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Biochemical, Histopathological, and Microbiological Assessment of Herbal Bitters-Induced Hepatotoxicity and Gut Health Alterations in Albino Rats.

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Abstract

Herbal bitters formulations are traditionally consumed for their purported benefits on digestion and liver health. This study investigated the potential hepatotoxic and gastrointestinal effects of two commercial herbal bitters (Bitters A and Bitters B) following subchronic administration in an albino rat model. Thirty-six rats were divided into nine groups (n=4). Group 1 served as a negative control. Groups 2-9 received varying doses and regimens of Bitters A or B over 30 days. Upon termination, body weight was recorded, blood collected for liver enzyme assay, and livers harvested for histopathological examination. Fecal samples were analyzed for microbial content and consistency. While most groups exhibited weight gain, Group 9 showed significant terminal weight loss. Serum biochemistry revealed no significant change in aspartate aminotransferase (AST) levels. However, significant alterations were observed in alanine aminotransferase (ALT) and alkaline phosphatase (ALP); ALT was significantly elevated in

Groups 4, 6, and 7, and ALP was markedly elevated in Groups 4 and 6 but severely suppressed in Group 7. Histopathological analysis confirmed hepatic injury across treated groups, with findings including portal inflammation, hepatocellular vacuolation (steatosis), sinusoidal congestion, vascular distortion, and architectural disruption. Microbiological assessment indicated an antidiarrheal effect and the presence of probiotic Lactobacillus species. The 30-day administration of these herbal bitters induced significant, dose-dependent hepatotoxicity characterized by biochemical imbalance and structural liver damage, raising substantial safety concerns regarding their chronic use.

Keywords: Biochemical, Histopathological, Microbiological Assessment, Herbal Bitters, Hepatotoxicity

1. Introduction

The liver is an essential organ in the human body that performs a variety of critical tasks, including digestion, detoxification, immune modulation, metabolism, and the storage of vitamins (Guyton et al., 2011). Comprising approximately 2% of adult body weight, the liver is unique due to its dual blood supply from the hepatic artery (~25%) and the portal vein (~75%) (Sembulingam et al., 2012). Its principal functions include bile production for lipid emulsification, bilirubin metabolism, and the biotransformation of xenobiotics via phase I and II enzymatic pathways, prominently involving the cytochrome P450 system (Almazroo et al., 2017). Additionally, the liver synthesizes plasma proteins, metabolizes fat-soluble vitamins such as A and D3, and contributes to endocrine balance through hormone conversion (Vernon et al., 2022). Given its central role in homeostasis, the liver is particularly vulnerable to injury from exogenous substances, including phytochemicals present in herbal formulations (Umarudeen et al., 2023).

In recent years, the use of herbal bitters formulations has grown in popularity due to their purported benefits for digestion, detoxification, and metabolic balance (Smith, 2023). However, this rise in consumption has been paralleled by emerging concerns regarding their potential hepatotoxicity (Jones et al., 2022). These formulations often contain complex blends of botanicals rich in bioactive compounds such as alkaloids, flavonoids, and terpenes, which may exert adverse effects on the liver when consumed in excess or by susceptible individuals (Jones et al., 2022). Notably, several studies have documented liver injury associated with herbal bitters, underscoring the need for scientific scrutiny (Uzoh et al., 2022; Agu et al., 2023). These safety concerns are amplified by the general lack of standardized regulation, quality control, and clear labeling of many herbal products (FDA, 2023).

From a public health perspective, investigating the hepatotoxic potential of these widely consumed products is imperative. Increased awareness of associated risks can empower consumers to make informed decisions (Brown & Green, 2021; Miller, 2020), while scientific evidence can guide regulatory authorities in developing safety guidelines (FDA, 2023). Furthermore, understanding the toxicological profile of these bitters can inform the development of safer therapeutic alternatives (Agudosi et al., 2023; Uwanta et al., 2023). Previous research by our group and others has explored related areas of microbial and environmental toxicology (Agu et al., 2022; Okeke et al., 2023; Umeoduagu et al., 2023), as well as the probiotic potential of lactic acid bacteria in fermented foods (Uzoh et al., 2022), highlighting the multidisciplinary approach needed to assess complex herbal formulations.

Therefore, this study was designed to systematically evaluate the hepatotoxic effects of two commonly used herbal bitters formulations (Bitters A and Bitters B) in an albino rat model. By integrating biochemical, histopathological, and microbiological analyses, this research aims to provide a comprehensive safety profile of these products and contribute to the evidence base

necessary for responsible use and effective regulation.

2. Methodology

2.1 Materials

The materials that were used in the study are: Iron cages, feeding troughs, automated weighing balance, feed, syringes and needles, oral galvage, gloves, water pipes, glass slides (plain), dissecting board, saw dust, plain bottles, specimen bottles, beakers, test tubes, cotton wool, microscope, drinking bottles, stopwatch, sample bottles, centrifuge, microtome, hot plate, containers, water bath, grater, and embedding machine.

2.1.2 Reagents

The reagents used in this study are: fixative (10% formal saline), distilled water, Haematoxilyn and Eosin (H&E) stain, paraffin wax, absolute ethanol, chloroform, xylene, alcohol (absolute, 95%, 75%, 70%) and Dibutylphthalate Polystyrene Xylene (DPX). Commercially available alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

2.2 Experimental Animal Care and Handling

For this investigation, thirty-six (36) healthy Albino rats weighing an average of 130g were used. These rats were acquired from the Department of Animals and Environmental Biology, Rivers State University, Port Harcourt.

The Albino rats were kept in nine (9) groups of four (4) rats each, with a temperature of 25℃ and a relative humidity of 60-70%. They were given free access to water and standard feed (Finisher) produced by Top Feeds Nig. Ltd., and their cages were cleaned and the bedding changed every day. The rats were given two weeks to acclimatize to their new surroundings with 12 hours light exposure.

2.3 Dosage Calculation

The dosage used for this study was dependent on the average weight of the rats in each group and the volume of the herbal bitters formulation.

Dosage Calculation for Extracts:

Volume of bitters A =150mls
Volume of bitters B =200mls
Average weight of rats =113g
Average body weight of humans = 70,000g
The quantity of herbal bitters formulations administered was calculated using the formula:

$$\text{Dose} = \frac{\text{Volume of bitters} \times \text{Average weight of rats per group}}{\text{Average body weight of humans}}$$

A group with an average weight of 112.8g was administered 0.2mls of bitters A.

2.4 Experimental Design

Thirty-six (36) albino rats were assigned into nine (9) groups of four (4) rats each as follows:

Group 1 (Control group):was given commercial feed and water only for 30 days.
Group 2 wasadministeredorally with a normal dose of bittersAdaily alongside commercial feed and water for 30 days.
Group 3 was administered orally with a normal dose of bittersA once a week alongside commercial feed and water for 30 days.
Group 4 was administered orally with an overdose of bittersA daily alongside commercial feed and water for 30days.
Group 5 was administered orally with an overdose of bitters B once a week alongside commercial feed and water for 30 days.
Group 6 was administered orally with a normal dose of bitters B daily alongside commercial feed and water for 30 days.
Group 7 was administered orally with a normal dose of bitters B once a week alongside commercial feed and water for 30 days.

Group 8 was administered orally with an overdose of bitters B daily alongside commercial feed and water for 30 days.
Group 9 was administered orally with an overdose of bitters B daily alongside commercial feed and water for 3026 days.

2.5 Sample Collection

The final body weight of the rats was obtained at the end of the study which lasted for 30 days using a digital weighing balance. The animals were anaesthetized with chloroform and humanely sacrificed, the live of each animal was harvested.

Blood samples were collected through cardiac puncture and were put into Lithium Heparin bottles for liver function test. The livers of each Albino rat were preserved in 10% formal saline for histological analysis.

2.6 Laboratory Analysis.

2.6.1 Histopathological investigation

Procedure for Tissue Processing

Harvested tissue samples (liver) were fixed in 10% formal saline solution for 72 hours. Tissue samples were grossed, labelled, placed in tissue cassettes, and processed for histology using increasing grades of alcohol (70%, 75%, 90%, 95%, Absolute) for dehydration and cleared with two changes of Xylene for two hours each to increase the refractive index of the tissue. Tissues were infiltrated and impregnated in two changes of molten paraffin wax for two hours each to provide internal support for the tissue and blocked on ice pack. The prepared tissues were sectioned using a rotary microtome and the ribbons were picked on a clean grease free slide. Slides were drained and heat fixed on a hotplate at 70^{o}C. Tissues were further dewaxed in xylene, hydrated in decreasing concentrations of alcohol.

Principle of Haemtoxylin and Eosin Staining Technique

Haemtoxylin is a basic dye that stains the acidic components of the cell i.e., the nucleus whereas eosin is acidic dye that stains the acidic components of the cell i.e., cytoplasm.

Procedure for Haemtoxylin and Eosin Staining Technique

The issue sections were taken to water, and stained in Haematoxylin for 5 minutes. Tissue sections were differentiated in 1% acid alcohol and rinsed in water. Tissue sections were blued in Scott's tap water for 3 minutes. Tissue sections were counterstained in 1% eosin for 3 minutes and dehydrated in ascending grades of alcohol (70%, 75%, 90%, 95%, Absolute). Tissue sections were cleared in xylene and mounted using Dibutylphthalate Polystyrene Xylene (DPX) mountant and were observed under the microscope. Photomicrographs of the sections were obtained using Leica DM750 microscope with digital camera ICC50E LASEZ software installed in HP 8th gen ore i5 laptop.

2.6.2 Biochemical Analysis

(a)Determination of Plasma Alkaline Phosphatase by Roy Method.

Principle

Alkaline Phosphatase reacts with sodium Thymolphthalein Monophosphate buffered in 2-amino-2-methyl-1-propanol (AMP) to release phosphate. The addition of Sodium Hydroxide (0.1M) and sodium carbonate (0.1M) as alkaline and color developer respectively stops the enzyme activity and simultaneously generates blue coloration. The intensity of the blue color is proportional to the concentration of ALP in the sample.

Procedure

Test tubes appropriately labelled standard (S), control (C), samples (T) and blank (B) were set up. 50µl of test sample, standard and control were added into their respective test tubes followed by addition of 500µl of alkaline phosphatase substrate reagent and gently mixed before incubating for 10 minutes at 37°C. After the incubation period, 2.5ml of stop and color developer reagent mixture was dispensed into test tubes labelled S, C, T and B and mixed. The absorbance of the blue color was measured spectrophotometrically at 590nm against reagent blank (B).

(b) Determination of Plasma Alanine Aminotransferase (ALT) by Reitman and

Frankel Method

Principle

ALT transfers amino group from alanine to α-ketoglutarate producing glutamate and pyruvate. The rate of formation of pyruvic acid is determined by coupling the ALT catalyzed reaction. Oxoacid generated, quantified by coupling oxo-derivatives formed with 2. 4-dinitrophenylhydrazine to form an oxo-acid hydrazine which in the presence of alkaline medium is seen as a reddish-brown color.
ALT: x-oxoglutarate + L-alanine GPT L-glutamate+ pyruvate.

Procedure

Test tubes appropriately labelled standard (S), control (C), samples (T) and blank (B) were se9t up. 100 µl of respective test sera and control samples were dispensed into appropriately labeled tubes followed by the addition of 500µl of ALT substrate-buffer solution. The content of the tubes was mixed and incubated at 37^{o}C for 30 minutes. After the incubation, 500µl of 2, 4-dinitrophenylhydrazine was dispensed into all the test tubes. The content of the tubes was mixed and incubated at 25^{o}C for 20 minutes. After the incubation, 5.0ml of 0.4N sodium hydroxide (NaOH) was added to all the tubes to stop the reaction and absorbance read at 540nm wavelength against reagent blank. The concentration of ALT in the test samples were extrapolated from calibration curve already prepared.

(c) Determination of Plasma Aspartate Aminotransferase (AST) by Reitman and

Frankel Method

AST transfers amino groups from aspartate to α-ketoglutarate forming oxaloacetate and glutamate. The oxaloacetate reacts with 2, 4-dinitrophenylhydrazine which in an alkaline medium gives a reddish coloration. The intensity of the color produced is directly proportional to the concentration of AST in the specimen. The absorbance of the color produced was read at 546nm wavelength spectrophotometrically.
AST: x – Oxoglutarate + L – aspartate GOT L – glutamate oxoalogcetate.

Procedure

Test tubes appropriately labelled as control (C), samples (T), and blank (B) were set up. 100µl of respective test sera and control samples were dispensed into appropriately labelled tubes followed by the addition of 500µl of AST substrate-buffer solution. The content of the tubes was mixed and incubated at 37^{o}C for 30 minutes. After the incubation, 500µl of 2, 4-dinitrophenylhydrazine was dispensed into all the test tubes. The content of the tubes was mixed and incubated at 25^{o}C for 20 minutes. After the incubation, 5.0ml of 0.4N sodium hydroxide (NaOH) was added to all the tubes to stop the reaction and absorbance read at 540nm wavelength against reagent blank. The concentration of ALT in the test samples were extrapolated from calibration curve already prepared.

Characterization and Identification of bacteria

Identification of the bacterial isolates was accomplished by the observation of colonial

characteristics, Gram reaction and biochemical tests (Chessbrough, 2006). The characterization of the isolates was performed, by employing Gram staining reaction, Catalase test, Citrate test, Sugar fermentation test, Coagulase test, Motility test, Oxidase test, Urease test, Indole test, Methyl Red and Vogesproskauer test as described by Bergey's Manuel of Determinative Bacteriology, 9th edition (2006).

Gram reaction

Thin smear of the isolate was made on clean, non-greasy, dust-free slides, air dried and heat fixed. The smear was flooded with crystal violet and allowed to remain on the slide for 60 seconds. Thereafter, the crystal violet was washed off with gentle running water. Again, the slide was flooded with slide with Gram's iodine, allowed to remain for 60 seconds and washed off. The slide was decolourized with acetone-alcohol mixture. The slide was counter-stained with safranin for 60 seconds and rinsed with tap water and allow to air dry. The slide was then viewed under oil immersion lens microscope (×100). Purple colour indicated Gram–positive organisms while red or pink colour indicated Gram-negative organisms.

Catalase test

Exactly 3ml of 3% solution of hydrogen peroxide (H_2O_2) was transferred into a sterile test tube. Then, 3 loopful of a 24-hour pure culture of the test bacteria were inoculated into the test tube. The tube was observed for immediate bubbling indicative of a Positive, while no bubbling indicated a negative reaction.

Motility test (Hanging Drop Method)

A loopful of 18-24-hour broth culture of the test bacteria was placed at the centre of a clean grease-free cover-slip. Carefully, the cover slip was inverted and placed over the concave portion of a hanging drop slide. The cover-slip/slide arrangement was observed for motility at X100 magnification on a compound microscope. Care was taken to not interprete "drift" or "Brownian motion" as motility. Results were recorded as motile or non-motile.

Oxidase Test

All bacteria that are oxidase positive are aerobic, and can use oxygen as a terminal electron acceptor in respiration. This does NOT mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport.

Whatmann No.1 filter paper was soaked with the substrate tetramethyl-p-phenylenediamine-dihydrochloride. The filter paper was moistened with sterile distilled water. Then the test colony was picked with wooden or platinum loop and smeared in the filter paper. The inoculum was by observing the area around the inoculated paper for a color change to deep blue or purple within 10-30 seconds. Positive and negative quality controls were also set up (Positive control: *Pseudomonas aeruginosa*; B. Negative control: *Escherichia coli*). **Positive** was indicated by development of dark purple color (indophenols) within 10 seconds. **Negative**: Absence of color.

Urease Test using Christensen's Urea Agar

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive *Proteeae* from other *Enterobacteriaceae*.

Heavy inoculum from an 18-to24-hour pure culture was used to streak the entire **Christensen's Urea Agar** slant surface. Adequate care was taken not to stab the butt as it will serve as a color control. The tubes were incubated loosened caps at 35^{o}C. The slants were observed for a color change at 6 hours, 24 hours, and every day for up to 6 days. Urease production would be indicated by a bright pink (fuchsia) color on the slant that may extend into the butt. Note that any degree of pink is considered a positive reaction. Prolonged incubation may result in a false-positive test due to hydrolysis of proteins in the medium. To eliminate protein

hydrolysis as the cause of a positive test, a control medium lacking urea was also set up. Rapidly urease-positive *Proteae*(*Proteus* spp., *Morganellamorganii*, and some *Providencia stuartii* strains) will produce a strong positive reaction within 1 to 6 hours of incubation. Delayed-positive organisms (e.g., *Klebsiella* or *Enterobacter*) will typically produce a weak positive reaction on the slant after 6 hours, but the reaction will intensify and spread to the butt on prolonged incubation (up to 6 days). The culture medium will remain a yellowish color if the organism is urease negative

Indole Test

A loopful of an 18-24-hour culture was used to inoculate the test tube containing 3 ml of sterile tryptone water. Incubation was done at 35–37 ^{o}C first for 24 hours and further for up to 48 hours. Test for indole was done by adding 0.5 ml of Kovac's reagent, shaken gently and then examined for a ring of red colour in the surface layer within 10 minutes, indicative of a positive reaction. Absence of red colour indicated a negative reaction.

Methyl Red test

Exactly 5 drops of methyl red indicator were added to an equal volume of a 48hours culture of the isolate in Methyl red–VogesProskauer (MR-VP) broth. The production of a bright red colour indicates a positive test while yellow colour indicates a negative test after vigorous shaking.

Voges-Prausker test

Exactly 2ml of the 18-24 hours' culture of the test organism growing on MR-VP broth was asceptically transferred into a sterile test tube. Then 0.6ml of 5% α-naphthol was added, followed by 0.2ml of 40% KOH (NB: It was essential that this reagent was added in this order). The tube was shaken gently to expose the medium to atmospheric oxygen and then allowed to stand undisturbed for 15-30 minutes. A positive test was indicated by the presence of a red colour after 15-30 minutes, indicative of the presence of diacetyl, the oxidation product of acetoin (Test was always considered invalid after one hour because VP-negative cultures may produce a copper-like colour, false positive), lack of pink-red colour denoted a negative reaction.

Citrate test

A 24h old culture was inoculated into test tubes containing sterile Simmons Citrate agar slant and then incubated for 24hours. A positive test was indicated by a change from green to blue colour on the surface of the Simmons Citrate agar slant. No colour change indicated a negative reaction.

Sugar Fermentation Test

Each of the isolate was tested for its ability to ferment a specific sugar. 1g of the sugar and 1g of peptone water were dissolved in 100ml of water. 5ml of the solution were transferred into clean test-tubes using sterile pipettes. The test-tubes containing peptone water and sugar were added Durham's tube which were placed inversely and bromothymol blue as an indicator. These were sterilized for 10minutes and allowed to cool before inoculating the inocula. The test-tubes were incubated for 3days. The production of acid and gas or acid only indicated utilization of sugars. Acid production was indicated by change in colour of the medium from green to yellow while gas production was observed by presence of gas in the Durham's tubes.

In-vivo assay for Diarrhea Treatment Using Laboratory Mice

Isolates of lactic acid bacteria that are found to possess probiotic properties would be inoculated on MRS broth and incubated at 37 °C for 72 hours, under anaerobic conditions. After incubation, the bacterial culture would be centrifuged at 5000× g for 30 minutes. Culture supernatant would be filtered out and the pellets washed twice with PBS, before being re-suspended in 20mL of PBS (pH 6.8). The suspension would be kept at 4°C until use.

In the evaluation of the effects of the LAB in diarrhea treatment. The rats would be fasted for 18 hours prior to the experiment as described by Shoba and Thomas (2001). each animal in each group received 0.5 ml of castor oil orally. Following the castor oil delivery, each group's total number and weight of diarrheal drops were determined over the course of a 4-h observation period. The rats would be divided into five groups (n=4). Group I would serve as the control group and would be given distilled water only. Group II would be given 20mL of Diphenoxylate hydrochloride (5 mg/kg of body weight) orally as suspension. Group III would orally be given 300µL of the LAB culture suspension. Group IV would orally be given 400µL of culture suspension. Group V would orally be given 500µL of LAB culture suspension. All solutions given to the animals would be administered daily for the duration of the experiment. Following the castor oil delivery, each group's total number and weight of diarrheal drops were determined over the course of a 4-h observation period. The time for the onset of diarrhea in each animal was also determined as the interval between the castor oil delivery and the appearance of the first diarrheal feces. Then, the percentage inhibition of diarrhea from the negative control group was determined using the following formula.

3.7 Data Analysis

The data generated from this study were analyzed using SPSS version 23. Results were exressed as mean± SD with p-values less than 0.05 being considered statistically significant.

Results

4.1 Morphological Results

Figure 4.1, demonstrates the changes in the body weight of rats during and after the administration of herbal bitters formulations (bitters A and Bitters B). Animals in group 1 which is the control group gained weight during the course of the study. Group 2, 3, 4, 5, 6, 7 and 8 animals gained significant weight during the course of the study but there was a decrease in weight of the Group 9 animals towards the end of the study.

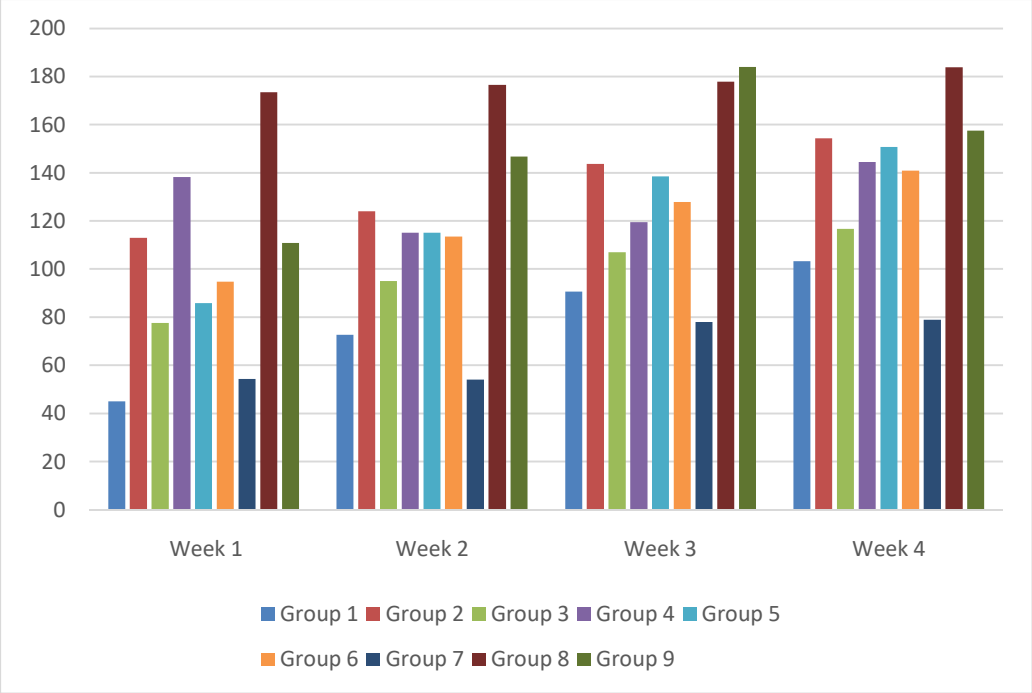

Fig 4.1: Changes in Body Weight of Rats According to Weeks

4.2 Effects of Herbal Bitters Formulations (A Bitters and B Bitters) on Liver Enzymes of Albino Rats

There is no significant difference in AST levels when compared to group 1, suggesting no significant hepatocellular damage across study groups. The ALT levels of group 2, 3, 8 are significantly reduced while that of groups 4, 6 & 7 are significantly raised when compared to the negative control group. ALP levels show the most variability, with significant reductions in groups 2 and 7, and elevations in group 4.

Table 4.2: Effect of Herbal Bitters Formulations (A Bitters and B Bitters) on the Liver Enzymes.

	AST **IU/L**	ALT **IU/L**	ALP **IU/L**
Group 1	6.00±1.00	4.33±0.58^{a}	94.33±11.72^{a}
Group 2	5.67±1.53	3.00±1.00^{c}	80.66±13.32^{b}
Group 3	7.33±2.08	3.66±1.53^{c}	108.33±10.96^{c}
Group 4	5.50±1.29	6.00±0.82^{d}	110.00±11.58^{c}
Group 5	5.25±0.96	4.25±0.50^{a}	107.00±10.42^{c}
Group 6	6.50±1.73	6.00±0.82^{d}	119.75±8.38^{d}
Group 7	5.00±1.00	5.33±1.53^{e}	32.66±44.47^{e}
Group 8	5.25±1.25	3.50±0.58^{c}	106.25±11.59^{c}
Group 9	6.00±0.82	4.25±0.50^{a}	101.75±17.31^{c}
p-value	0.470	0.001	<0.001
F-value	0.989	5.201	6.993

Values in the same column with different superscripts are significantly different from each other ($p \leq 0.05$)
Key: AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase

4.3 Histological Examination of Liver Albino Rats

Histological examination of the liver of albino rats treated with herbal bitters formulation (Bitters A and Bitters B) for 30 days with their respective controls.

Groups	Photomicrograph Observations	H & E
Group 1	Section showed unremarkable features which is composed of interlobular vessels, portal area, central vein, hepatocytes, and sinusoids.	H & E x 100
Group 2	Section shows distortion or portal area, hepatic degeneration and vacuolation, inflammatory cells infiltrate, distortion of interlobular vessels, sinusoids expansion and infiltration by inflammatory cells and red blood cells.	H & E x 40
Group 3	Section shows compressed sinusoids with red blood cells and endothelial lining cells, dilated interlobular vessel, mild hepatic vacuolation, increase in inflammatory cells infiltrate within the portal area and vessel wall as well as bile duct distortion. There is increase in inflammatory cells and vessel wall fibrosis and distortion.	H & E x 100

Group 4	Section shows increase in lymphoid aggregates when compared to that seen in group 3. These aggregates are seen mostly within the portal area. The area shows mild hepatic vacuolation, muscular components distortion, sinusoids congestion with red blood cells, vessels congestion and reactive hepatic cells nuclei.	H & E x 40
Group 5	Section shows unremarkable features and sinusoids expansion..	H & E x 100
Group 6	Section distorted hepatic architecture with increase in inflammatory cells aggregate within the portal area and vascular channels. This area shows vessels congestion, sinusoidal congestion, hepatic vacuolation and reactive hepatic nuclei changes as well as increase inflammatory cells admixed with red blood cells within the sinusoidal spaces.	H & E x 400
Group 7	Section shows portal area distortion with its muscular wall degeneration. Area shows degenerative hepatic cells, inflammatory cells infiltrate distorting the portal area and hepatic vessels.	H & E x 40
Group 8	Section shows congested vessel with red blood cells, sinusoid congestion with red blood cells, reactive hepatic cell nuclei, and distortion of the portal area by increase in inflammatory cells aggregate within the portal areas.	H & E x 100
Group 9	Section shows mild hepatic vacuolation, vessel wall infiltration by inflammatory cells, vessel congestion, sinusoids expansion containing red blood cells within its spaces which appears admixed with mild inflammatory cells. Area shows lymphoid aggregates exhibiting hepatic structure distortion mostly within the portal area.	H & E x 40

Table 4.4: Microbial Assay of Mice (NUMBER OF WET AND DRY FEACES) for 6hrs period

	N1	N2	N3	N4					
GROUP 1 (distilled water)	WF: 13 DF: 0	WF: 11 DF: 2	WF: 13 DF: 0	WF: 12 DF: 1					
GROUP 2: (BITTER A)	WF: 3 DF: 10	WF: 1 DF: 12	WF: 2 DF:11	WF: 1 DF: 12					
GROUP 2: (BITTER B)	WF: 1 DF: 12	WF: 0 DF: 13	WF: 0 DF:13	WF: 1 DF: 12					
1					11				
GROUP 3: (300µl)	WF: 5 DF: 8	WF: 2 DF: 11	WF: 4 DF: 9	WF: 3 DF: 9	WF: 4 DF: 11	WF: 2 DF: 11	WF: 6 DF:5	WF: 5 DF: 8	
GROUP 4 (400 µl)	WF: 3 DF: 8	WF: 4 DF: 13	WF: 2 DF: 6	WF: 3 DF: 8	WF: 2 DF: 9	WF: 2 DF: 10	WF: 1 DF: 8	WF: 4 DF: 10	
GROUP 5 (500ul)	WF: 3 DF: 7	WF: 2 DF: 8	WF: 0 DF: 9	WF: 2 DF:11	WF: 1 DF: 9	WF: 2 DF: 9	WF: 0 DF:8	WF: 0 DF: 9	

Table 4.5: Morphological and Biochemical Identifications of the Various Lactic Bacterial Isolates.

Isolate	Form	Surface	Colour	Margin	Elevation	Opacity	Gram	Cat	Mot	Ind	MR	VP	Cit	Lac	Glu	Suc	Fru	Mal	Oxi	Ure	Probable Identity
1	Circular	Smooth	Cream	Entire	Convex	Translucent	+Rod	-	-	var	+	+	-	+	+	+	+	+	-	-	*Lactobacillus pentosus*
11	Circular	Smooth	White	Entire	convex	Translucent	+Rod	-	-	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*

Key (Table Legend):
Isolate = Sample code number;
Form = Colony shape;
Surface = Texture of colony surface;
Colour = Pigmentation of colony;
Margin = Edge of the colony;
Elevation = Vertical profile of the colony;
Opacity = Degree of light transmission through the colony;
Gram = Gram reaction and cell morphology;
Cat = Catalase test;
Mot = Motility test;
Ind = Indole test;
MR = Methyl Red test;
VP = Voges–Proskauer test;
Cit = Citrate utilization test;
Lac = Lactose fermentation;
Glu = Glucose fermentation;
Suc = Sucrose fermentation;
Fru = Fructose fermentation;
Mal = Maltose fermentation;
Oxi = Oxidase test;
Ure = Urease test;

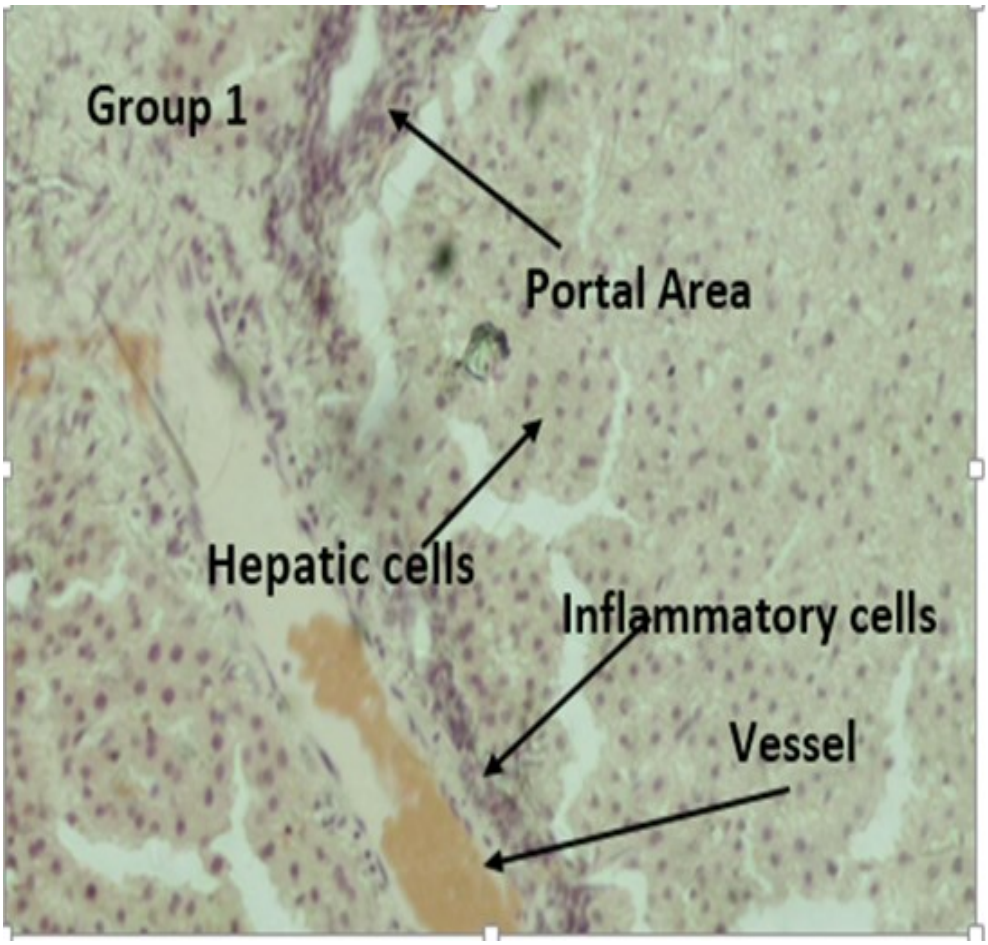

Plate 4.1: Photomicrograph of a section taken from liver of albino rats in group 1.
Section showed unremarkable features which is composed of interlobular vessels, portal area, central vein, hepatocytes, and sinusoids. H & E x 100.

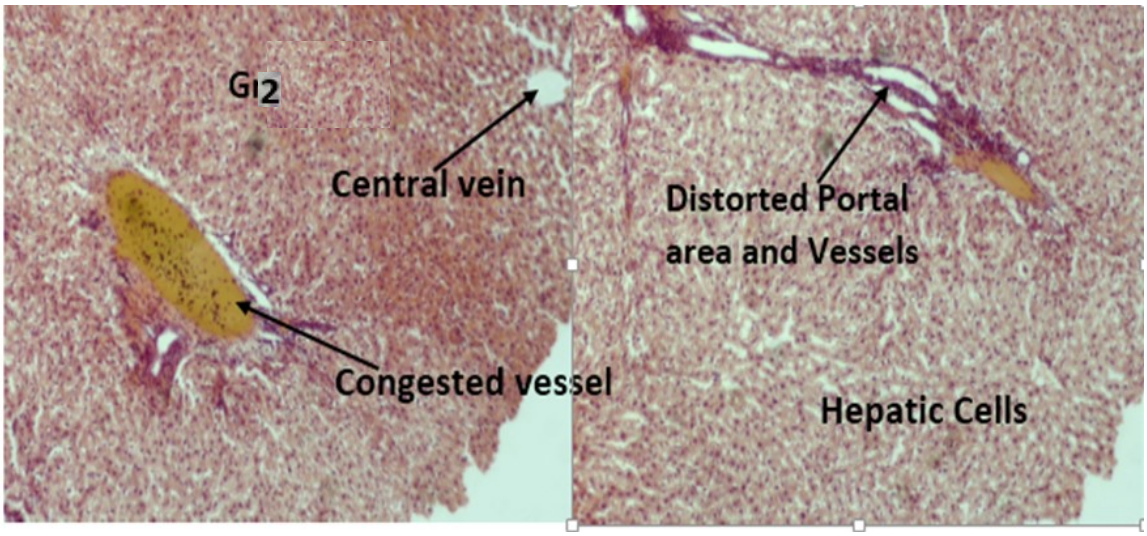

Plate 2: Photomicrograph of a section of liver tissue from albino rats in group 2. Section shows congested vessel with red blood cells, sinusoid congestion with red blood cells, reactive hepatic cell nuclei, and distortion of the portal area by increase in inflammatory cells aggregate within the portal areas. H & E x 40.

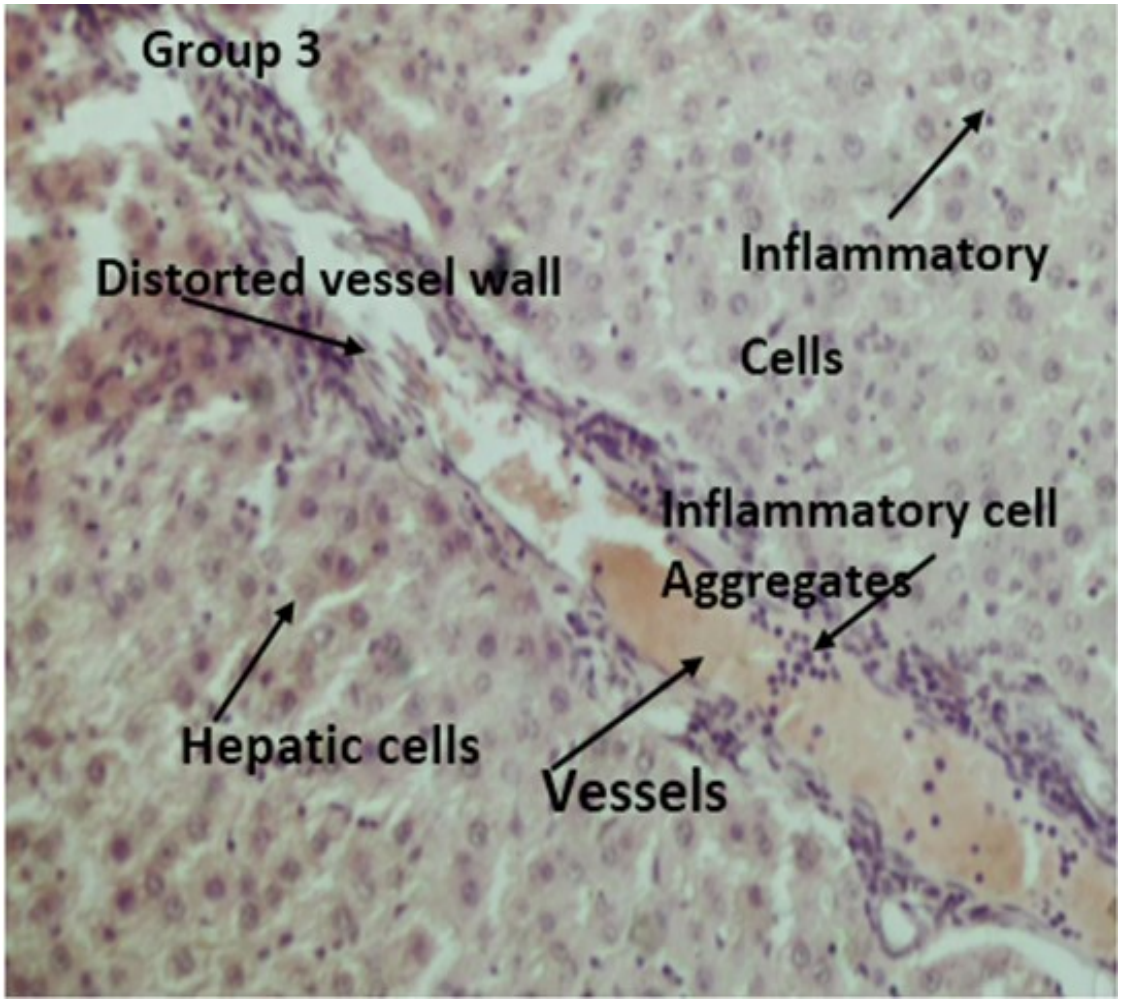

Plate3: Photomicrograph of a section of liver tissue from group albino rats in group 3. Section shows compressed sinusoids with red blood cells and endothelial lining cells, dilated interlobular vessel, mild hepatic vacuolation, increase in inflammatory cells infiltrate within the portal area and vessel wall as well as bile duct distortion. There is increase in inflammatory cells and vessel wall fibrosis and distortion. H & E x 100.

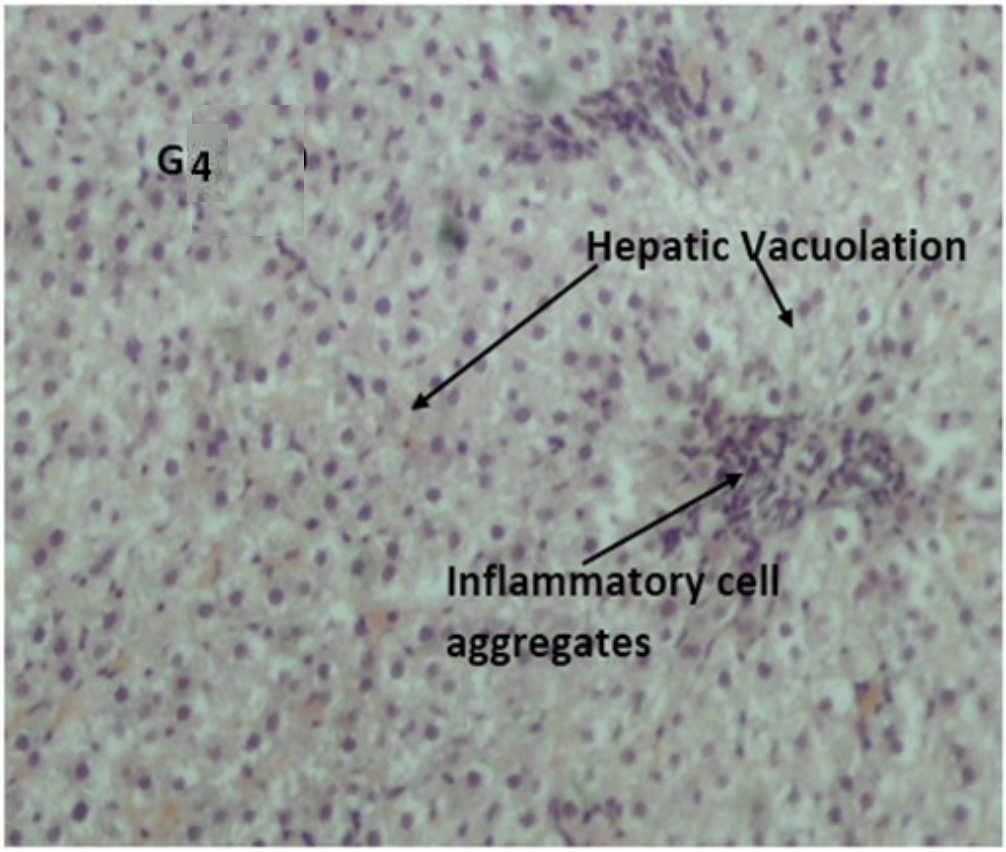

Plate 4: Photomicrograph of a section of liver tissue from albino rats in group 4. Section shows mild hepatic vacuolation, vessel wall infiltration by inflammatory cells, vessel congestion, sinusoids expansion containing red blood cells within its spaces which appears admixed with mild inflammatory cells. Area shows lymphoid aggregates exhibiting hepatic structure distortion mostly within the portal area. H & E x 100.

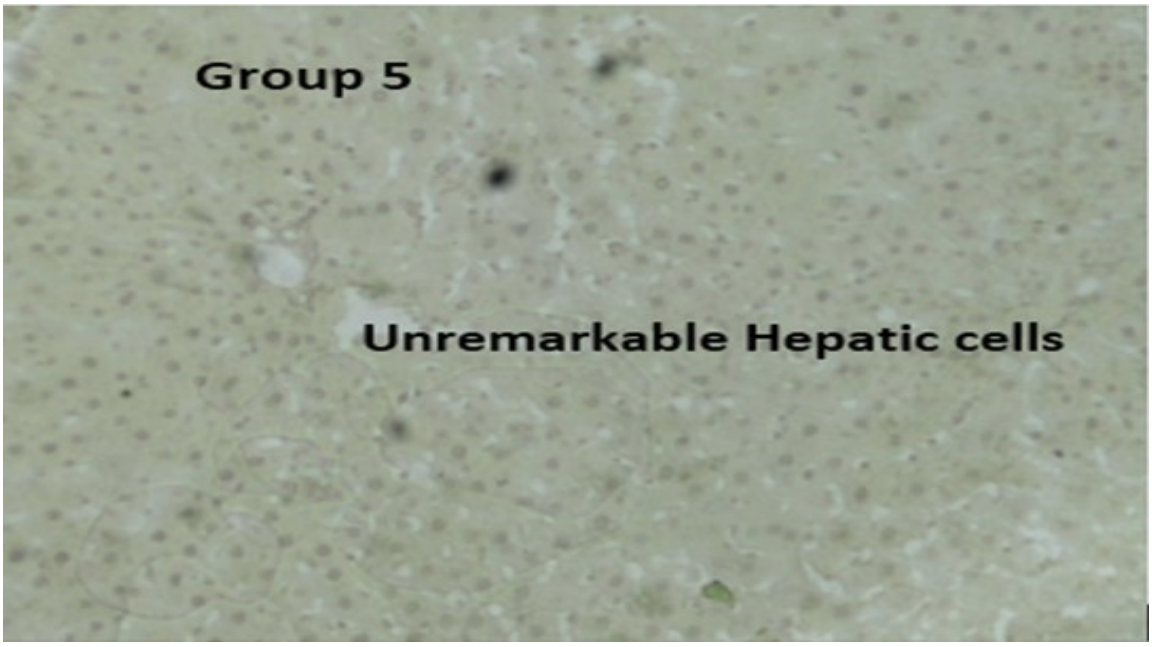

Plate 5: Photomicrograph of a section taken from liver of albino rats in group 5. Section shows unremarkable features and sinusoids expansion.H & E x 100.

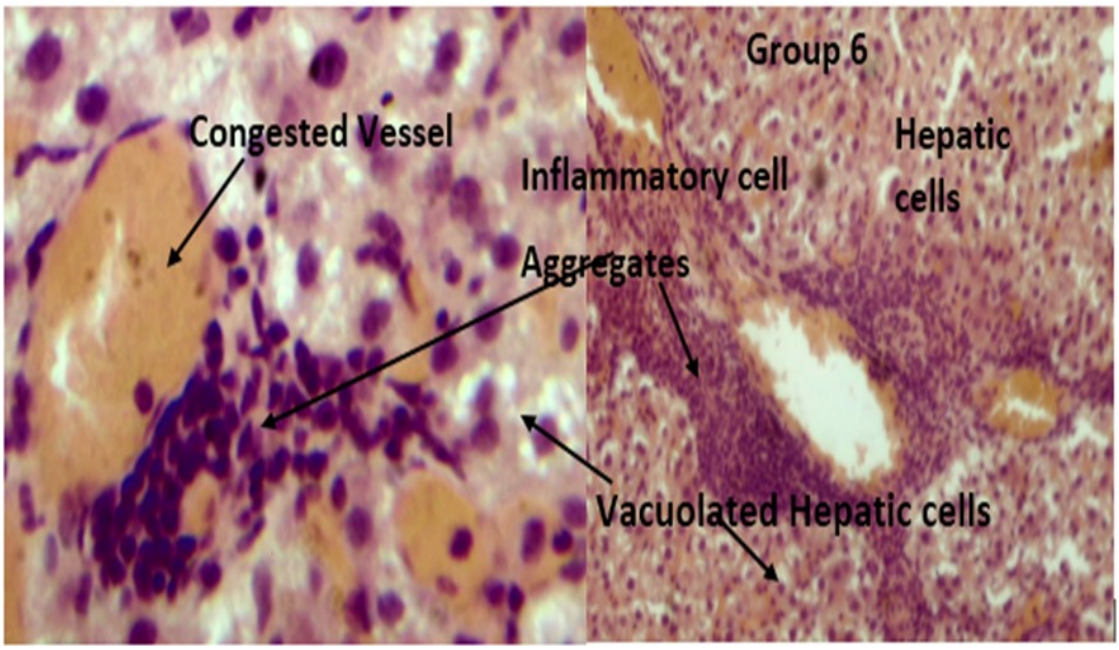

Plate 6: Photomicrograph of a section of liver tissue from group albino rats in group 6. Sectiondistorted hepatic architecture with increase in inflammatory cells aggregate within the portal area and vascular channels. This area shows vessels congestion, sinusoidal congestion, hepatic vacuolation and reactive hepatic nuclei changes as well as increase inflammatory cells admixed with red blood cells within the sinusoidal spaces. H & E x 400.

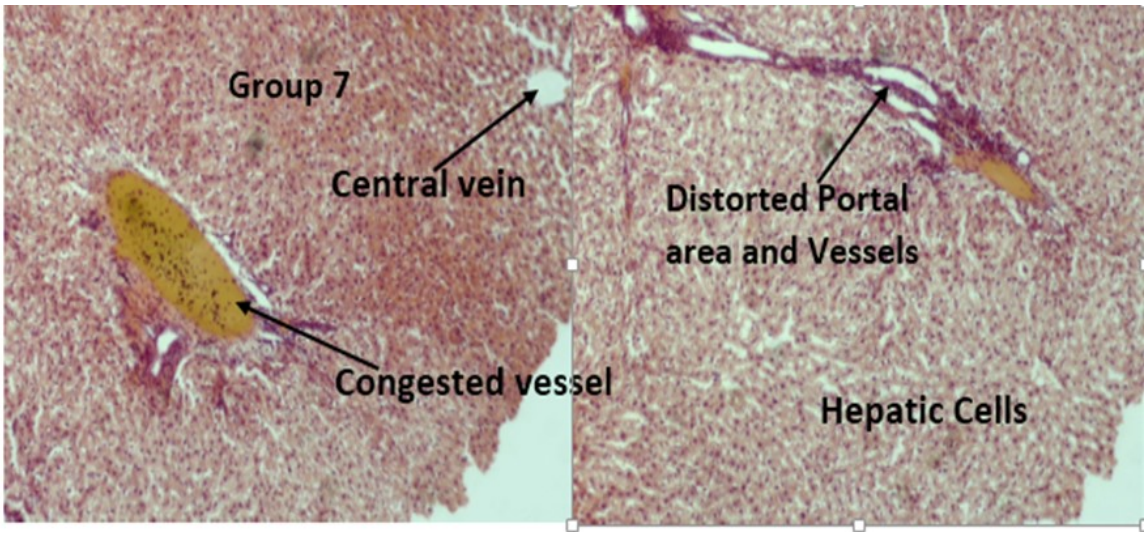

Plate 7: Photomicrograph of a section of liver tissue from albino rats in group 7. Section shows portal area distortion with its muscular wall degeneration. Area shows degenerative hepatic cells, inflammatory cells infiltrate distorting the portal area and hepatic vessels. H & E x 40.

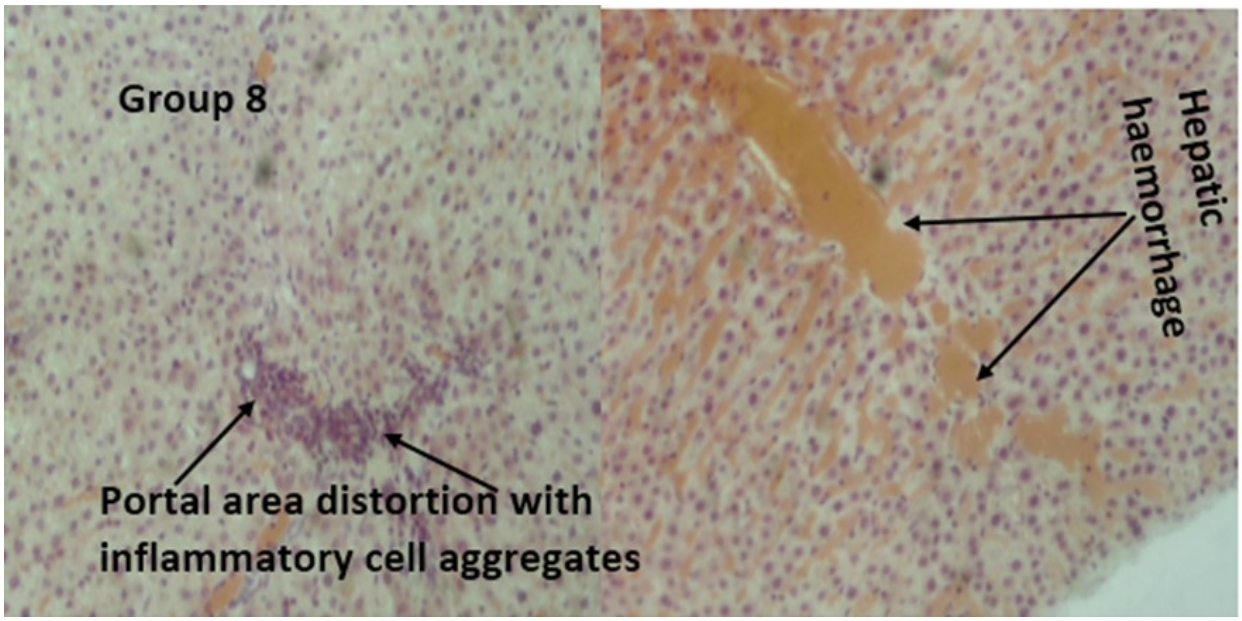

Plate 8: Photomicrograph of a section of liver tissue from albino rats in group 8. Section shows congested vessel with red blood cells, sinusoid congestion with red blood cells, reactive hepatic cell nuclei, and distortion of the portal area by increase in inflammatory cells aggregate within the portal areas. H & E x 100.

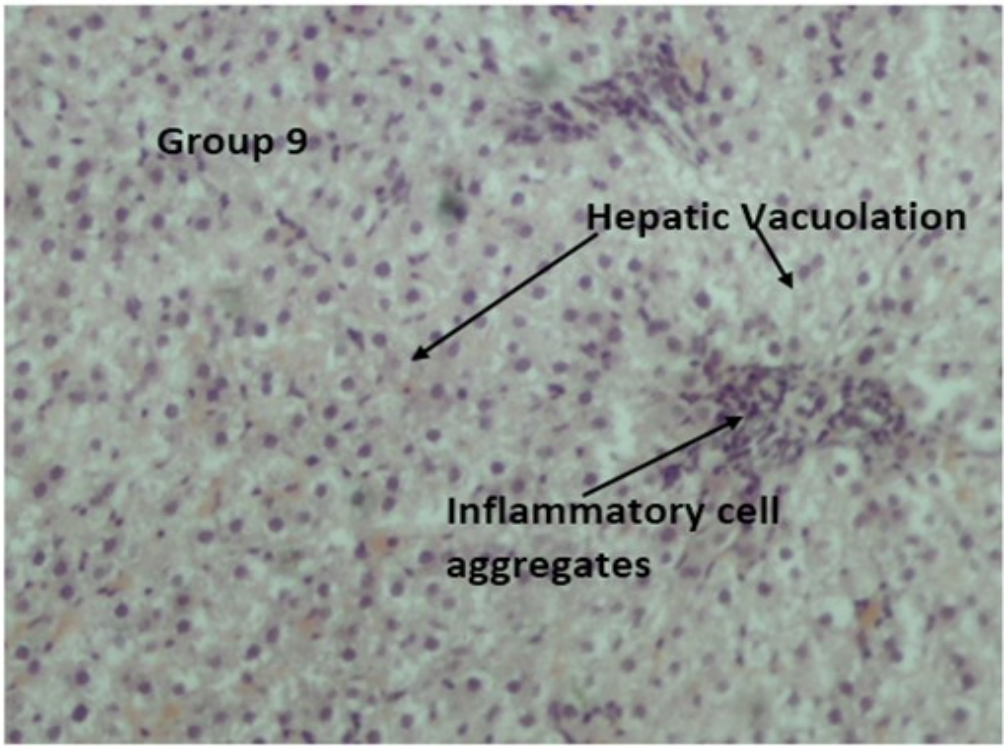

Plate 9: Photomicrograph of a section of liver tissue from albino rats in group 9. Section shows mild hepatic vacuolation, vessel wall infiltration by inflammatory cells, vessel congestion, sinusoids expansion containing red blood cells within its spaces which appears admixed with mild inflammatory cells. Area shows lymphoid aggregates exhibiting hepatic structure distortion mostly within the portal area. H & E x 40.

Discussion

The chronic administration of the investigated herbal bitters formulations elicited a complex toxicological profile in the experimental model, characterized primarily by significant hepatotoxicity, secondary systemic effects, and notable gastrointestinal activity. The integrative analysis of morphological, biochemical, histopathological, and microbiological data reveals a concerning disconnect between potential local gastrointestinal benefits and demonstrable systemic organ damage.

The observed weight dynamics serve as an initial indicator of systemic physiological impact. While the weight gain in most treatment groups paralleled that of the control, suggesting the bitters did not induce acute wasting, the significant terminal weight loss in Group 9 is a critical toxicological signal. In rodent studies, sustained weight loss or reduced weight gain compared to controls is a primary, non-specific indicator of toxicity, often reflecting decreased feed and water intake, malabsorption, or a metabolic shift towards catabolism due to organ dysfunction or systemic illness (OECD, 2008). The specific effect in Group 9 highlights a dose- or formulation-dependent threshold where the adverse effects of the bitters begin to overtake basal metabolic processes, marking this regimen as particularly deleterious.

The serum biochemistry panel provides more specific evidence of target organ toxicity, with the liver emerging as a primary site of injury. The stability of aspartate aminotransferase (AST) levels across groups suggests the absence of widespread, acute necrotic events, as AST is released during severe cellular disruption (Giannini et al., 2005). In contrast, the significant alterations in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) paint a picture of nuanced hepatic insult. ALT is a more liver-specific cytosolic enzyme, and its elevation in Groups 4, 6, and 7 is a canonical biomarker of hepatocellular injury and increased membrane permeability (Ozer et al., 2008). The paradoxical and significant reduction of ALT in Groups 2, 3, and 8 is an unusual finding that warrants caution. While decreased ALT can sometimes reflect improved hepatic health, in this context of concomitant histopathological damage, it more likely suggests an inhibitory effect of the bitters' constituents on enzyme synthesis or activity, rather than a hepatoprotective outcome (Kwo*et al*., 2017).

The most dramatic biochemical perturbations were in ALP levels. The marked elevations in Groups 4 and 6 are strongly indicative of cholestatic injury, biliary epithelial damage, or ductular proliferation, as ALP is highly concentrated in the membranes of bile duct cells (Moss & Henderson, 1999). This aligns with histological observations of bile duct distortion. Conversely, the profound suppression of ALP in Group 7 (32.66 ± 44.47 IU/L) is a severe and anomalous finding. Such extreme reduction may point to significant impairment of hepatic synthetic function, severe malnutrition, or a direct inhibitory effect on the enzyme, which could itself be a toxicological endpoint of concern (Price and Alberti, 1979). The statistically robust p-values ($p<0.001$ for ALP) underscore the biological, not random, nature of these enzymatic disruptions.

Histopathological analysis provides the definitive morphological confirmation of hepatotoxicity. The liver sections from treated groups exhibited a spectrum of lesions consistent with chemical-induced injury. The persistent inflammatory infiltrates in the portal and lobular regions (Groups 2, 3, 4, 6, 7, 8, 9) indicate a sustained immune response to ongoing cellular irritation or damage, implicating the bitters or their metabolites as pro-inflammatory agents (Ishak et al., 1995). Hepatic vacuolation (micro- or macro-vesicular steatosis), observed in multiple groups, signifies a direct disruption of intrahepatocytic lipid metabolism, a common pathway for many hepatotoxicants that can lead to lipid accumulation and oxidative stress (Bessone*et al*., 2018). The widespread vascular congestion and sinusoidal dilation suggest compromised hepatic circulation, potentially leading to localized hypoxia and secondary parenchymal damage.

Most critically, the architectural distortion seen in groups like 6 and 7 represents a failure of the liver's regenerative capacity to maintain its functional micro-anatomy, signifying advanced injury. The correlation between the most severe histopathology (e.g., Groups 4, 6, 7) and the most significant biochemical deviations strengthens the conclusion of direct, formulation-specific hepatotoxicity.

The gastrointestinal and microbiological data present a contrasting, yet subordinate, narrative. The consistent shift from wet to dry fecal output across treatment groups demonstrates a significant antidiarrheal or constipating effect. This is likely mediated by bioactive constituents in the bitters that reduce gut motility, enhance water reabsorption in the colon, or modify secretory processes (Izzo*et al.,* 2016). The isolation and phenotypic identification of probiotic lactic acid bacteria (Lactobacillus pentosus and L. plantarum) from the test subjects is noteworthy. These species are well-documented for their beneficial roles in gut health, including pathogen inhibition, immune modulation, and stabilization of the intestinal barrier (Walter, 2008; Hill *et al.,* 2014). Their presence could be endogenous or potentially promoted by the bitters, possibly contributing to the observed firming of stools. However, this potential local gastrointestinal benefit is critically overshadowed by the concurrent, unequivocal evidence of hepatic injury. This dichotomy underscores a fundamental principle in toxicology: beneficial effects on one organ system do not preclude serious toxicity in another, and systemic safety must be evaluated holistically (Ekor, 2014).

Conclusion

The 30-day subchronic exposure to Bitters A and Bitters B induced significant, dose-dependent hepatotoxicity in the albino rat model. The toxicological profile is multifactorial, encompassing hepatocellular injury (elevated ALT, vacuolation), cholestatic components (elevated ALP, bile duct changes), inflammatory response, and vascular disturbances. While an antidiarrheal effect linked to possible gut flora modulation was observed, it does not mitigate the central finding of liver damage. These results raise substantial safety concerns regarding the chronic consumption of these particular herbal formulations. They highlight the imperative for rigorous, evidence-based toxicological screening of traditional remedies to fully characterize their risk-benefit profiles. Future work should focus on the bioassay-guided fractionation of these bitters to identify the specific hepatotoxic compounds, followed by mechanistic studies to elucidate their pathways of toxicity at the molecular level.

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