significantly lowered than the AST value (27.40±0.51 iu/L) of rats treated with PCM (Table 2).

Considering the ALT, it was observed that the ALT values of Wistar rats treated with 200 and 400 mg/kg TP (23.25±0.95; 40.33±1.45 iu/L respectively) were significantly lowered than the ALT value (44.80±1.39 iu/L) of rats treated with PCM (Table 2).

Table 2: Result of serum chemistry of Paracetamol- induced hepatotoxic male Wistar rats treated and not treated with ethanolic extract of TP

GROUPS	AST (iu/L)	ALT (iu/L)	ALP (iu/L)
A: NS	10.00±2.00	14.00±1.00	32.50±0.50
B: PCM	27.40±0.51[a]	44.80±1.39[a]	47.00±0.89 [a]
C: SLY	7.50±0.50 [b]	10.50±0.50 [b]	32.50±0.50 [b]
D: 100mg/kg of TP	15.75±1.38 [b c]	19.50±0.65[b c]	37.00±0.91 [b]
E: 200mg/kg of TP	17.50±1.56 [b c]	23.25±0.95 [a b c]	42.50±1.56 [a b c]
F: 400mg/kg of TP	20.00±2.65[a c]	40.33±1.45 [a c]	41.00±0.58 [a c]

NS=Normal saline; PCM= Paracetamol; SLY= Silymarin; TP= *Tridax procumbens*
Mean ± SEM Mean ± SEM= mean and standard error of mean; a = significantly difference compared with NS; b = significantly difference compared with PCM; c = significantly difference compared with SLY.

Histopathology of Paracetamol- induced hepatotoxic Wistar rats liver treated and not treated with Tridax procumbens

The section of Wistar rat liver treated with PCM, showed mild inflammatory infiltrations of the periportal area (arrowed a) and hydropic degeneration of the hepatocytes (arrowed b), (Plate 1).

The section of Wistar rat liver treated with SLY, showed focal area of (arrowed) mild inflammatory infiltration of the hepatocytes (Plate 1).

The section of Wistar rat liver treated with 100 mg/kg of TP, showed hydropic degeneration of the hepatocytes, (Plate 1).

The section of Wistar rat liver treated with 200 mg/kg TP, showed mild inflammatory infiltrations around the blood vessel (arrowed), Plate 1.

The section of Wistar rat liver treated with 400 mg/kg TP, showed inflammatory infiltrations of the portal area and focal infiltration of the hepatocytes (Plate 1).

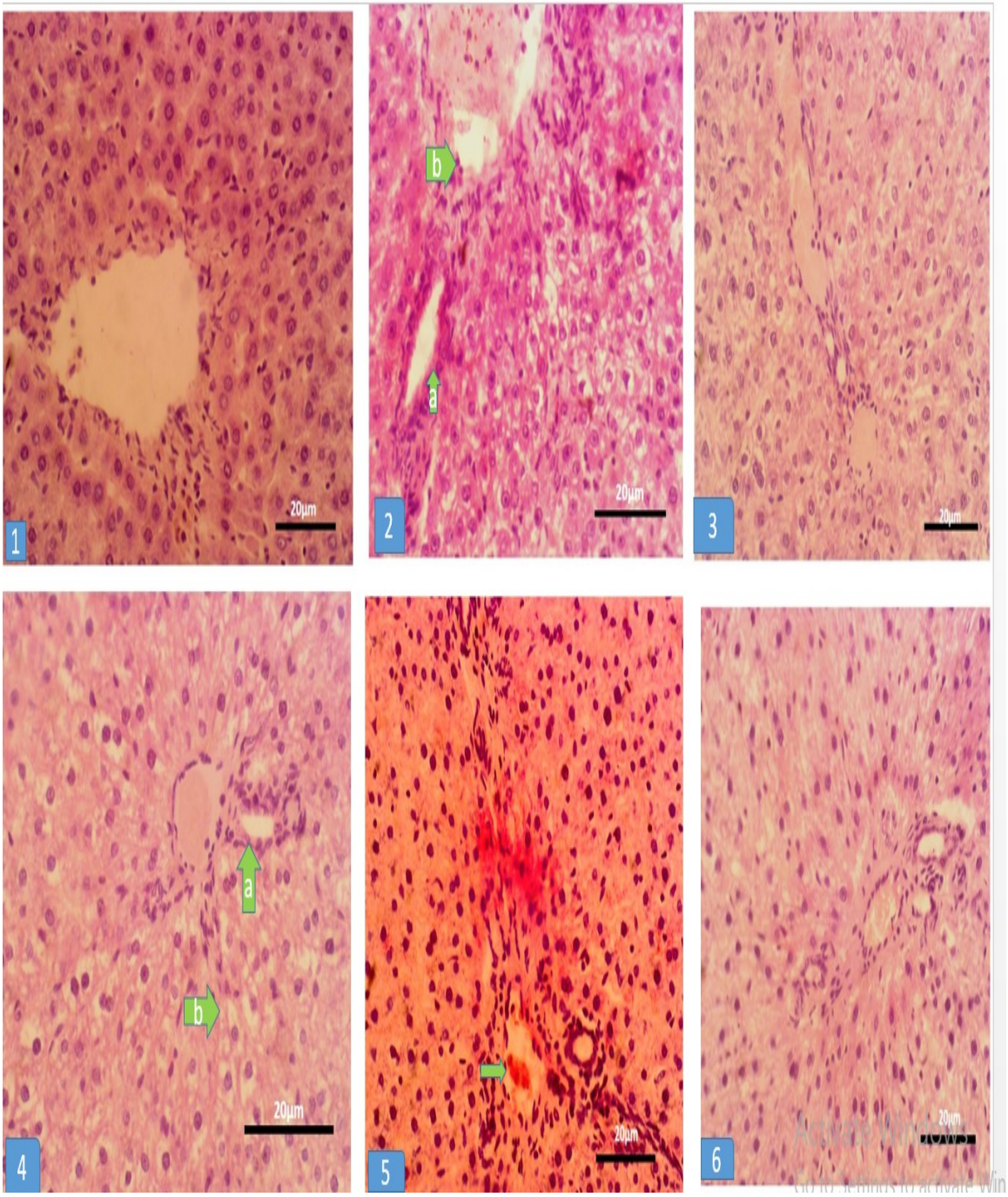

Plate 1: Photomicrographs with H& E Stain Section of Rat livers treated with: 1. Normal saline; 2. Paracetamol; 3. Silymarin; 4. 100 mg/kg TP, 5. 200 mg/kg TP; 6. 400 mg/kg TP.

TP= *Tridax procumbens*

Effects of Cyptochrome P450 antibody and antigen reactions with Tridax procumbens on Paracetamol-induced hepatotoxic Wistar rats

The immunohistochemistry image of paraffin-embedded liver tissue using Cytochrome P450 antibody at dilution rate of 1:200, with Warp red Chromogen. The red arrow indicated (b) represents antigen-antibody reactions. The reaction showed severe antigen-antibody expressions in PCM – induced tissue (2) which was not seen in liver tissue of rats treated with normal saline (1) and SLY (3), but mildly antigen- antibody expressions seen in liver tissue treated with 400 mg/kg (6)- Plate 2.

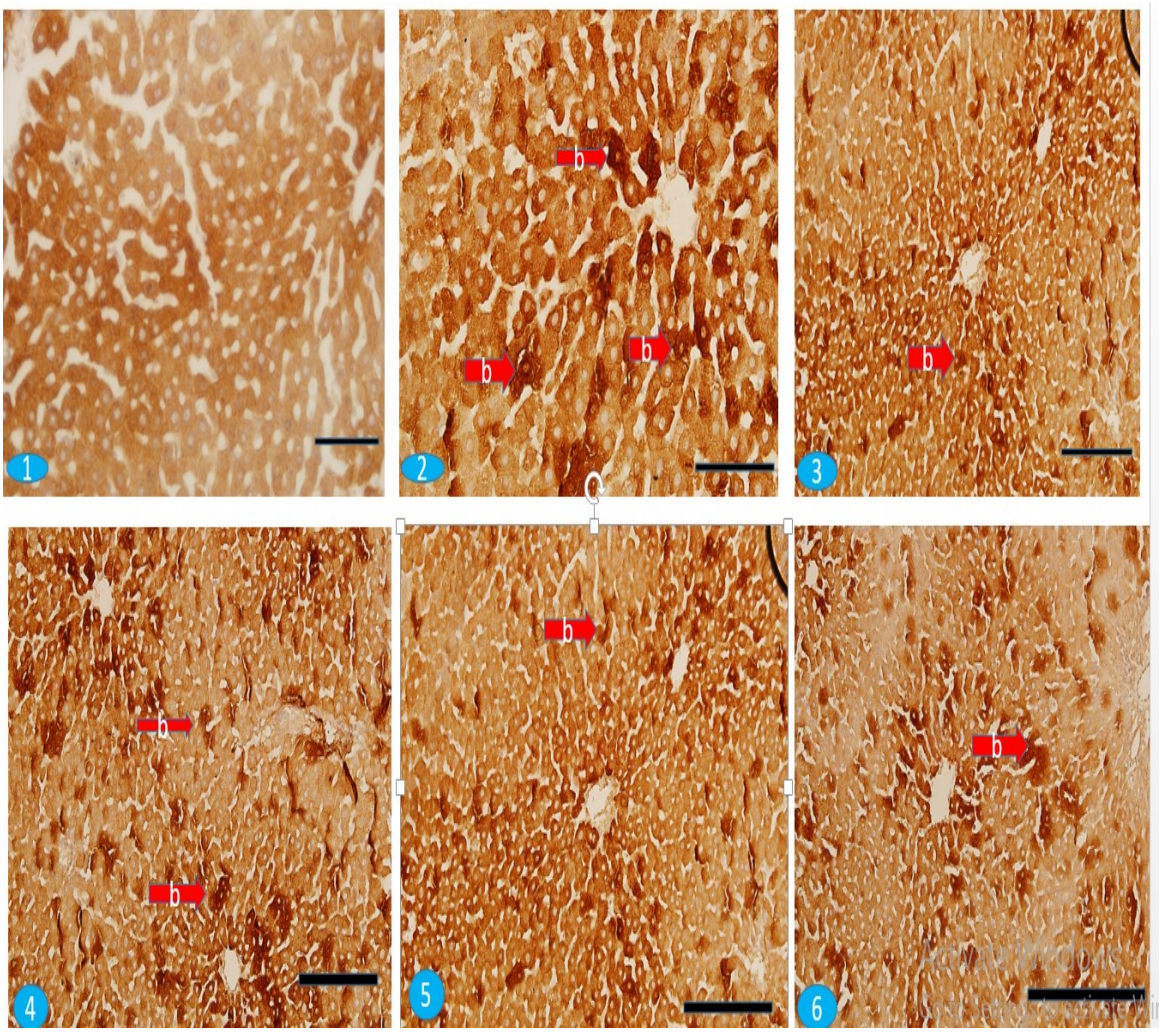

Plate 2: Immunohistochemistry image of liver tissue using Cytochrome P450 antibody at dilution rate of 1:200, Scale bar= 20µm.

Photomicrographs 1-6: Rat hepatocyte treated with NS; PCM; SLY; 100mg/kg TP; 200 mg/kg TP and 400 mg/kg TP respectively.

NS=Normal saline; PCM= Paracetamol; SLY= Silymarin; TP= *Tridax procumbens*.

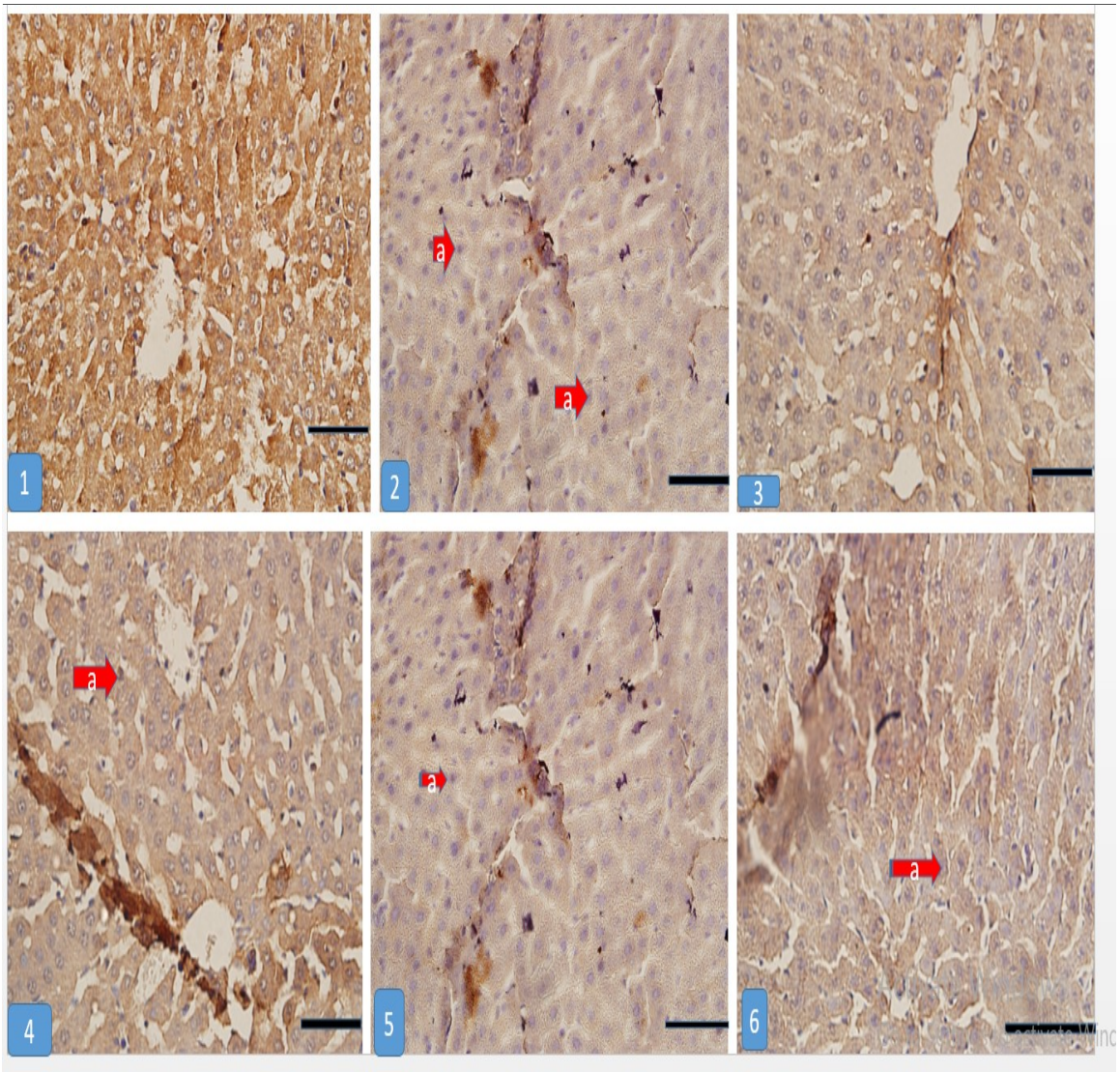

Plate 3: Immunohistochemistry image of rat hepatocytes using TNF-α antibody at dilution rate of 1:200, Scale bar= 20μm.
Photomicrographs 1-6: Rat hepatocyte treated with NS; PCM; SLY; 100mg/kg TP; 200 mg/kg TP and 400 mg/kg TP respectively.
NS=Normal saline; PCM= Paracetamol; SLY= Silymarin; TP= *Tridax procumbens*
The red arrow indicated (a) represents antigen-antibody reactions. The reaction was high in PCM – induced hepatocyte (2) which was not seen in hepatocyte of rats treated with normal saline (1) and SLY (3), but mildly seen in hepatocyte treated with 400 mg/kg (6).

DISCUSSION

The increase in live body weight of rats treated with 200 mg/kg of *T. procumbens* compared with rats treated with normal saline in this study may be due to the antioxidant effects of *T. procumbens*. This is in con-

firmation to the declarations of Varsharani *et al.* (2022) that *T. procumbens* is rich in phytochemicals which may be due to the presence of flavonoids and sterols (Vinod *et al.,* 2013*).* The significant increase of AST seen in rats treated with PCM in this study may be due to the hepatotoxic potentials which were decreased in rats treated with 200 and 400 mg/kg of *T. procumbens*. This supports the assertions in previous studies that *Tridax procumbens* has been commonly used for pharmacological purposes, due to the presence of myriad of phytochemical properties (Varsharani *et al.,* 2022; Sucheta *et al.,* 2023). Similar trends were seen in ALT and ALP values of rats treated with 100 and 200 mg/kg of *T. procumbens* in this study. Thus, establishing the fact that ethanolic extract of *T. procumbens* showed lowered serum levels of ALP, AST and ALT in rats treated with 100 and 200 mg/kg of *T. procumbens* in this study which was also observed in the study of Faryal *et al.,* (2022). This suggests hepatoprotective effects of 100 and 200 mg/kg of *T. procumbens*. The liver toxicity seen in this study in rats treated with PCM may be due to direct toxicity of the primary compound with the reactive metabolite or immunologically-mediated response (Gulati *et al*., 2018), while the hepatoprotective action of *T. procumbens* exhibited may be due to anti-inflammatory and analgesic activity in animal studies with the protective actions which may be due to the presence of flavonoids and sterols found in *T. procumbens* as reported by Vinod *et al.* (2013). The histopathology of rats' liver treated with PCM in this study with mild inflammatory infiltration in the periportal area and hydropic degeneration of the hepatocytes is an indication of hepatotoxic potentials of PCM in this study as seen in the study of Gulati *et al.,* (2018). The section of rats liver treated with ethanolic extract of 200 mg/kg and 400 mg/kg *T. procumbens* that showed mild inflammatory infiltrations around the blood vessels with mild distension of the sinusoids in the hepatocytes and the restoration of the liver architecture may be due to antioxidant and pharmacological effects of *T. procumbens* as shown by Bhagat and Kondawar, (2019). The observed restoration of liver architecture in rats treated with 200 mg/kg and 400 mg/kg *T. procumbens* may be due to hepatoprotective effects of *Tridax procumbens* which is similar to the claims of Gulati *et al*., (2018) and Sucheta *et al.,* (2023). It was observed that the Cytochrome P450 enzymes antigen and antibody reaction was more pronounced in the PCM-treated rats. This may be due to the crucial role of Cytochrome P450 enzymes played in the metabolism of PCM, leading to the generation of toxic N-acetyl-p -benzoquinone imine (NAPQI) (Rotundo and Pyrsopoulos, 2020). The NAPQI can overwhelm the antioxidant defense mechanisms of the liver, contributing to inflammation and hepatotoxicity (Rotundo and Pyrsopoulos, 2020). It was suspected that Cytochrome P450 antigen-antibody reactions were reduced in the 200 and 400 mg/kg doses of *T. procumbens*. This may be due to the situation that Cytochrome P450 antigen-antibody reaction which is always involved in xenobiotic reactions in the body with the target of establishing hemostasis might have reduced reactive oxygen species in the process (Reigner *et al.,* 2013).

The TNF-α antigen- antibody reactions seen in the PCM-treated rats' hepatocytes in this study may be due to multiple pathways of inflammation, proliferation, and apoptosis (Aly *et al*., 2020). while there were mild inflammatory reactions in the TNF-α antigen-antibody reaction of rat hepatocytes treated with 400 mg/kg of TP which may be due to recovery from the inflammatory reactions caused by PCM. This may be due to release

of cytokines by the administration of 400mg/kg of TP to resolved the induced reactive oxygen species generated by the PCM administration (Del campo *et al.,* 2018). These observations seen in 400mg/kg *T. procumbens*-treated rats' hepatocytes may be a confirmation of the anti-inflammatory activity of *T. procumbens* previously shown in animal studies by Vinod *et al*., (2013).

CONCLUSION

This study emphasized the anti-inflammatory responses expressed by reducing inflammatory cytokines (Cyctochrome P450 and TNF-α) in liver of rats treated with different doses of *T. procumbens* compared with the expressions in liver of PCM-treated rats in this study. It can be concluded that 400 mg/kg *T. procumbens* extract may provide potent and safer approach to the treatment of paracetamol-induced hepatotoxicity by inhibiting inflammatory response.

RECOMMEDATION

The knowledge seen from this study reviewed that further study can be explored to understand the mechanism of action of *T. procumbens* in establishing the hepatoprotective potentials from the inflammatory changes. It can also be suggested that *T. procumbens* can be incorporated into herbal preparation as a hepatoprotective medicinal plant for livestock production.

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