Omomule *et al.*, ***Journal of Medicinal Herbs***, Vol. 12, No. 2: 47-56, 2021
doi: 10.30495/MEDHERB.2021.684235

Journal of Medicinal Herbs
journal homepage:**www.jhd.iaushk.ac.ir**

Hepatic function status of acidified ethanol induced Wister rats treated with ethylacetate extract of *Abrus precatorius* Linn. seeds

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ARTICLE INFO

Type: *Original Research*
Topic: *Medicinal Plants*
Received *February 08th 2021*
Accepted *June 09th 2021*

Key words:
- ✓ *Ethanol*
- ✓ *Liver*
- ✓ *mRNA*
- ✓ *Cytochrome*

ABSTRACT

Background & Aim: Ethanol metabolism by the liver differentially impairs hepatic functions. This study investigated the attenuating potential of *Abrus precatorius* seeds on liver damage in HCl/EtOH induced rats and evaluated the prophylactic potential of ethylacetate extract of *A. precatorius* on the hepatic function of acidified ethanol induced rats.

Experimental: Rats were pre-treated with ethylacetate extract of *A. precatorius* seeds and standard drugs for eight consecutive days and 0.15M HCl/EtOH (60%) (1:1) was administered once on the 8th day. Biochemical assay, mRNA expression and liver histo-pathological studies were performed using standard procedures.

Results: HCl/EtOH induction significantly ($P<0.05$) raised hepatic alanine amino transferase and aspartate amino transferase activities that were ameliorated by pre-treatment with 100 and 200mg/kg b.w of *A. precatorius* seeds extract. The significant ($P<0.05$) up-regulation of CYP2D3, CYP3A4 and GGT mRNA expression in the liver tissues of HCl/EtOH induced rats were successfully down-regulated upon pre-treatment with the extract. The micro-morphological alteration characterized by severe congestion of central venules and sinusoids, fibrosis at the portal veins and presence of inflammatory cells observed in HCl/EtOH induced rats was reversed by pre-treatment with *A. precatorius* seeds.

Recommended applications/industries: Acidified ethanol used for ulcerative induction could result in liver injury in rats; pre-treatment with *A. precatorius* seeds extract could exert hepato-protective efficacy.

1. Introduction

The liver remains the largest most complex organ, contributing almost 2% of the total body weight (Rajaratnam *et al*., 2014). It is associated with major biochemical and physiological processes of the body system such as breaking down of the erythrocytes, storage of glycogen, production and detoxification of hormoneand synthesis of plasma proteins (Omoboyowa *et al*., 2015). Hepatic penetration might be a rare but severe complication of peptic ulcer (Kayacetin and Kayacetin, 2004). Therefore, induction of ulcer by acidified ethanol possesses major threat to the hepatic functions.

Chronic exposure to alcohol increases sterol regulatory element-binding protein-1 activity invariably induce fatty liver, elevating NADH/NAD+ ratio, reducing peroxisome proliferator-activating receptor-α (PPAR-α) activity and elevating complement-3 hepatic levels (Lee *et al*., 2017). These physiological alteration results in hepatic disorders, several studies have reported alcohol-induced injury in animal models (Lee *et al*., 2017; Zhou *et al*., 2017; Yu *et al*., 2019). The significance of phytochemicals and natural antioxidants in medicinal plants has prompt scientific investigation in the bioactive relevance in attenuating life threatening diseases.

Abrus precatorius (AP) Linn, commonly known as rosary pea belongs to the family fabaceae (Arora *et al*., 2011). It is described as a climbing shrub grows in subtropical region of India, South America and Africa (Rajani *et al*., 2013). Various parts of *A. precatorius* have been reported for its different pharmacological values. The leaves are effective in the management of wounds, herpes zoster, rheumatoid arthritis, cough and fever (Parthasarathy and Kumar, 2019). In Nigeria, leaves from *A. precatorius* has been used for the management of malaria, respiratory tract infections, typhoid and hepatitis (Parthasarathy and Kumar, 2019; Saganuwan and Onyeyili, 2010). Abrin has been reported as a poisonous protein present in the seeds of *A. precatorius* which gets converted to its mutagenic form upon refrigerated storage but is denatured by cooking to render the seeds safe for consumption because of its medicinal values (Arora *et al*., 2011; Khare, 2004). The phytochemical compositions, antioxidants and anti-inflammatory properties of AP seeds have been reported by Arora *et al.* (2011). Monago and Alumanah, (2005) reported the anti-diabetes efficacy of AP seeds. This present study is aimed to ascertain ameliorating potential of *A. precatorius* seeds on hepato-cellular injury resulting from ulcerative induction using acidified ethanol.

2. Materials and methods

2.1. Plant collection and identification

Seeds of *A. precatorius* were obtained from the university medicinal plant garden. The authentication of seed was carried out by a botanist from Plant Science and Biotechnology Department of our institution with a voucher specimen no: AAUA/2072.

2.2. Processing of plant sample

Fresh *A. precatorius* seeds were air-dried at room temperature for 21 days, after which it was pulverized with an electric blender. The ground seed (138 g) was soaked in 2 L of ethylacetate for two days, filtered with whatman No 1 filter paper, the filtrate was concentrated using Büchirotavaporator R-200 (Switzerland) at 45°C to give extract yield of 3.2 g (2.31%).

2.3. High performance liquid chromatography analysis

The reference compounds used for this analysis were quercetin, caffeic acid, chlorogenic acid, apigenin and luteolin purchased from Sigma inc (St. Louis, USA). HPLC analysis of ethylacetate extract of *A. precatorius* seed was obtained using Shimadzu HPLC (Japan) with Prominence pump (LC-20AD), column oven (EC 250/4), Prominence auto-sampler (NUCLEOSIL-100-5), fluorescence detector (RF-10AXL) and Hypersil Gold column (250×4.6 cm, 5 µm, Thermo scientific, USA).

2.4. Sample preparation

Sample under investigation was prepared by dilution with the mobile phase: acetonitrile and 0.1% acetic acid in water 1:9. The same method was followed to prepare the standard stock solution. The sample and standard solutions were passed through 0.22 µm PTFE-syringe filter. Exactly 3 ml of the filtrate was injected into the HPLC and the compounds were detected at 254 nm (Tapan, 2016) at 30°C oven temperature. The flow rate was 1 ml/min for 20 min running time.

2.5. Validation of the observed compounds from HPLC method

The validation parameters used were specificity and accuracy according to International Conference on Harmonisation (ICH) guideline (Santos *et al*., 2020)

2.6. Specificity

The retention periods and corresponding UV spectra for compounds in the extract and the standards were compared to validate peaks of the compounds.

2.7. Accuracy

To assess the accuracy, the extract was preparedin three different dilutions, at concentration 200 µg/mL and analyzed. The mean and standard deviation of the concentration was recorded.

2.8. Animal treatment and induction

Thirty Wistar albino rats were obtained from the Biochemistry Animal House, Biochemistry Department, Adekunle Ajasin University, Nigeria. The animals'groupings were done according to Akinloye *et al*. (2020). The rats were randomized and distributed into 6 groups of 5 animals each (n = 5):

Group 1: Healthy rats given normal feed and water *ad libitum*.

Group 2: Healthy rats administered 1 ml of distilled water orally for 8 days and 0.15 M HCl/ ethanol (60%)

(ratio 1:1) once on day 8 and were sacrificed after 5 hours.

Group 3: Healthy rats were pre-treated with 50 mg/kg b. w. Diclofenac orally for 8 days and0.15 M HCl/ ethanol (60%) (ratio 1:1) once on day 8 and were sacrificed after 5 hours.

Group 4: Healthy rats were pre-treated with 100 mg/kg b. w. *A. precatorius* seed extract orally for 8 days, 0.15 M HCl/ ethanol (60%) (ratio 1:1) once on day 8 and were sacrificed after 5 hours.

Group 5: Healthy rats were pre-treated with 200 mg/kg b. w. *A. precatorius* seed extract orally for 8 days, 0.15 M HCl/ ethanol (60%) (ratio 1:1) once on day 8 and were sacrificed after 5 hours.

Group 6: Healthy rats were pre-treated with 50 mg/kg b. w. cimetidine orally for 8 days, 0.15 M HCl/ ethanol (60%) (ratio 1:1) once on day 8 and were sacrificed after 5 hours under 1% chloroform anesthesia (Aledani *et al*., 2020).

2.9. Collection of tissue and serum

The whole blood of the treated and control animals were collected through the abdominal vein in a plain bottle, centrifuged at 2500 rpm for 10 min to separate the serum from the blood cells. The liver tissue was excised by sterilized scissors; cut into pieces and preserved in TRIzol for mRNA expression while the remainders were embedded in 10% formaldehyde for histo-pathological study.

2.10. Biochemical assays

Biochemical indices were estimated using Randox commercial test kits including; Alanine and aspartate aminotransferaseswere assayed according to Reitman and Frankel (1957). Total protein was determined according to Tietz (1995) using Randox test kits as reported in the manufacturer's manuals.

2.11. mRNA expression using RT-PCR

The liver tissues were used for CYP2D3, CYP3A4 and GGT mRNA expression following the method described by Omoboyowa *et al.* (2020) with modification. Briefly, RNA was isolated using 1 ml of TRIzol (Zymo Research, USA) from 70 mg of rats'liver tissue. Contamination with DNAs was removed by treatment with DNAse I (Thermo Fisher Scientific) following the manufacturer's procedure. Chloroform (2 ml) was added and the samples were vortex for 15 seconds, incubated at room temperature for 3 minutes. The mixtures were centrifuged at 12000× g for 15 min. The aqueous phase that contains the RNA was transfer into another vial. Isopropyl alcohol (0.5 ml) was used to precipitate the RNA from the aqueous phase and the RNAs were washed with 75% ethanol (Chomczynski and Mackey, 1995). 1 µg of the RNA was reconstituted in nuclease free water and quantified using spectrophotometer (Hitachi-U1900) at 280 nm and 260 nm. The A_{260}: A_{280} ratio was 1.86.

Exactly 1µg of the extracted RNA was used during the reverse transcription to generate cDNA using ProtoScript ll First Strand cDNA synthesis kit (BioLabs, New England) in a 3- step reaction: 65°C for 5 min, 42°C for 1 hour and 70^0C for 5 min.

Polymerase chain reaction amplification was carried out using OneTaq® 2X Master Mix (BioLabs, England). The reactions were carried out on a Labgene thermocycler using the primers listed in the table below. The chain reaction conditions includes: 1 cycle at 95°C for 5 min, 30 cycles at 95°C for 30 sec, 30 cycles at 55°C for 30 sec, 30 cycles at 72°C for 30 sec. cDNA was quantified using Image J software and the gene expression was normalized with β-actin gene as housekeeping gene.

Table 1. Primers used for RT-PCR.

S/N	Gene	Forward primersequence	Sequence for reverse primer
1	CYP2D3	GGCCAGTGGTCTTTGGTAGC	GGCAGCCACAGAACTGTTTTA
2	CYP-3A4	CTGCATTGGCATGAGGTTTG	CTTACAAGGCTGGAAGGAGAAG
3	GGT	CAGTTCCTCCAGGACAAAGAC	GATGCATCAGCTCCGTGATAG
4	β-actin	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

2.6. Liver histopathology

Completely fixed liver tissues in 10% formaldehyde formedblocks sliced into 0.2 cm × 0.2 cm approximately and hydrated by graded alcohol solutions. The tissues were cleared in 2 changes of xylene, infiltrated in paraffin wax for 2 h. Sections sliced at 5 µm, stained with haematoxylin and eosin. The photomicrographs were obtained using an Amscope microscope camera attached to a light microscope (Amscope microscope) at ×100 and ×200 magnifications (Avwioro, 2010).

2.12. Analysis of laboratory data

Analyses of data were carried outusing one-way analysis of variance (ANOVA); turkey test was used for compare means with graph pad prism 9. Results generated were presented as Mean ± SD. Significance difference was at P<0.05.

3. Results and discussion

Although very rare cases of histological study proven liver penetration by gastric ulcer has been reported (Kayacetin and Kayacetin, 2004), but induction of gastric injury by acidified ethanol is a major concern of hepatocellular injury. Investigations have revealed that excessive exposure to alcohol ingestion might result to hepatic damage associated with alcohol liver diseases pathogenesis (Husain *et al*., 2001; Altamirano and Bataller, 2011). Many drugs for reversal of alcohol liver related diseases such as chelating agents, opiate antagonists, hemorheologic agents, TNF-α antagonist, antioxidants etc. have been presented with renal toxicity and other side effects (Lee *et al*., 2017). Therefore, thispresent study investigated the attenuating potential of ethylacetate extract of *A. precatorius* seeds against hepato-cellular injury in HCl/EtOH induced rats.

3.1. HPLC analysis of ethylacetate extract of A. precatorius seed

The HPLC chromatogram of ethylacetate extract of *A. precatorius* seed is shown in Figure 1. Table 1 gives the concentration of the flavonoids compounds present in the extract.

The HPLC analysis of the extract reveals the presence of luteolin (76.489 ± 0.030 ng/ml) and quercetin (1.280±0.283ng/ml) among other flavonoids. Luteolin is a naturally occurring polyphenolic flavone and luteolin-riched plants have been reported to play diverse pharmacological activities through anti-oxidant and anti-inflammatory pathways (Zhang *et al*., 2016). Brusselmans *et al.* (2005) reported that luteolin reduces lipogenesis via inhibition of fatty acid synthase therefore, luteolin-riched plant is capable of preventing alcohol fatty liver. Quercetin has been reported to possess anti-oxidant activity (Chen, 2010). The pharmacological activities of these flavonoids present in this plant might contribute to the hepato-protective activity of ethylacetate extract of *A. precatorius* seed.

Table 2. Flavonoids present in the ethylacetate extract of *Abrus precatorius* seeds**.**

Retention Time	Compounds	Concentration (ng/ml)
1.532	Caffeic Acid	9.832 ± 0.014
2.082	Luteolin	76.489 ± 0.030
3.890	Apigenin	2.222 ± 0.141
4.315	Quercetin	1.280 ± 0.283
4.698	Chlorogenic Acid	5.751 ± 0.007

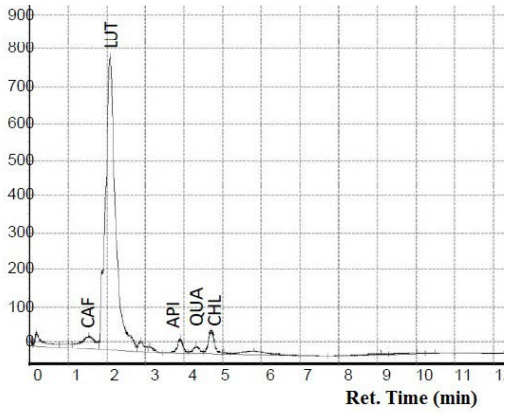


Fig. 1. HPLC chromatogram of flavonoids presents in *A. precatorius* seeds.

3.2. Biochemical assays

Figure 2 a-c showed the results of biochemical assays of HCl/EtOH induced ulcerative animals administered ethylacetate extract of *A. precatorius* seeds (EEAPS). The HCl/EtOH induced rats showed elevated AST and ALT activities compared to the normal rats, pre-treatment with the extract (EEAPS), the enzymes activities were observed to reduced significantly (P<0.05). Pre-treatment of HCl/EtOH induced rats with standard drugs (diclofenac and cimetidine) showed significant (P<0.05) reduction in ALT and AST activities compared toHCl/EtOH induced rats (Figure 2 a-b).

Figure 2c revealed significant (P<0.05) reduction in protein level of HCl/EtOH induced animals compared to the untreated control rats. Pre-treatment with EEPAS and standard drugs, the protein concentration increases compared to HCl/EtOH induced rats. Acidified ethanol induced liver impairment in rats as shown by elevation of serum AST and ALT activities. Elevated serum aminotransferases activities have been reported as biomarkers of liver impairment induced by ethanol (Masano *et al*., 2003). These enzymes leak into circulation in conformity with the extent of hepatocellular injury when there is hepatopathy or hepatocyte necrosis of abnormal membrane

permeability (Nkosi *et al*., 2005; Battua and Kumara, 2009).

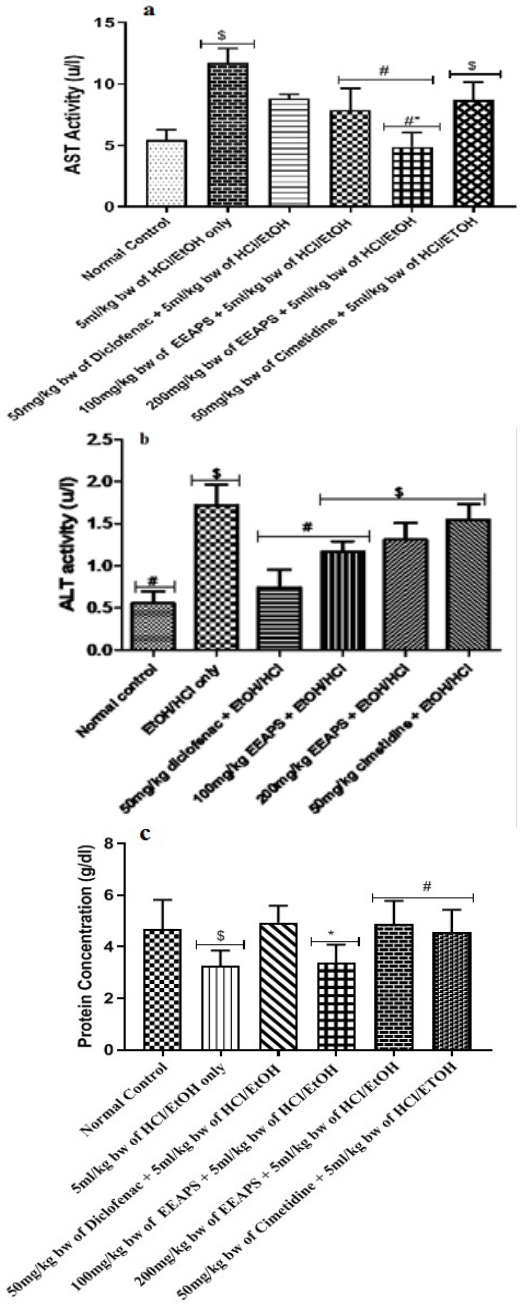


Fig 2a-c. Analyses of the biochemical parameters of HCl/EtOH-induced rats treated with ethylacetate extract of *A. precatorius* seeds (EEAPS)(a) Aspartate transaminase (AST) activity; (b) Alanine transaminase (ALT) activity; (c) Protein concentration. $^{\$}$(P<0.05) significant compared to normal control; $^{\#}$(P<0.05) significant compared to negative control rats; *(P<0.05) significant when compared to 50 mg/kg bw of diclofenac treated rats administered 5mg/kg bw of HCl/EtOH.

3.3. mRNA expression of liver tissue

Figures 3-5 shows liver mRNA expression of cytochrome P isoforms (CYP2D3 and CYP3A4) and garmma glutamyl transferase (GGT) in HCl/EtOH induced rats treated with EEAPS andstandard drugs. The rats induced with HCl/EtOH without treatment showed significant (P<0.05) up-regulation of CYP2D3, CYP3A4 and GGT compared to normal control rats. Treatment with EEAPS significantly (P<0.05) down-regulated the genes, treatment with 200 mg/kg b.w EEAPS were shown to be more effective in ameliorating the expression of the genes. The standard drugs (diclofenac and cimetidine) also down regulated the genes compared to HCl/EtOH induced rats without pre-treatment.

The acidified ethanol induction of liver damage was confirmed by mRNA expression of cytochrome-P (CYP) and gamma glutamyltransferase (GGT) in the liver. It is well recognized that CYPs have an important role in the biotransformation of many xenobiotics and coverts same to reactive intermediates (Abdou *et al*., 2020). Figure 3 and 4 revealed significantly (P<0.05) up-regulation of CYP2D3 and CYP3A4 in HCl/EtOH induced rats compared to the untreated control rats but pre-treatment with ethylacetate extract of *A. precatorius* seeds (EEAPS) ameliorated this effect by down-regulation of these genes. Ethanol and other alcohols have been shown to induce CYP3A4 in several experimental models (Liangpunsaku *et al*., 2005; Louis *et al*., 1994; Roberts *et al*., 1995) which is believed to cause hepatotoxicity (Thummel *et al*., 1993).

Cimetedine, an H2-histamin receptor antagonist inhibits hepatic mixed function oxidase (MFO) activity (Baird *et al*., 1987). CYP in liver microsomes has binding site with high affinity for cimetidine, the interaction of the cyano and imidazole locations of cimetidine and the iron in the heamegroup of CYP has been reported (Caballero *et al*., 2002). Diclofenac on the other hand is oxidized to 4-hydroxylated derivative by CYP 2C9 and CYP 3A4 after complete absorption from the intestine (Krasniqi *et al*., 2016). The hydroxylated metabolite undergoes glucoronidation or sulphation (Kirchheiner *et al*., 2002). The interactions of both drugs with CYP might result into up-regulation of CYP 2D3 and CYP 3A4 observed in this present study.

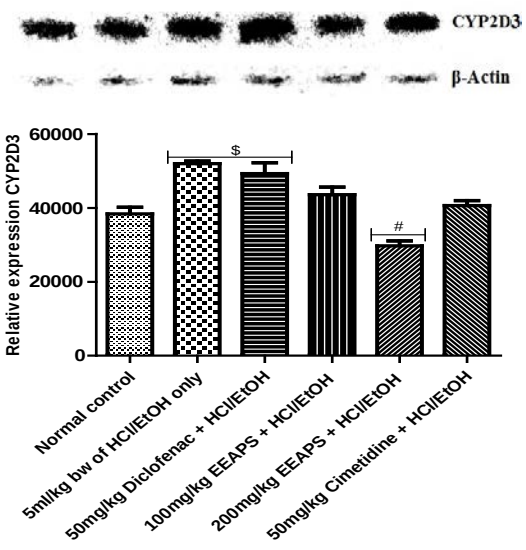


Fig. 3. Relative expressions of CYP2D3 mRNA in the Liver of HCl/ethanol-induced ulcerogenic rats expressed as Mean ± SEM (n = 5 per group). $^{\$}$P<0.05 significant compared to normal control animals; $^{\#}$P<0.05 compared to negative control. EEAPS: Ethylacetate extract of *A. precatorius* seeds.

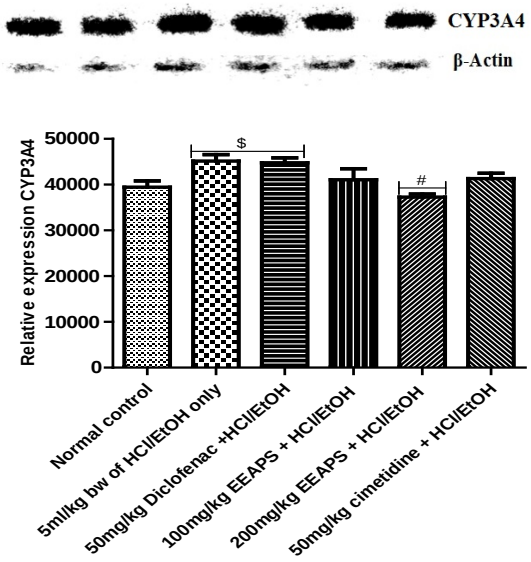


Fig. 4. Relative expressions of CYP3A4 mRNA in the Liver of HCl/ethanol-induced ulcerogenic rats expressed as Mean ± SEM (n = 5 per group). $^{\#}$P<0.05 significant compared to negative control; EEAPS: Ethylacetate extract of *A. precatorius* seeds.

Gamma glutamyltransferase (GGT) catalyzes the transfer of λ-glutamyl functional groups from molecules such as glutathione to an acceptor e. g. amino acids or water (Whitfield, 2001). Determination of GGT is commonly used as a screening maker for alcoholism (Nishimura and Teschke, 1983).

Figure 5 showed up-regulation of GGT in HCl/EtOH induced rats compared to normal control rats; this indicates hepatotoxicity which was attenuated by pre-treatment with EEAPS as evident by down-regulation of GGT gene.

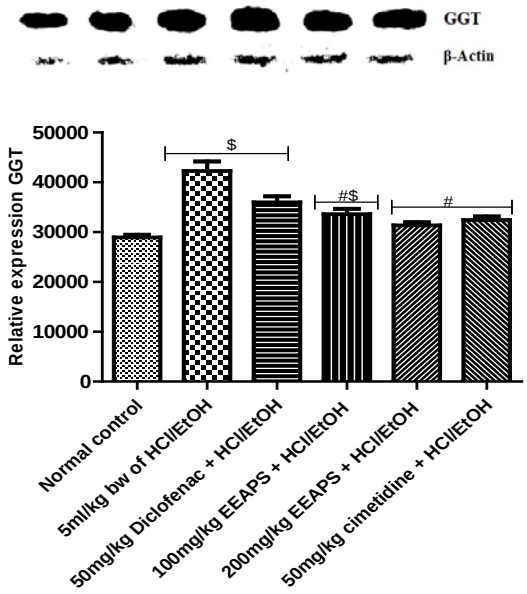
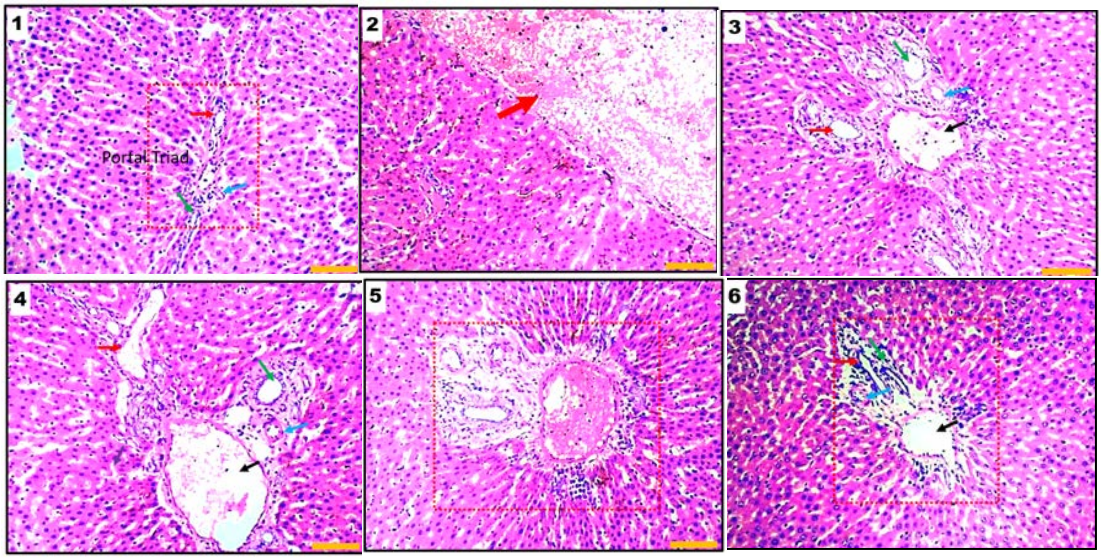


Fig. 5. Relative expressions of GGT mRNA in the Liver of HCl/ethanol-induced ulcerogenic rats expressed as Mean ± SEM (n = 5 per group). $^{\$}$P<0.05 significant compared to normal control; $^{\#}$P<0.05 compared to negative control. EEAPS: Ethylacetate extract of *A. precatorius* seed.

3.4. Histopathology study

From Figure 6, the normal control rats revealed normal central venules without congestion, the morphology of the liver cells (hepatocytes) appear normal with distinct layering, the sinusoids appear normal and not infiltrated, portal triad appear intact with no observable dilation, no signs of fibrosis nor hemorhage. The liver morphology of rats induced with HCl/EtOH shows altered micromorphology that is characterized by central venules and sinusoids with severe congestion (red arrow), portal tract show signs of fibrosis/ulceration with infiltration as well as some presence of red inflammatory cells, the morphology of the hepatocytes appear pyknotic with loss of cytoplasmic contents, the sinusoids appear congested and infiltrated, portal triad appear dilated. HCl/EtOH

induced rats treaed with 50 mg/kg b.w of diclofenac shows a mild altered micromorphology that is characterized by central venules and sinusoids with mild congestion (red arrow), portal tract show signs of mild fibrosis/ulceration with infiltration as well as some presence of mild red inflammatory cells, the morphology of the hepatocytes appear mildly pyknotic with some loss in cytoplasmic contents, the sinusoids appear mildly congested and infiltrated, portal triad appear dilated and distorted. Liver morphology of HCl/EtOH induced rats treated with ethylacetate extract of *A. precatorius* seeds and cimetidine show normal central venules without congestion except for a mild congestion observable in 100 mg/kg b.w of EEAPS treated rats treatment (red big arrow), the morphology of the hepatocytes appear normal with distinct layering, the sinusoids appear normal and not infiltrated except for a mild infiltration in 100 mg/kg b.w of EEAPS treated rats, portal triad appear intact with no observable dilation but a mild distortion in 100mg/kg b.w of EEAPS treated rats, no signs of fibrosis nor hemorhage.



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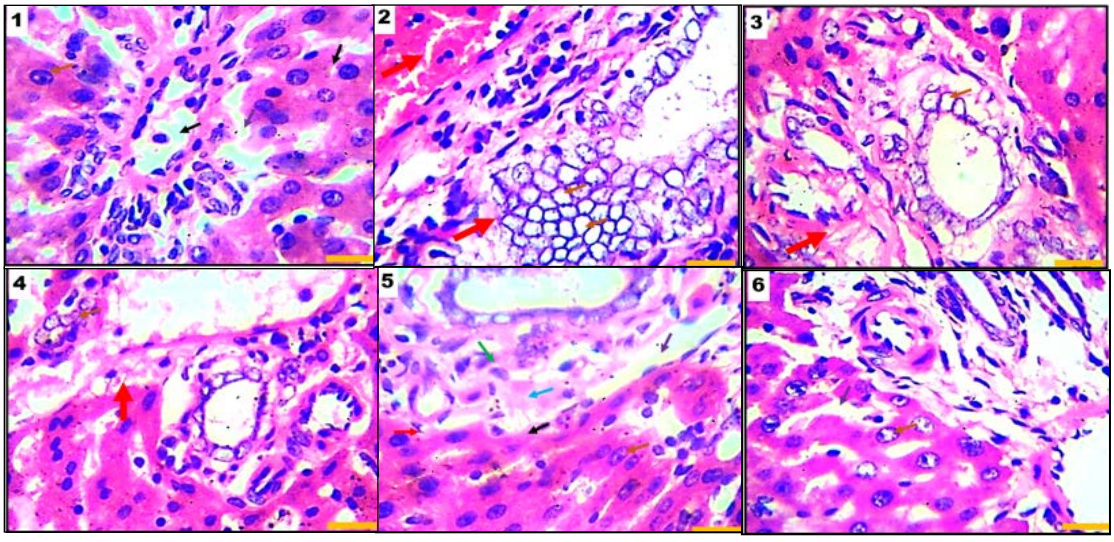

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Fig. 6. Magnified views of liver micromorphological organization in HCl/EtOH-induced ulceragenic rats treated with ethylacetate extract of *A. precatorius* seeds.

Figure 7 shows the hepatic injury score of HCl/EtOH induced rats treated with ethylacetate extract of *A. precatorius* seeds. The rsult showed increase in the hepatic injury score of HCl/EtOH inuduced rats compared to un-treated animals. Pre-treatment with extract and standard drugs ameliorated the hepatic injury score with the highest effect observed at 200 mg/kg b.w of ethylacetate extract of *A. precatorius* seeds and 50 mg/kg b.w of cimetidine .

The hepatic damage was further confirmed by liver histo-pathological examination in this study. Several micro-morphological alterations characterized by severe congestion of central venules and sinusoids, fibrosis at the portal veins and presence of inflammatory cells were observed in HCl/EtOH induced rats which were absent in HCl/EtOH induced rats pre-treated with EEAPS. This further confirmed the hepato-protective activity of *A. precatorius* seeds in acidified ethanol induced liver damage in rats. The results were in agreement with previous findings that show the hepato-protective of lemon juice on liver injury induced by alcohol in mice (Zhou *et al*., 2017).

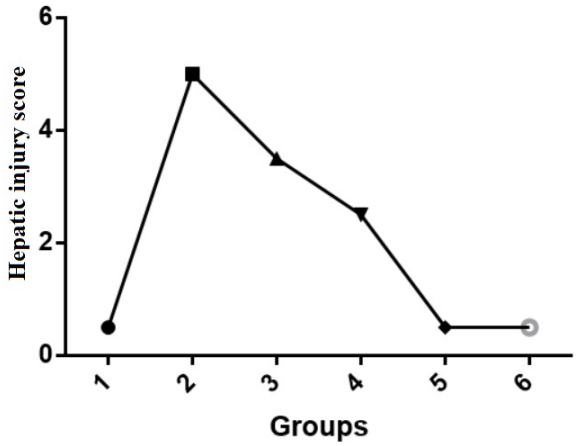

Fig. 7. Effect of ethylacetate extract of *A. precatorius* seeds on hepatic injury score of HCl/EtOH induced ulcerogenic rats. Group 1: Normal control; Group 2: 5 ml/kg bw of HCl/EtOH only; Group 3: 50 mg/kg b.w of diclofenac + 5 ml/kg bw of HCl/EtOH; Group 4: 100 mg/kg bw of ethylacetate extract of *A. precatorius* + 5ml/kg bw of HCl/EtOH; Group 5: 200 mg/kg bw of ethylacetate extract of *A. precatorius*+ 5 ml/kg bw of HCl/EtOH; Group 6: 50 mg/kg b.w of cimetidine+ 5 ml/kg bw of HCl/EtOH.

4. Conclusion

Administration of acidified ethanol for the induction of ulcer in animal models could induce hepatic injury. Pre-treatment with *A. precatorius* seeds extract exerted hepato-protective potential on the hepatocellular damage through reduction of serum ALT and AST activities, down-regulation of CYP2D3, CYP3A4 and GGT mRNA expression and reversal of liver micro-morphological alterations. The results revealed that, seeds of *A. precatorius* might be a potential therapeutic agent for the prevention and management of hepatocellular injury related to alcohol consumption.

5. Acknowledgments

The Authors are grateful to Dr. Elekofihinti O.O., Department of Biochemistry, Federal University of Technology, Akure for providing the facilities to perform the gene expression and Mrs. Oyewale of Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko for her contributions during the biochemical assays.

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