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Protective and regenerative effects of the methanol stem bark extract of *Harungana madagascariensis* in combination with ascorbic acid, selenium and allopurinol in acetaminophen-induced toxicities in albino rats

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Abstract
Aim: The protective and regenerative properties of *Harungana madagascariensis* were evaluated in Albino rats because the plant possess medicinal properties and has been used in folkloric for the treatment of several diseases. The effects of the Methanol extract of this plant in combination with Ascorbic acid, Selenium and Allopurinol were evaluated on the liver and kidney because the plant is known to possess phenolic compounds and other phytochemicals that has anti-oxidant and free radical scavenging activities.
Methods: Albino rats (Wistar) were used in this study and were grouped into 21 of five animals per group for both protective and regenerative studies. Acetaminophen at 800mg/kg dose was used to induce hepatotoxicity and nephrotoxicity while different doses of methanol extract in combination with three antioxidants (Ascorbic acid 200mg/kg, Selenium 1mg/kg and Allopurinol 50mg/kg) were used to ameliorate these effects in a 9-day study. Serum Biochemical assays and histopathology were evaluated in the study.
Results: The results from this study showed that Acetaminophen induced-stress on the liver and kidney caused increased in all the liver biomarkers such as the Aspartate transaminase (ALT), Alanine transaminase, alkaline phosphate, creatinine, blood urea, total protein and gamma-glutamyl transferase while the combination of the stem bark extract of *H. madagascariensis* along with ascorbic acid, selenium and allopurinol at different doses caused protective and regenerative effects on the biomarkers and reduced the increased levels of the liver enzymes seen in acetaminophen group. In the acetaminophen group, the histopathology of the liver showed moderate portal congestion with mild periportal cellular infiltration and a moderate to severe portal fibrosis, while the kidney shows severe diffuse tubular degeneration and necrosis. The combination of MHM (500mg/kg) with these antioxidants caused remarkable reversal of the histopathological changes caused by acetaminophen in both organs.
Conclusion: The synergy between MHM and the antioxidants especially Ascorbic acid and Selenium may have increase the efficacy of the plant extract, as it may both protect and treat both organs at lower dose of 200mg/kg and 100mg/kg when combine with this two antioxidants in the case of liver or kidney damage induced by acetaminophen as indicated in this work.

Keywords: *Harungana madagascariensis* extracts; hepatoprotection, anti-oxidants, histopathology, regenerative

Introduction
Liver is a vital organ in the body that is responsible for a large number duties that are vital for the normal body functioning (Poynard *et al*., 2003) [27] such as carbohydrate metabolism, proteins and fatty acid metabolism, production of clotting factors, it is also involve in vitamin storage (Lavoie *et al.*, 2005) [17]. Damage to the liver can be as a result of intake of alcohol, smoking, inhalation of harmful substances and intake of drugs either at normal dosage or at overdose (Oluwadunni *et al.*, 2019; Zakim *et al*., 2002) [26, 33].
Drug-induced liver toxicity is always be the cause of liver injury and this result in about one-half of the cases of acute liver failure and also mimics other forms of acute and chronic liver disease (Kaplowitz 2004) [16]. Some therapeutic agents, administered at large doses or even within therapeutic ranges, can injure the liver (Olaleye *et al*., 2014) [25]. Some chemical agents that are used in laboratories, industries, natural chemicals like microcystins and herbal remedies may also induce hepatotoxicity (Abdel- Misih *et al.*, 2010) [1].
Acetaminophen is metabolically activated by cytochrome P450 enzymes to a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) that depletes glutathione (GSH) and

covalently binds to protein. It was shown that repletion of GSH prevented the toxicity (Mitchell *et al.* 1973), and this led to the development of N-acetylcysteine used as antidote (Lee *et al.*, 2005) [18]. Hepatotoxicity of acetaminophen was observed in rodents treated with large doses of acetaminophen (Boyd and Bereczky 1966) [9], where the rats were not as sensitive to the hepatotoxicity as both mice and hamsters (Davis *et al.* 1974) [34].

Antioxidants are agents, which can protect the liver against damage by interacting with and neutralizing free radicals, thereby preventing them from causing damage (Machala *et al.*, 2001) [20]. The body synthesis some of the antioxidants which are endogenous antioxidants like catalase, superoxide dismutase and Glutathione systems. External sources of some other antioxidants that the body needs is mainly from the diet (Diplock *et al.*, 1998, Bouayed *et al.*, 2010) [13, 8] and these include: Acetyl cysteine, Allopurinol, Vitamin A, Vitamin C, Vitamin E, Beta-carotene, Chrysin, Lycopene, Selenium, Manganese and Zeaxanthin (Dabelsteien *et al.*, 2007).

Ascorbic acid which is also known as Vitamin C can both act as a strong, efficient, and cheap antioxidant agent and, at the same time, behave as a radical promoter. It is an electron donor and water-soluble antioxidant, and this property accounts for all its known functions (Jose *et al.*, 2013) [15].

Selenium (Se) is an important trace element in humans, and its deficiency has caused serious health effects, such as Keshan disease (Tapiero *et al.*, 2003) [31]. Foods are the major natural source and its levels generally depend on soil Se levels (Suzuki *et al.*, 2005) [30]. It has been discovered as an important component of antioxidant enzymes, such as glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinases (IDD) in the body (Tapiero *et al.*, 2003; Brenneisen *et al.*, 2005) [31, 10].

Allopurinol is an inhibitor of xanthine oxidase enzyme, which is a major source of reactive oxygen species (ROS) that triggers the release of several inflammatory mediators (Maria *et al.*, 2018) [21]. Allopurinol catalyzes the formation of uric acid from xanthine, which is indicated for treatment of chronic gout and for recurrent attacks of acute gout (Wang *et al.*, 2013) [32]. Besides the hypouricemic effect, there are direct and indirect evidences of antioxidant effect of allopurinol. It has been shown to scavenge superoxide anion and hydroxyl radicals in *in vitro* and experimental studies, where it improves endothelial dysfunction caused by oxidative stress in patients of congestive heart failure, type 2 diabetes, and coronary artery disease, and in smokers (Wang *et al.*, 2013; Antonella *et al.*, 2018) [32, 6].

Harungana madagascariensis is a monotypic medium to tall tree species commonly called dragon's blood tree. It is used in folkloric treatment of jaundice, diarrhea, dysentery, typhoid fever, constipation, liver problems, anaemia and malaria, river blindness, ulcer, asthma, hepatitis, dysmenorrhea, and toothache (Moronkola *et al.*, 2015; Oluwadunni *et al.*, 2019) [24, 26]. Several scientific reports validating the folkloric use of the leaves and stem bark of this plant in the treatment of anaemia, diabetes, oxidative stress related diseases, bacterial infections, diarrhoea and gastro-intestinal disorders are documented (Anita *et al.*,2015; Agnaniet *et al.*, 2016) [5, 2]. The phytochemistry of the roots leaves and stems bark of *Harungana madagascariensis* have been investigated extensively and have several bioactive compounds like: anthracene derivatives with anti-plasmodial and antibacterial flavonoids (Machala *et al.*, 2001; Malan *et al.*, 2015) [20, 23]. Documented scientific studies indicate isolation of compounds like anthrones, anthraquinones, xanthones, flavonoids, and essential oils from this plant (Biapa *et al.*, 2013; Moronkola *et al.* 2015; Oluwadunni *et al.* 2019) [7, 24, 26]. The study was therefore carried out to assess the combinative effects of the plant extract along with each of vitamin C, selenium and allopurinol in laboratory animals.

Material and Methods

Plant material

About 2000g of fresh stem bark of *Harugana madagascariensis* was collected from Molete market, Ibadan, Nigeria. The bark was rinsed in distilled water and air-dried completely at room temperature (30 ± 2 ºC), protected from heat and direct sunlight for about 4 weeks. The dried plant stem bark was homogenized to fine powder using Laboratory Hammer mill (Zhen Chang Equipment SFS P66) and solvent to solvent extraction of fine powder of the plant was done using N-hexane, Ethyl acetate and Methanol respectively.

Pilot Toxicity Study

5000mg/kg, 3000mg/kg and 1000mg/kg body weight of the methanol and Ethyl acetate extract was used for acute toxicity test (Lorke, 1983) [19]. The animals were observed for changes in behavior and mortalities for 24hours and a week.

Experimental Animals and their care

A total of 105 Albino rats (Wistar) of both sexes weighing 180-220g were purchased from the Faculty of Veterinary Medicine, University of Ibadan and the study was carried out in Experimental Animal Unit of the Department of Veterinary Pharmacology and Toxicology of the same University. The animals were handled in accordance with international principles guiding the Use and Handling of experimental animals (United States National Institutes for animal Health) after an ethical approval was obtained from the Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan. The approval number is UI-ACUREC/19/0121. The rats were maintained on standard rat feed (Vital Feeds from Grand Cereals Limited, Jos (a subsidiary of UAC Nigeria) and portal of acclimatization and throughout the experimental period. The rats were also maintained at an ambient temperature between 28-30Oc, humidity of 55±5%, and standard (natural) photoperiod of approximately 12 hours of lighting (06:30 hours – 18:30 hours) alternating with approximately 12 hours of darkness (18:30 hours – 06:30 hours).

Acetaminophen induced toxicity in animals

The animals were grouped into 21 with each group having 5 Albino rats for both protective study and regenerative study.

Group 1 received acetaminophen 800mg/kg for the last two days, while Group 2 served as normal control received distill water orally for ten days, Group 3 received Sylimarin 100mg/kg for 8 days before acetaminophen 800mg/kg on day 9, Groups 4-12 are the protective groups; Group 4 received methanol extract 500mg/kg and ascorbic acid 200mg/kg for 8 days before given acetaminophen 800mg/kg on day 9. Group 5 received Methanol extract 200mg/kg and ascorbic acid 200mg/kg for 8days before given acetaminophen 800mg/kg on day 9. Group 6 animals were administered methanol extract 100mg/kg and ascorbic acid (200 mg/kg) for 8 days before given acetaminophen 800mg/kg on day 9; same for selenium 1mg/kg and allopurinol 50mg/kg was given in combination with methanol extract at 500mg/kg, 200mg/kg and 100mg/kg respectively and were in groups 7-12.

Groups 13-21 formed the Hepatoregenerative group

Group 13 animals were administered acetaminophen 800mg/kg on day 1 and 2 before given methanol extract 500mg/kg and ascorbic acid 200mg/kg till day 9, group 14 animals were administered acetaminophen 800mg/kg on day 1 and 2 before given methanol extract 200m g/kg and ascorbic acid 200mg/kg till day 9, group 15 animals were given acetaminophen 800mg/kg on day 1 and 2 before given methanol extract 100mg/kg and ascorbic acid 200mg/kg till day 9. It was the same for selenium 1mg/kg and allopurinol 50mg/kg that were given in combination with methanol extract at 500mg/kg, 200mg/kg and 100mg/kg respectively and were placed in groups 16-21.

Biochemical Analysis

On day 10, animals were anaesthesized with diethyl ether and blood samples were collected from retro-orbital vein into plain bottles for the determination of biochemical parameters such as Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT), Blood urea (BUN), Creatinine (CRT), Total bilirubin (T. Bil) and Total protein (T.PRO). Well-labeled plain bottles were used to collect 10 mL of blood sample, which was allowed to clot for 4 hours before centrifuging using Uniscope Laboratory Centrifuge (Model SM 112, Surgifriend Medicals, England) at 2000 revolution per minute for 15minutes to separate the sera from clotted blood cells. Each serum was carefully separated in the plain bottles that were well label accordingly at room temperature of 23-26 ºC. The activities of AST, ALP and ALT were estimated as described by Reitman and Frankel (1957). The activities of T. Bil were determined by colorimetric method using a kit supplied by Randox test kit (UK). The blood urea was determined using urease-Berthelot (enzymatic) colorimetric method and serum total protein was evaluated based on the Gornall et al. (1949) method.

Gross and histopathology

The organs were identified (kidney and liver), sectioned and rinsed in normal saline. The tissue was fixed in 10% formal-saline, dehydrated with 100% ethanol solution and embedded in paraffin and sectioned to 5µm, stained using H&E method, cleared in xylene and mounted in a mountant (Akanbi & Taiwo 2014) [3].

Results

In the protective group, Methanol extract 500mg/kg and ascorbic acid 200mg/kg combination decreased the level of ALT, CRT and T.BIL ($p<0.05$) and ALP, BUN ($p<0.01$), showing significant different compared to acetaminophen group. Methanol 200mg/kg group in combination with Ascorbic acid, shows decreased in the level of ALP, AST, ALT and T.BIL ($p\geq0.005$) but increased in CRT and GGT when compared with Acetaminophen group as described on table 1. In Methanol extract group 100mg/kg in combination with Ascorbic acid 200mg/kg, there was decreased in the level of AST, ALT and BUN ($p\geq0.01$), ALP (p=0.0001) but there was increased in T.PRO, CRT and GGT comparing both groups with Acetaminophen.

Except for ALP ($p\geq0.0001$) of the 200 and 100 mg/kg groups, there was no significant difference in all the parameters in the 500, 200 and 100 mg/kg doses of the extract when compared with the Sylimarin group (Table 1).

Table 1: Protective Effects of Methanol stem bark extract (500mg/kg, 200mg/kg and 100mg/kg) in combination with Ascorbic Acid (200mg/kg).

Parameters	Groups					
	I	II	III	IV	V	VI
T.P RO	7.47±0.67	6.67±0.15	6.95±0.35	7.13±0.11	7.33±0.50	8.06±0.11
ALB	3.27±0.38	2.9±0.1	2.85±0.21	3.13±0.35	2.83±0.32	2.93±0.05
GLO	4.67±0.78	3.7±0.05	4.1±0.14	4.0±0.45	4.5±0.51	5.13±0.15
A.G ratio	0.7±0.22	0.7±1.36	0.65±0.07	0.76±0.21	0.6±0.1	0.53±0.05
AST	47±1.41	37.3±1.53	41±2.82	45±2	43.33±3.78	44.6±1.5
ALT	35.25±0.95	26±1.00	31±2.82	33.3±3.05[a]	31.66±3.21	31.6±1.15
ALP	127.75±0.5	80±6.08	101.5±6.36	110±13.9[c]	118.33±10.1	110.66±4.04
BUN	19.05±0.19	15.63±0.15	18±0.42	18.2±0.53[c]	18.1±0.45	17.16±0.28
CRT	0.75±0.05	0.533±0.05	0.6±0	0.7±0.1[a]	0.8±1.35	0.7±1.35
T.BIL	0.475±0.05	0.23±0.05	0.35±0.07	0.36±0.05[a]	0.33±0.05	0.3±0
GGT	0.375±0.019	0.23±0.02	0.26±0.02	0.32±0.07	2.76±0.20	2.86±0.05

I-Acetaminophen group; II- Normal group; III-Silymarin 100mg/kg; IV-Methanol extract (500mg/kg) and Ascorbic acid; V-Methanol extract 200mg/kg and Ascorbic acid; VI-Methanol extract 100mg/kg and Ascorbic acid

In the regenerative group as shown in Table 2, the combination of methanol extract 500mg/kg and ascorbic acid 200mg/kg reduced the level of AST, ALT, ALP, T.BIL, and GGT ($p< 0.01$) and BUN ($p< 0.05$) when compared with acetaminophen group and there was only decreased in the level of AST, ALT and T.BIL in the Methanol 200mg/kg and Ascorbic acid combination when compared with Acetaminophen group. Methanol extract 100mg/kg and ascorbic group showed decreased in the level of ALP (p=0.0001), AST, ALT, BUN and T.BIL ($p\geq0.01$) when compared to Acetaminophen group as shown in table 2 above.

There was significant difference in AST, ALT and ALP ($p\geq0.0001$) while there was no significance difference in other parameters when 500mg/kg of Methanol extract in combination with ascorbic acid is compared with Sylimarin group while there was no significant difference in all the parameters except for ALP, AST and ALP ($p\geq0.001$) when 200mg/kg of methanol combination was compared with Sylimarin group and there was significant difference in AST, ALT, ALP in both groups when 100mg/kg of the extract was compared with Sylimarin group ($p\geq0.0001$).

Table 2: Regenerative effect of the combination of Methanol extract of *H. madagascariensis* 500mg/kg, 200mg/kg and 100mg/kg extracts with Ascorbic acid 200mg/kg.

Parameters	Groups					
	I	II	III	IV	V	VI
T.P RO	7.467	6.67±0.15	6.15±0.49	7.03±0.25	7.65±0.07	8.16±0.47
ALB	3.27±0.38	2.9±0.1	2.55±0.07	3.16±0.15	3.15±0.07	3.06±0.23
GLO	4.67±0.78	3.7±0.05	3.6±0.42	3.86±0.11	4.5±0.14	5.1±0.45
A/G ratio	0.7±0.22	0.7±1.36	0.75±0.07	0.76±0.05	0.65±0.07	0.53±0.05
AST	47±1.41	37.3±1.53	35.5±0.07	41.6±0.5	45.5±2.12	43±1
ALT	35.25±0.95	26±1.00	26±1.41	32±1	33.5±0.7	32±0
ALP	127.75±0.5	80±6.08	77.5±2.12	106.3±3.5	127±0	104.66±4.50
BUN	19.05±0.19	15.63±0.15	16.1±0.70	18.5±0.25	18.45±0.21	17.06±0.41
CRT	0.75±0.05	0.533±0.05	0.5±0	0.36±0.05	0.75±0.07	0.66±0.05
T.BIL	0.475±0.05	0.23±0.05	0.15±0.07	0.6±0	0.35±0.07	0.36±0.05
GGT	0.375±0.019	0.23±0.02	0.22±0.02	0.26±0.03	3.15±0.35	2.7±0.28

I-Acetaminophen group; II- Normal group; III- Silymarin 100mg/kg; IV- Methanol extracts 500mg/kg and Ascorbic acid; V- Methanol extracts 200mg/kg and Ascorbic acid; VI-Methanol extract 100mg/kg and Ascorbic acid.

In the protective group (Table 3), the combination of Methanol extract 500mg/kg with Selenium 1mg/kg decreased the level of ALP and BUN ($p<0.01$) and CRT and GGT ($p<0.05$) while there was decreased in the level of AST, ALT, ALP and BUN ($p\geq0.01$) in the 200mg/kg group but increase in CRT and GGT when compared with Acetaminophen group. In the Methanol extract 100mg/kg group with Selenium 1mg/kg, there was decreased in the level of ALP (p=0.0001), AST, ALT, BUN and T.BIL ($p\geq0.01$) and increased in GGT; when compared group with Acetaminophen group as shown in table 3. There was no significant difference in all the parameters of the 500mg/kg, 200mg/kg and 100mg/kg groups except ALP ($p\geq0.0001$) when compared with Sylimarin group as indicated in table 3.

Table 3: Protective effects of Methanol extract (500mg/kg, 200mg/kg and 100mg/kg) in combination with Selenium 1mg/kg.

Parameters	Groups					
	I	II	III	IV	V	VI
T.P RO	7.47±0.67	6.67±0.15	6.95±0.35	7.55±0.07	7.23±0.68	7.6±0.81
ALB	3.27±0.38	2.9±0.1	2.85±0.21	3.0±0.28	2.9±0.1	3±0.4
GLO	4.67±0.78	3.7±0.05	4.1±0.14	4.55±0.21	4.33±0.58	4.6±0.43
A.G ratio	0.7±0.22	0.7±1.36	0.65±0.07	0.6±0.14	0.66±0.05	0.63±0.05
AST	47±1.41	37.3±1.53	41±2.82	46±0	43±6.24	41±2.8
ALT	35.25±0.95	26±1.00	31±2.82	35±0	31.66±3.78	30±3.46
ALP	127.75±0.5	80±6.08	101.5±6.36	117±2.82[c]	122±7.93	108±5.19
BUN	19.05±0.19	15.63±0.15	18±0.42	16.5±0.21[c]	18.43±1.10	16.8±0.87
CRT	0.75±0.05	0.533±0.05	0.6±0	0.65±0.07[a]	0.83±0.23	0.66±0.05
T.BIL	0.475±0.05	0.23±0.05	0.35±0.07	0.45±0.07	0.33±0.05	0.33±0.11
GGT	0.375±0.019	0.23±0.02	0.26±0.02	0.33±0.02[a]	3.06±0.23	2.5±0.47

I-Acetaminophen group; II- Normal group; III-Silymarin 100mg/kg; IV- Methanol extract (500mg/kg) and selenium; V- Methanol extract 200mg/kg and selenium 1mg/kg; VI- Methanol extract 100mg/kg and selenium 100mg/kg

In the combination of Selenium with Methanol extract 500mg/kg group (Table 4), there was reduced in the level of ALP, BUN and GGT ($p<0.01$) and T.BIL ($p<0.05$) but there is no significant difference in all the biochemical parameters in the Methanol extract 200mg/kg group in combination with Selenium when compared with Acetaminophen group. In the Methanol extract 100mg/kg with Selenium 1mg/kg group, there was decreased in AST, ALT, BUN, T.BIL ($p\geq0.01$), ALP level decreased (p=0.0001) and increased in T.PRO, CRT and GGT when compared with Acetaminophen group. There was no significant difference in all the parameters of Methanol extract 500mg/kg combination with Selenium 1mg/kg expect for ALP ($p\geq0.0001$) when compared with Sylimarin group but there was significant difference in AST, ALT and ALP ($p\geq0.0001$) due to their increase when Methanol 200mg/kg combination with Selenium group is compared with Sylimarin group. There was significant difference in AST, ALT because of slight increase and ALP $p\geq0.0001$ when compared with Sylimarin group.

Table 4: Regenerative effects of Methanol extract (500mg/kg, 200mg/kg and 100mg/kg) in Combination with Selenium (1 mg/kg).

Parameters	Groups					
	I	II	III	IV	V	VI
T. PRO	7.47±0.67	6.67±0.15	6.15±0.49	7.2±0.7	8.36±1.43	8.1±0.45
ALB	3.27±0.38	2.9±0.1	2.55±0.07	2.95±0.07	3.56±0.09	3.1±0.3
GLO	4.67±0.78	3.7±0.05	3.6±0.42	4.25±0.77	4.83±0.89	5±0.17
A.G ratio	0.7±0.22	0.7±1.36	0.75±0.07	0.7±0.14	0.7±0.17	0.43±0.28
AST	47±1.41	37.3±1.53	35.5±0.07	45±2.82	48.66±6.65	43±1.7
ALT	35.25±0.95	26±1.00	26±1.41	34±2.82	34±2.64	33.6±1.15
ALP	127.75±0.5	80±6.08	77.5±2.12	113±7.07[b]	123±11.1	111±1.7
BUN	19.05±0.19	15.63±0.15	16.1±0.70	16.5±0.21[c]	19.53±1.7	17.7±0.05
CRT	0.75±0.05	0.533±0.05	0.5±0	0.65±0.07	1±0.34	0.7±1.35

T.BIL	0.475±0.05	0.23±0.05	0.15±0.07	0.45±0.07[a]	0.05±0.46	0.36±0.05
GGT	0.375±0.019	0.23±0.02	0.22±0.02	0.33±0.02[b]	3.86±1.15	2.46±0.25

I-Acetaminophen group; II-Normal group; III-Silymarin 100mg/kg; IV-Methanol extract 500mg/kg and Selenium; V-Methanol extract 200mg/kg and Selenium 1mg/kg; VI-Methanol extract 100mg/kg and Selenium 1mg/kg

In this protective group (Table 5), the combination of Methanol extract 500mg/kg with Allopurinol 50mg/kg decreased the level of ALP and BUN ($p< 0.01$) and GGT ($p< 0.05$) while there is also decreased in the level of AST, ALT, ALP and T.BIL ($p\geq0.001$) and no significant difference in CRT and GGT in Methanol extract 200mg/kg in combination with Allopurinol 50mg/kg when compared with Acetaminophen group, methanol extract 100mg/kg group did not show significant difference in all the biochemical parameters when compared to that of the Acetaminophen group. There was no significant difference in all the parameters of the groups 500mg/kg and 200mg/kg except for ALP ($p\geq0.0001$) when compared with the Sylimarin group but there was significance difference in AST and ALP ($p\geq0.0001$) when compared with Sylimarin group.

Table 5: Protective effects of Methanol Extract 500mg/kg, 200mg/kg and 100mg/kg in combination with Allopurinol (50 mg/kg)

Parameters	Groups					
	I	II	III	IV	V	VI
T.P RO	7.47±0.67	6.67±0.15	6.95±0.35	7.1±0.87	7.56±1	9.06±0.60
ALB	3.27±0.38	2.9±0.1	2.85±0.21	3.06±0.32	3.06±0.3	3.8±0.45
GLO	4.67±0.78	3.7±0.05	4.1±0.14	4.03±0.55	4.5±0.72	5.26±0.20
A.G ratio	0.7±0.22	0.7±1.36	0.65±0.07	0.73±0.057	0.66±0.05	0.66±0.05
AST	47±1.41	37.3±1.53	41±2.82	45±4.35	42.33±5.68	52±6.08
ALT	35.25±0.95	26±1.00	31±2.82	33±4.35	31.66±1.52	36.3±2.08
ALP	127.75±0.5	80±6.08	101.5±6.36	109±4.72[c]	114.33±11.5	128±9.64
BUN	19.05±0.19	15.63±0.15	18±0.42	16.06±0.92[c]	18.7±1.05	18.83±1.66
CRT	0.75±0.05	0.533±0.05	0.6±0	0.63±0.11	0.93±0.32	0.76±0.05
T.BIL	0.475±0.05	0.23±0.05	0.35±0.07	0.4±0.1	0.33±0.15	0.43±0.11
GGT	0.375±0.019	0.23±0.02	0.26±0.02	0.26±0.069[a]	3.46±0.49	3.7±1.02

I-Acetaminophen group; II-Normal group; III- Silymarin 100mg/kg; IV-Methanol extract (500mg/kg) and allopurinol; V-Methanol extract 200mg/kg and allopurinol; VI-Methanol extract100mg/kg and allopurinol

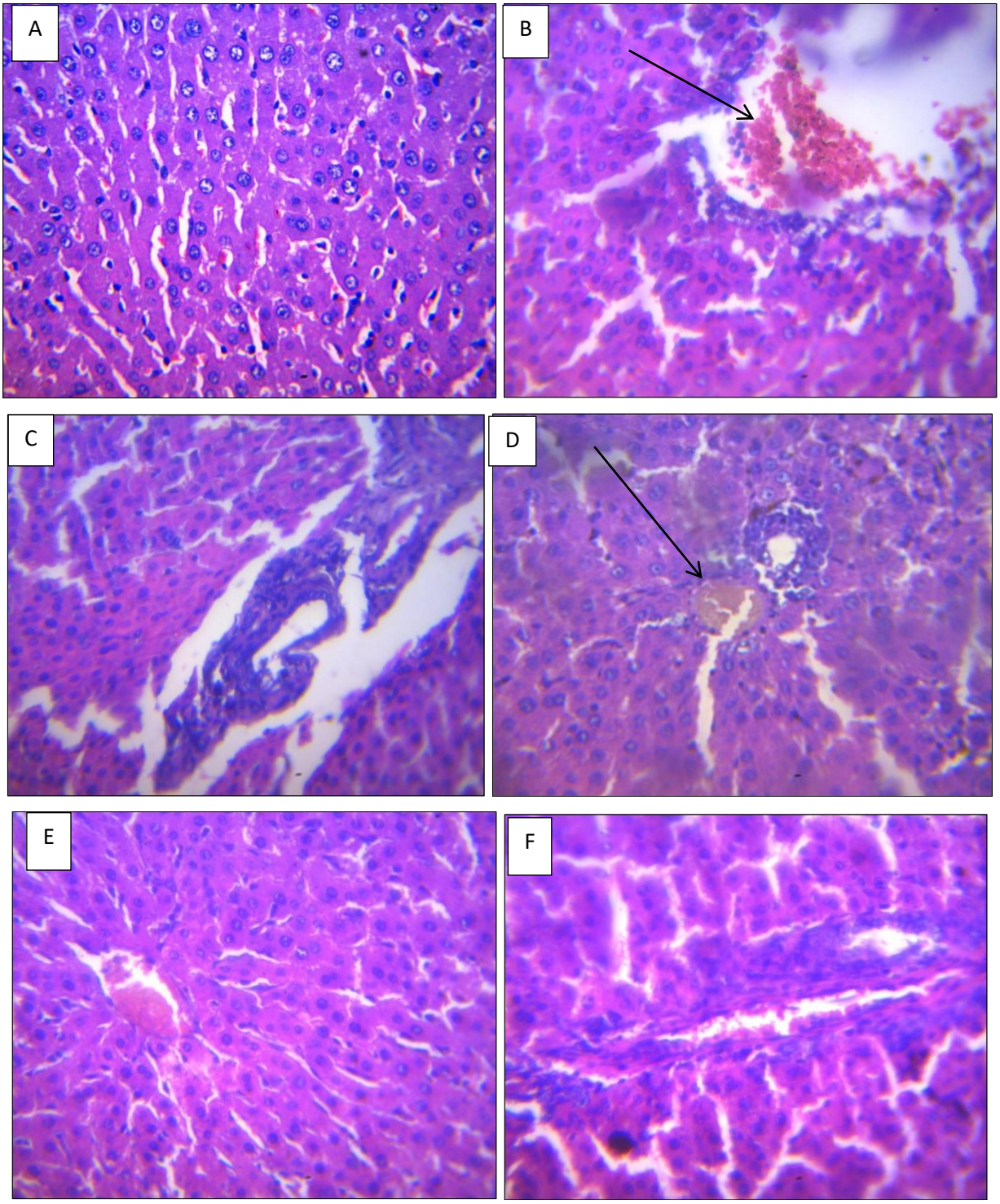
In Table 6, the combination of Methanol extract 500mg/kg with Allopurinol 50mg/kg decreased the level of ALP, BUN, CRT and GGT ($p< 0.01$) and T.BIL ($p< 0.05$) in their comparison with Acetaminophen group but there was no significant difference in all the biochemical parameters of Methanol extracts 200mg/kg and 100mg/kg in combination with Allopurinol 50mg/kg. There was no significant difference in all the parameters of methanol extract 500mg/kg in combination with allopurinol except for ALP ($p\geq0.0001$) when compared with Sylimarin group but there was significant difference in AST, ALT and ALP ($p\geq 0.0001$) in the 200mg/kg and 100mg/kg when compared with Sylimarin group.

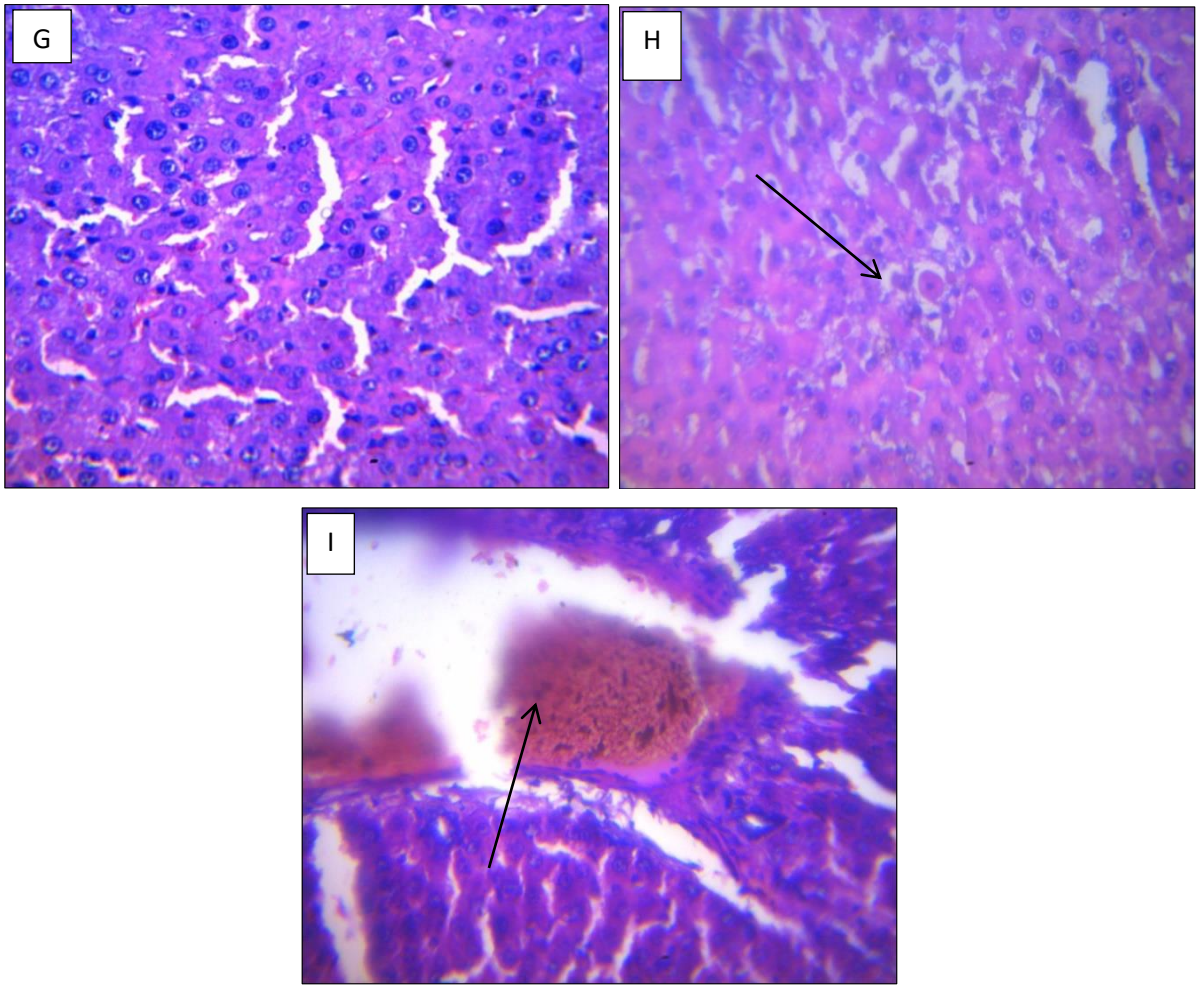
Table 6: Regenerative effects of Methanol extract 500mg/kg, 200mg/kg and 100mg/kg in combination with Allopurinol (50 mg/kg)

Parameters	Groups					
	I	II	III	IV	V	VI
T.P RO	7.47±0.67	6.67±0.15	6.15±0.49	6.86±0.23	8.26±1.1	9.03±0.45
ALB	3.27±0.38	2.9±0.1	2.55±0.07	2.73±0.15	3.7±0.7	3.93±0.20
GLO	4.67±0.78	3.7±0.05	3.6±0.42	4.15±0.25	4.56±0.55	4.76±0.80
A.G ratio	0.7±0.22	0.7±1.36	0.75±0.07	0.67±0.1	0.8±0.1	0.8±0.17
AST	47±1.41	37.3±1.53	35.5±0.07	42.6±3.51	46±5.29	51±4.35
ALT	35.25±0.95	26±1.00	26±1.41	32.66±3.05	34±2.6	36.3±1.52
ALP	127.75±0.5	80±6.08	77.5±2.12	112.3±4.04[c]	122.6±2.3	130±2
BUN	19.05±0.19	15.63±0.15	16.1±0.70	16.06±0.58[c]	19.3±1.02	18.6±1.2
CRT	0.75±0.05	0.533±0.05	0.5±0	0.6±0[b]	1±0.2	0.76±0.05
T.BIL	0.475±0.05	0.23±0.05	0.15±0.07	0.37±0.057[a]	0.53±0.25	0.46±0.05
GGT	0.38±0.019	0.23±0.02	0.22±0.02	0.25±0.02[c]	4.13±0.70	3.9±0.78

I-Acetaminophen group; II-Normal group; III- Silymarin 100mg/kg; IV-Methanol extract 500mg/kg and Allopurinol; V-Methanol extract 200mg/kg and allopurinol; VI-Methanol 100mg/kg and allopurinol

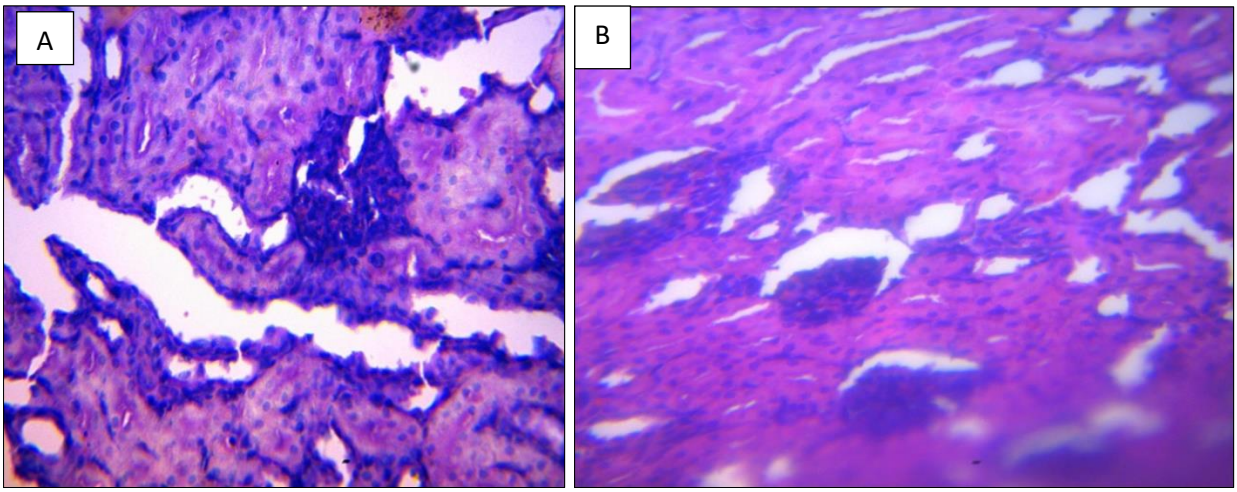
Histolopathological results of the combinations of methanol extract and antioxidants (ascorbic acid, selenium and allopurinol)

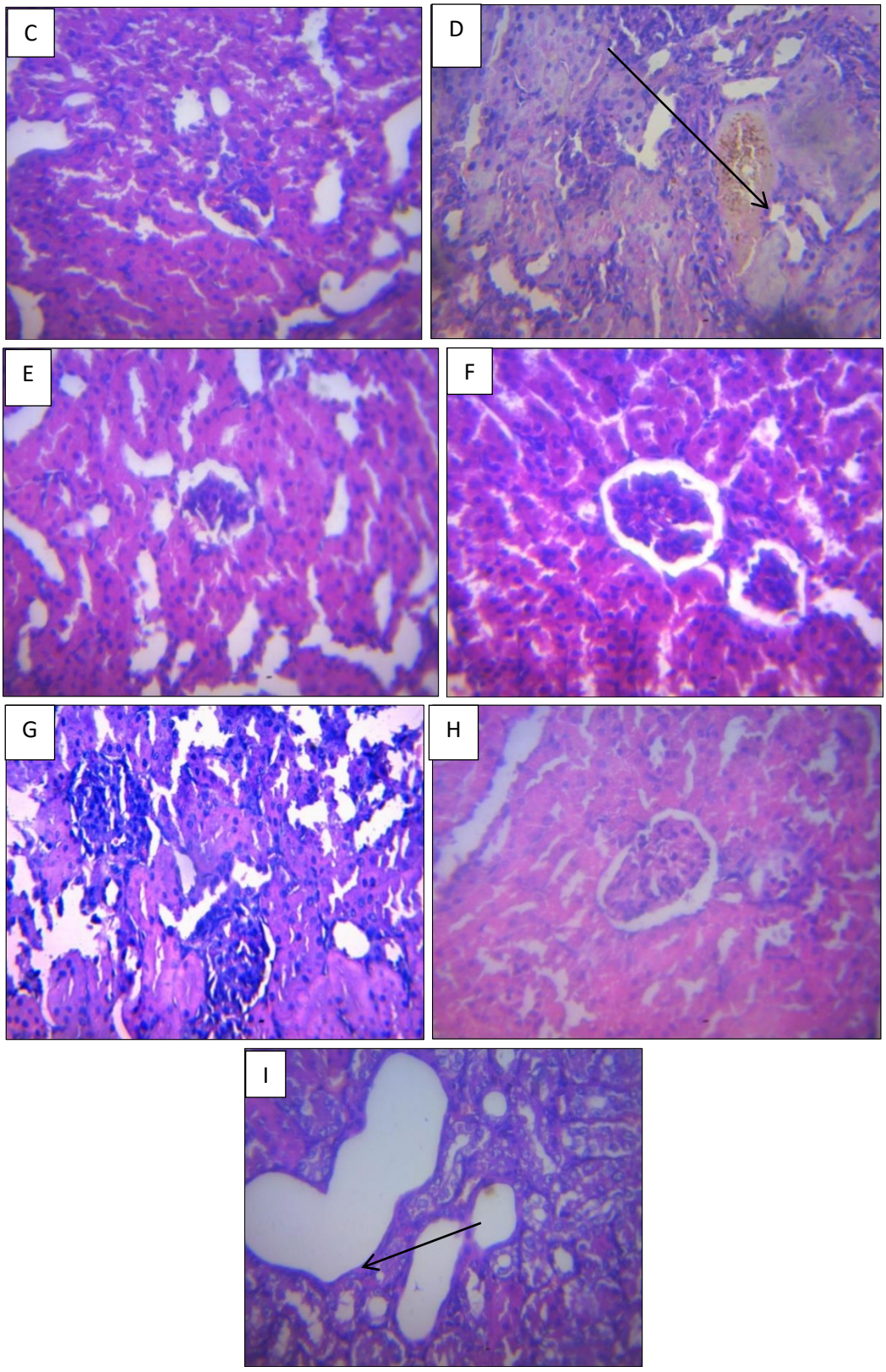




A- Liver of MHA 500mg/kg showing no visible lesion, stained with H&E with magnification of x400
B- Liver of MHA 200mg/kg showing mild portal congestion with periportal cellular infiltration, stained with H&E with magnification of x400.
C- Liver of MHA 100mg/kg showing bile ductules appearing prominent
D- Liver of MHS 500mg/kg showing mild to moderate periportal cellular infiltration
E- Liver of MHS 200mg/kg showing no visible lesion
F- Liver of MHS 100mg/kg showing no visible lesion
G-Liver of MHAL 500mg/kg showing no visible lesion
H- Liver MHAL 200mg/kg showing few hepatocytes are rounded and degenerated
I- Liver of MHAL 100mg/kg showing there is mild congestion of the portal vessel

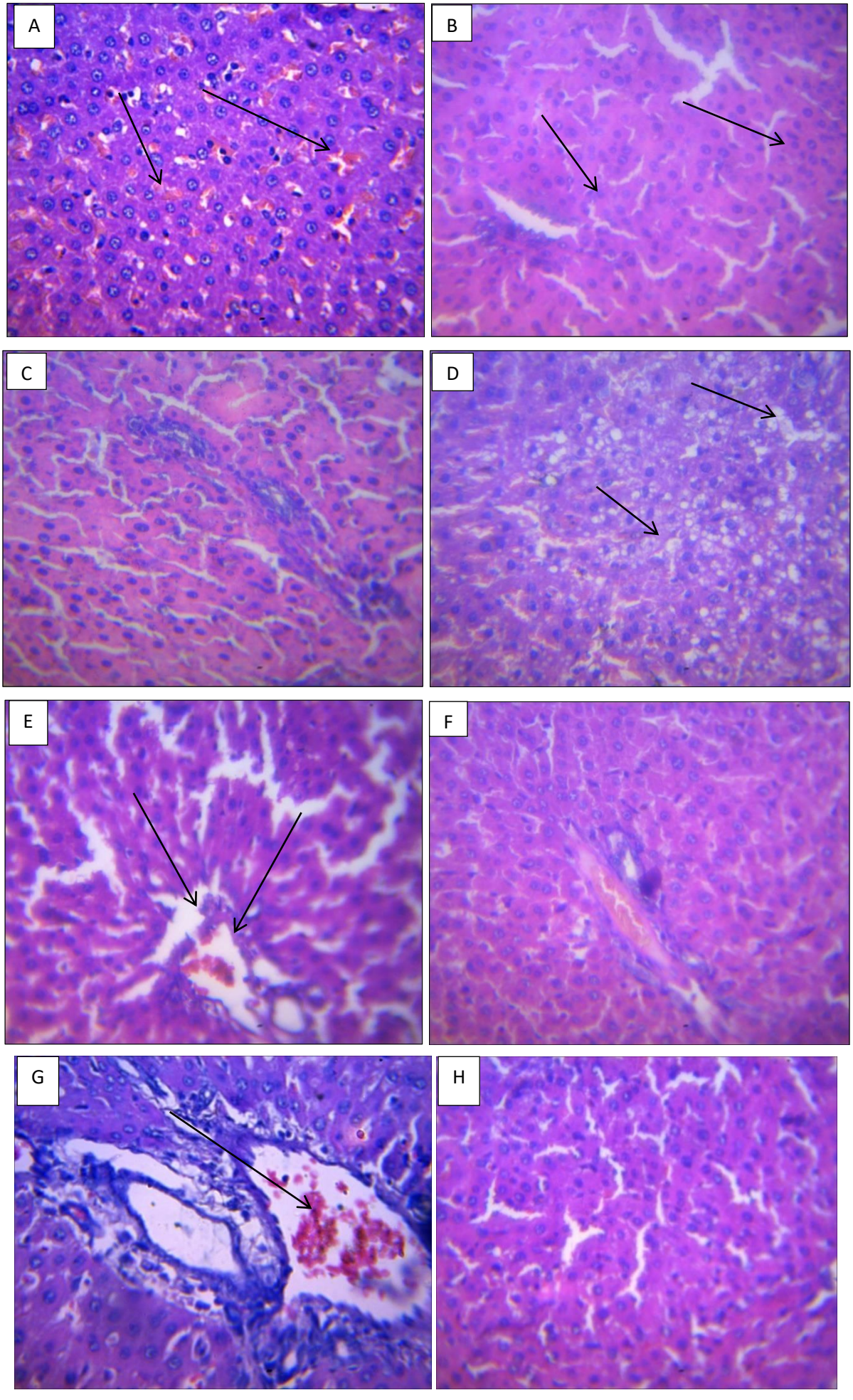
Fig 1: Protective effects of *H. madagascariensis* Methanol extract in combination with Ascorbic acid, Selenium and Allopurinol.





A- Kidney of MHA 500mg/kg show no visible lesion, stained with H&E stained with magnification of x400; B- Kidney of MHA 200mg/kg show there is no visible lesion, stained with H&E stain with magnification of x400; C- Kidney of MHA 100mg/kg showing no visible lesion, stained with H&E with magnification of x400
D- Kidney of MHS 500mg/kg showing very mild interstitial congestion
E- Kidney of MHS 200mg/kg showing no visible lesion
F- Kidney of MHS 100mg/kg showing no visible lesion
G- Kidney of MHAL 500mg/kg showing no visible lesion
H- Kidney of MHAL 200mg/kg showing no visible lesion
I- Kidney of MHAL 100mg/kg showing moderate to severe tubular degeneration and the tubules are greatly expanded, with degenerate epithelium

Fig 2: Protective effects on the kidneys


A
B
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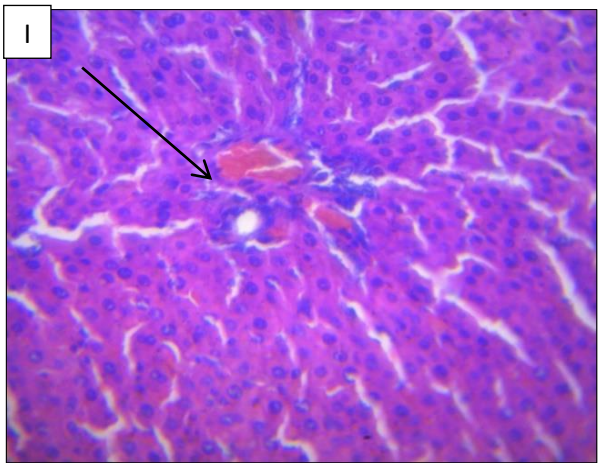
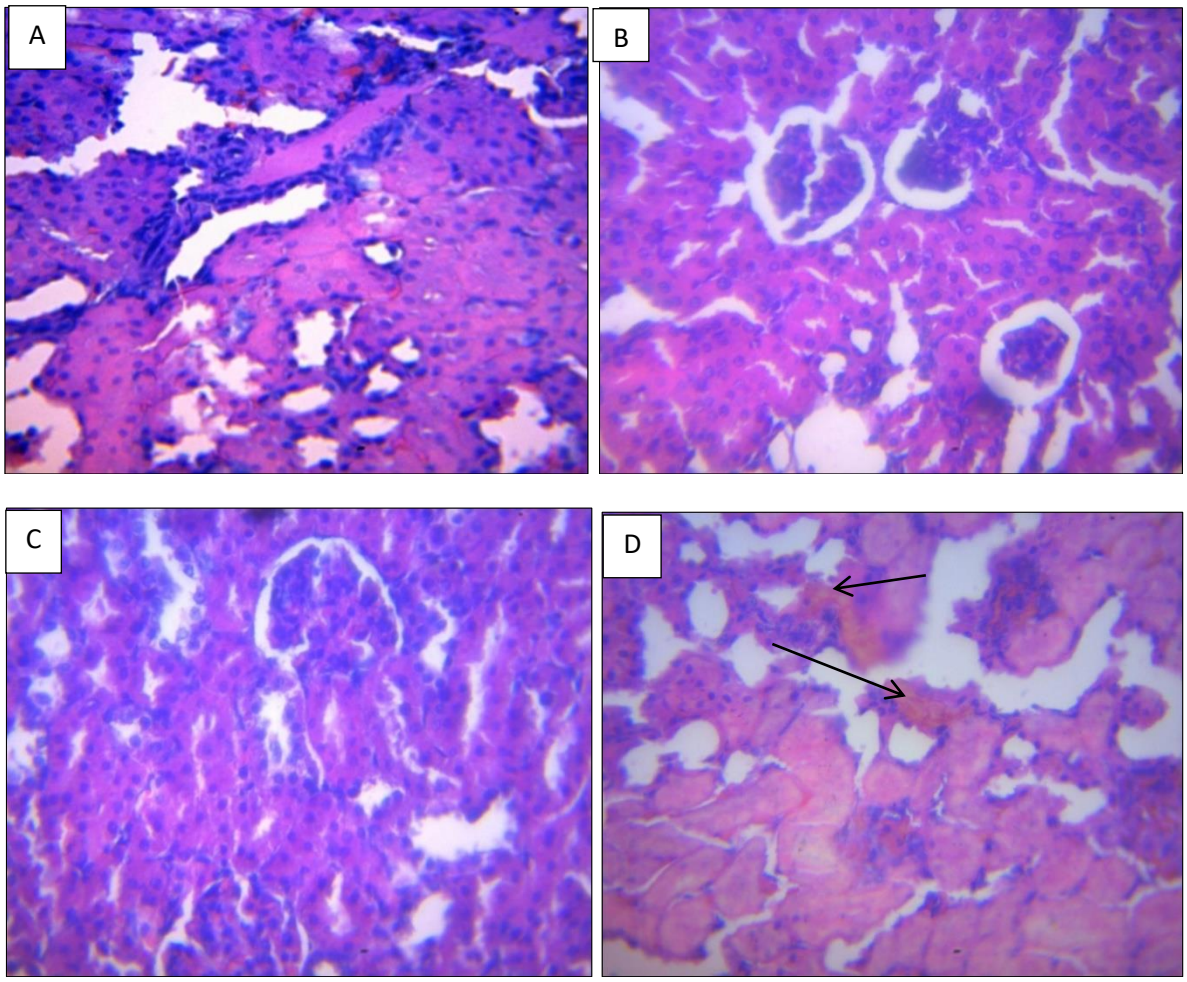
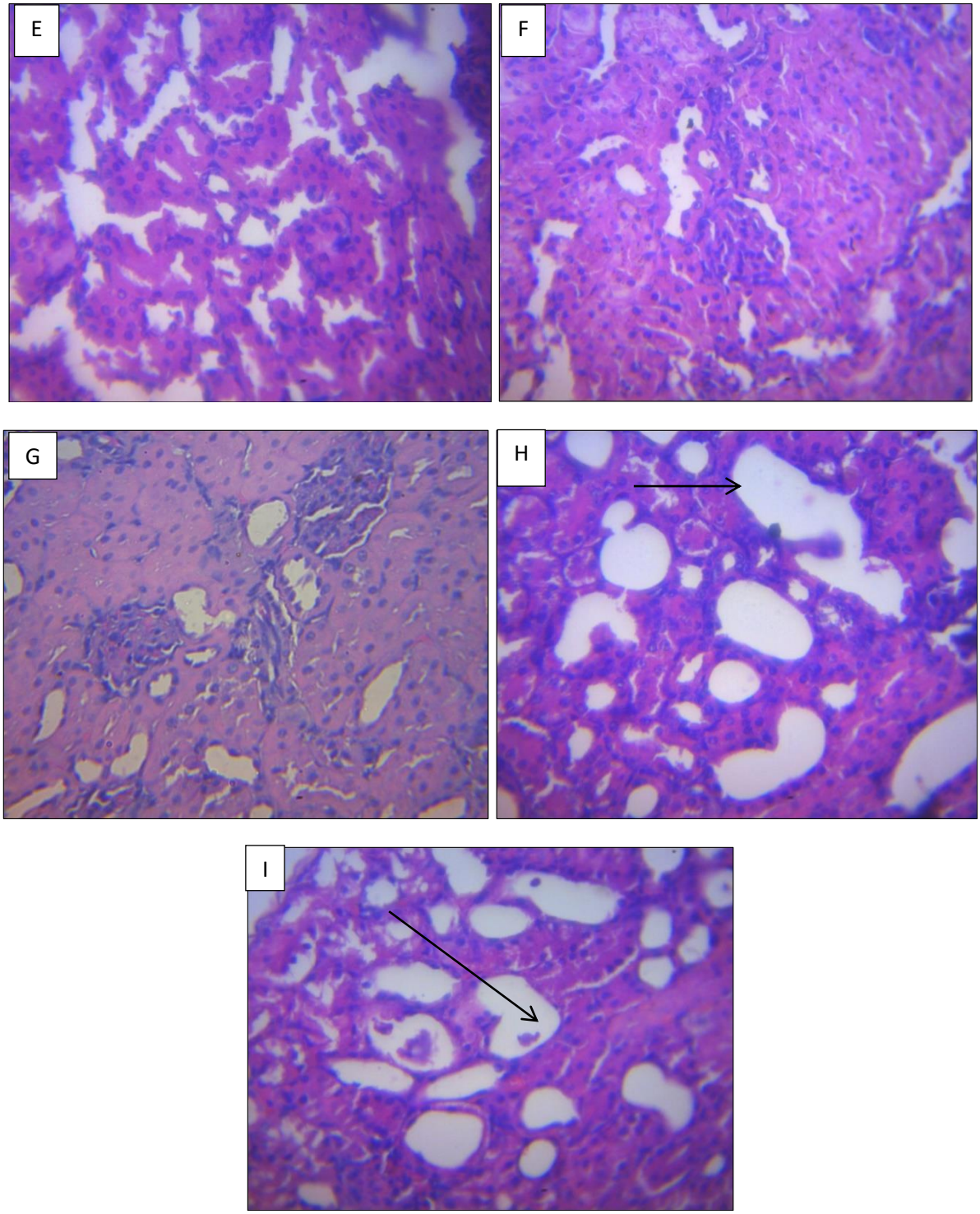


A- Liver of MHA 500mg/kg showing sinusoids appearing prominent with very mild congestion
B- Liver of MHA 200mg/kg showing no visible lesion
C- Liver of MHA100mg/kg showing no visible lesion
D- Liver of MHS 500mg/kg showing a moderate diffuse vacuolar degeneration
E- Liver of MHS 200mg/kg showing hepatocytes appear shrunken degenerated
F- Liver of MHS 100mg/kg showing no observable lesion
G- Liver of MHAL 500mg/kg showing mild to moderate portal and sinusoidal congestion with mild periportal cellular infiltration
H- Liver of MHAL 200mg/kg showing no visible lesion
I- Liver of MHAL showing mild congestion of the portal vessel

Fig 3: Regenerative effects of Methanol extract of *H.magascariensis* 500mg/kg, 200mg/kg, 100mg/kg in combination with the antioxidants on the liver.





A- Kidney of MHA 500mg/kg showing no visible lesion, stained with H&E and magnification of x400
B- Kidney of MHA 200mg/kg showing no visible lesion
C- Kidney of MHA 100mg/kg showing no visible lesion
D- Kidney of MHS 500mg/kg showing mild interstitial congestion
E- Kidney of MHS 200mg/kg showing no visible lesion
F- Kidney of MHS100mg/kg showing no visible lesion
G- Kidney of MHAL 500mg/kg showing no visible lesion
H- Kidney of MHAL 200mg/kg showing many tubules have expanded lumen
I- Kidney of MHAL 100mg/kg showing moderate to severe tubular degeneration with tubules greatly expanded and many tubules have clumps/cast in the expanded Lumina.

Fig 4: Regenerative effect of *H. madagascariensis* Methanol extract in combination with Ascorbic acid, Selenium and Allopurinol on the kidneys

Discussion

This study evaluated the protective effect of Methanol extract of *H. madagascariensis* (MHM) in combination with three antioxidants (Ascorbic acid, Selenium and Allopurinol) on liver and kidney damage induced by acetaminophen. Acetaminophen induced-stress on the liver and kidney caused damage as indicated in table 1 and figure 1 above. It causes increased in all the liver biomarkers such as the Aspartate

transaminase (ALT), Alanine transaminase, alkaline phosphate, creatinine, blood urea, total protein and gamma-glutamyl transferase. The histopathology of the liver shows there is moderate portal congestion with mild periportal cellular infiltration and a moderate to severe portal fibrosis, while the kidney shows severe diffuse tubular degeneration and necrosis.

The combination of MHM (500mg/kg) with Ascorbic acid (200mg/kg) decreases the level of ALT, CRT, ALP, T.BIL and BUN, 200mg/kg and 100mg/kg of MHM and also shows decreased in all the biomarkers but increase in GGT and CRT as shown in table 1. The histopathological analysis of the liver indicates no visible lesion at 500mg/kg, mild portal congestion with periportal cellular infiltration at 200mg/kg and bile ductules appearing prominent at 100mg/kg as shown in figure 1; while there was no visible lesion in the kidney at the three doses in the protective groups as indicated in figure 2. In the regenerative group, there was very mild congestion at 500mg/kg and no visible lesions in both organs at 200mg/kg and 100mg/kg as shown in figure 3 and 4. This shows that the combination may protect kidney at these doses but more effectively at 500mg/kg; and may treat both organs if damage by acetaminophen toxicity. This may be because of the Ascorbic acid that is antioxidant and able to reduce the stress induced by Acetaminophen by its ability to donate electron to free radical in the system thereby inactivating and neutralizing the effect of toxicants (Sabetian *et al.*, 2003). Its ability to participate in the liver for detoxification of toxic substances and boost blood level for immunity may also contribute to this (Fadime *et al.*, 2017) [14]. The combination of Selenium 1mg/kg and Methanol extract of *H. madagascariensis* in the protective groups reduced all the biomarkers as shown in table 3 at 500mg/kg but increase in CRT and GGT at both 200mg/kg and 100mg/kg, the histopathology shows mild interstitial congestion in the kidney and moderate periportal cellular infiltration in the liver at 500mg/kg but no visible lesion in both kidney and liver at 200mg/kg and 100mg/kg. There may be increase in CRT and GGT at 200mg/kg and 100mg/kg but these doses may be able to protect both organs in combination with MHM against damage induced by acetaminophen. Selenium 1mg/kg alone cannot reduce the level of the biomarkers and ameliorate the effect of acetaminophen on both organs as reported by Okwulu *et al.*, (2021).

In the regenerative groups, there was only reduction in ALP, BUN, GGT and T.BIL at 500mg/kg of MHM while at 200mg/kg there was no significant difference when compared with acetaminophen but 100mg/kg reduces the parameters but not as 500mg/kg. The histopathology analysis show no visible lesion in the liver but mild interstitial congestion in the kidney at 500mg/kg, the hepatocytes appear shrunken and degenerated at 200mg/kg and no visible lesion in both organs at 100mg/kg; the combination of MHM at 500mg/kg and 100mg/kg with Selenium 1mg/kg may treat liver damage induced by acetaminophen. Selenium being an important component of antioxidant enzymes, such as glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinases (IDD) may be the reason for the protective and regenerative effect that is seen in this work (Marija *et al.*, 2018) [22].

The combination of MHM with Allopurinol 50mg/kg in the protective group shows decrease in ALP, BUN and GGT at 500mg/kg, at 200mg/kg of MHM in combination with Allopurinol AST, ALT, ALP and T.BIL were reduced while at 100mg/kg there was no reduction of any parameter. Histopathology analysis shows no visible lesion in both organs at 500mg/kg while the liver shows few hepatocyctes rounded and degenerated at 200mg/kg. At 100mg/kg there was moderate to severe tubular degeneration and tubules are greatly expanded with degenerated epithelium in the kidney and the liver shows mild congestion of the portal vessel. The 500mg/kg of MHM and Allopurinol 50mg/kg may therefore be able to protect both organs in case of acetaminophen toxicity as shown in previous work (Oluwadunni *et al.*, 2019; Okwulu *et al.*, 2021) [26] and as seen in this work.

In the regenerative groups, 500mg/kg of MHM in combination with Allopurinol 50mg/kg was able to reduce the level of the biomarkers (ALP, BUN, CRT, GGT and T.BIL) both 200mg/kg and 100mg/kg could not reduce the level of the biomarkers. Histology shows mild to moderate portal and sinusoidal congestion with periportal cellular infiltration in the liver at 500mg/kg (fig 3), tubules have expanded lumen in the kidney at 200mg/kg and at 100mg/kg the kidney shows moderate to severe tubular degeneration with tubules greatly expanded (fig. 4) and the liver shows mild congestion of the portal vessels as seen in figure 3. Allopurinol though an antioxidant may not be effective alone (Okwulu *et al.*, 2021) and when combined with MHM at these doses to treat liver or kidney damage induced by acetaminophen may caused a regenerative and protective effects. *Harungana madagascariensis* has been shown to have phenolic compounds such as flavonoids and phenolic acids, alkaloids, saponins, tannins, anthraquinones and steroids but has no cardiac glycosides which are antioxidants that may be responsible for the pharmacological activities demonstrated in this work (Akpan *et al.*, 2012; Oluwadunni *et al*., 2019) [4, 26].

Conclusion

This work shows that the combination of Ascorbic acid 200mg/kg, Selenium 1mg/kg and Allopurinol 50mg/kg in combination with Methanol extract of *H. madagascariensis* at 500mg/kg can protect the liver and kidney against acetaminophen toxicity and may be used to manage toxicity effects on both organs caused by acetaminophen. It also shows that the combination with Ascorbic acid may be better than that of Selenium though it has shown to be effective also. This work indicates that Allopurinol combination with MHM may not be considered in the protection of both organs against acetaminophen toxicity or in the management of liver or kidney injury caused by acetaminophen. The synergy between MHM and the antioxidants especially Ascorbic acid and Selenium may have increase the efficacy of the plant extract, as it may both protect and treat both organs at lower dose of 200mg/kg and 100mg/kg when combine with this two antioxidants in the case of liver or kidney damage induced by acetaminophen as indicated in this work.

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