Toxicological Evaluation of *Rubia cordifolia* Root Extract on Liver Function in Rat Model

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

School of Pharmacy
BRAC University
October 2024

Declaration

It is hereby declared that.

1. The thesis submitted is my own original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all the main sources of help.

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Approval

The thesis titled "Toxicological Evaluation of *Rubia cordifolia* Root Extract on Liver Function in Rat Model" submitted by Begum Faijunnesa Abrittee 20146037, of Spring, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 13/11/2024.

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Ethics Statement

Ethical approval has been granted by the Biosafety, Biosecurity & Ethical Committee, Faculty of Biological Sciences, Jahangirnagar University, Savar, Dhaka, Bangladesh (Ref No: BBEC, JU/M 2024/ 11 (154)).

Abstract

R. cordifolia Linn., recognized for its extensive medicinal applications, contains a variety of bioactive compounds, yet its safety profile remains inadequately explored. This study investigates the toxicological effects of *Rubia cordifolia Linn*. (Manjistha) on liver function in male white albino (Sprague-Dawley) rats. The study was conducted for 28 days. The rats were divided into three groups: the control group was given 0.5 ml of water, the treated groups were given a at low dose (150 mg/kg) (T1), and a high dose (1500 mg/kg) (T2). Their biochemical analyses were done to evaluate the liver function, and after euthanasia, liver histopathological assessments were done. The results indicated significantly low deterioration in liver function markers (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and gamma glutamyl transpeptidase (GGT)) in both low (150 mg/kg) and high (1500 mg/kg) dose groups compared to controls. Histopathological evaluations revealed minor lymphocytic infiltration in high-dose groups without substantial liver damage, supporting the notion of *R. cordifolia* as a safe therapeutic agent. These findings reaffirm the safe use of Manjishtha in traditional and conventional treatment.

Keywords: *R. cordifolia Linn.,* Manjishtha, toxicological effects, ALP, ALT, GGT, AST, bilirubin, Histopathology, Biochemical analysis, Lymphocytic infiltration.

Dedication

I would like to dedicate this to my wonderful parents, whose love and support have shaped me into who I am today. Forever grateful for your unwavering guidance and endless sacrifices**.**

Acknowledgement

I would like to thank Almighty Allah, who helped me achieve everything I have today.

I would like to show my gratitude to my supervisor Professor Sharmind Neelotpol for guiding me in every step of this work. Without her support and motivation, I could not have passed this phase of my educational journey. A humble appreciation my remarkable friend and teaching assistant, Salwa Mahfuz Noha, whose support, insight, and companionship have consistently provided inspiration and strength. Again, I would also like to express my deepest and most sincere gratitude to A.F.M. Yusuf Haider, Professor and Acting Dean, School of Pharmacy, BRAC University for being an inspiration. In addition, I would like to show my sincere gratitude to all the faculty members of the School of Pharmacy, without whom I could not have accomplished my university journey.

Last but not the least I would like to express my love and gratitude to my parents who are truly deserved, And I appreciate the person I became today.

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List of Acronyms

RCL- *Rubia cordifolia Linn.*

AUB- Abnormal Uterine Bleeding

MNJ- Manjistha

H&E- Hematoxylin and Eosin

SEM- Standard Error of the Mean

ANOVA- Analysis of Variance

NIH- National Institute of Health

BCSIR- Bangladesh Council of Scientific and Industrial Research

AST - Aspartate aminotransferase

ALT- Alanine aminotransferase

ALP - Alkaline phosphatase

GGT- Gamma glutamyl transpeptidase

SGOT- Serum glutamic oxaloacetic transaminase

SGPT - Serum glutamate pyruvate transaminase

SALP - Serum alkaline phosphatase

Chapter 1
Introduction:

The family Rubiaceae contains trees, shrubs, and herbs, with over 450 genera and 6500 species. There are sixty species in Rubia and *Rubia cordifolia Linn*.(Indian Madder) is one of them. It is found up to 3,500 meters above sea level in wet temperate and tropical forests in the lower hills of the Indian Himalayas in the north, the Western Ghats in the south, as well as in Indonesia, Ceylon, Japan, Malay, Peninsula, Java, and Tropical Africa. It possesses extremely long, cylindrical, flexuous roots and thin red bark (Pawar et al., 2011).

R. cordifolia Linn. (RCL) commonly referred to as Manjistha, is extensively utilized in Ayurvedic medicine and is also incorporated in different conventional medicines to treat various medical conditions (Pawar et al., 2011). Its use has been observed in the treatment of renal hemorrhage, allergic purpura, irregular uterine bleeding, metritis and edema (Wen et al., 2022). It is greatly utilized to treat arthritis, rheumatism and dysmenorrhea (menstrual abdominal pain, lumbosacral pain, and bearing-down pain and discomfort in lower abdomen) and other diseases (Masullo et al., 2015;Yuan et al., 2019). A study finding showed that, *Rubia cordifolia Linn*. prevents the growth of ascite cancer cells, melanoma (Itokawa et al., 1993). Rubiae exerts antipsoriatic activity by suppressing cell development (Nyeem & Mannan, 2018). *R. cordifolia L.* extracts have antibacterial properties against the gram-positive strains of *P. aeruginosa.* From a study it was found that RCL extract fights abnormal uterine bleeding (AUB) by improving the coagulation pathway and downregulate some proteins that causes AUB (Wang et al., 2020). The following figure (Figure 1) showed the plant and roots of *R. cordifolia L.*

A B

Figure -1: (A) *Rubia cordifolia Linn*. Plant(Planet Ayurveda, 2019); (B) Rubia Cordifolia Roots (*Rubia cordifolia* Root Extract (Manjistha), 2024)

A review article stated that *R.cordifolia* is rich in more than 100 chemical constituents, mainly including quinones, primarily anthraquinone glycosides, found in the *R.cordifolia* plant (Dosseh et al., 1981). A high concentration of iridoids, 6-methoxy geniposidic acid, manjistin, garancin, and alizarin is noted (Pal, 2024). Together with arborane triterpenoids such as rubiarbonol A, B, C, D, E, and F, rubiprasin A, B, and C were additionally discovered (Nyeem & Mannan, 2018). The coloring material found in *R. cordifolia* roots is a combination of manjistin (xanthopurpurin-2-carboxylic acid) and purpurin (trihydroxy anthraquinone). Additionally, the plant includes rubilactone, mollugin, and dihydromollugin. The red dye alizarin, also known as 1, 2-dihydroxyanthraquinone or mordant red as first made from the madder plant's root. It was the first pigment from nature to be artificially synthesized in 1869 (Khan, 2019).

Although previous studies have largely focused on the bioactive compounds and therapeutic properties of *R. cordifolia* roots , there has been no investigation into the potential toxicity of

the roots. In an in-silico study, dibutyl phthalate that was isolated from *R. cordifolia* fruits was shown to have comprehensive toxicity. Over 1,000 mg/kg dose of *R. cordifolia* marked as the LD50 value (Anantharaman et al., 2015). According to Mapanga and Musabayane (2010), employing medicinal plants to treat a range of ailments may influence how the body functions. It might be risky to use medicinal herbs without understanding their toxicity profiles, which can have unforeseen effects on the liver and other organ capacity to function. Anthraquinones like alizarin and lucidin-3-O-primeveroside make a madder color (MC). It has been demonstrated that MC, a food coloring made from *Rubia tinctorum L.* roots, causes cancer in the liver of rats (Inoue et al., 2009).

1.1 Aim of the study:

The aim of this study is to evaluate the toxicological effects of *Rubia cordifolia* root extract in liver.

1.2 Objectives of the study:

The objectives of the study are to-

(i) review different articles evaluating whether *R. cordifolia Linn.* has any effect on liver.

(ii) evaluate the biochemical and histopathology properties of *R. cordifolia Linn.* to figure out if it produces any toxicity in liver.

Chapter 2

Methodology

The pharmacological and toxicological effects of *R. cordifolia* root extract on liver have been investigated by a literature review and histopathogical analysis. To fulfill the initial purpose of this research, a comprehensive assessment of some available studies was completed till date to analyze the biological effects of *R. cordifolia* roots on the liver. Furthermore, to accomplish the second objective a histopathological study was carefully designed and executed on a male rat model, utilizing different doses of *R. cordifolia* root extract to determine whether the extract exhibits any toxic effects at higher doses, if not at lower dose.

2.1 Literature review:

To achieve a comprehensive understanding of current research trends and findings in this field, search and review are being done on the Elsevier journals , Google Scholar, Scopus, PubMed, ResearchGate and MDPI. For searching relevant information certain keywords such as 'Manjistha, *Rubia cordifolia*, toxicity of *Rubia cordifolia*, therapeutic effects of manjistha, *Rubia cordifolia* constituents, hepatoprotective activity of *Rubia cordifolia*, liver toxicity caused by *Rubia cordifolia*, *Rubia cordifolia* and liver' were used.

2.2 Collection of plant material:

The roots of *R. cordifolia* were freshly collected from Sylhet, Bangladesh. The plant was authenticated at BCSIR, Dhaka with specimen number MGJRC-1 and a voucher specimen is deposited at Jahangirnagar University (Humbare et al., 2022).

2.3 Procedure of root extraction:

Analytical-grade ethanol solvents and reagents had been used for the experiment. Extracts of powders were prepared by solvent(ethanol) extraction process. In brief, the collected materials were thoroughly washed in water, cut into smaller parts, chopped and shed dried at 35º – 40º C for a week and pulverized in electric grinder to get extractable powder. 1g of powder was then extracted in 4.5ml of ethanol (96%). The collected mixture of active constituent with ethanol was dried with the help of a rotary evaporator under reduced pressure and the final root extract was obtained (*Plant Solvent Extraction Method Using Ethanol: 3 Steps - Cole-Parmer*, 2020)

2.4 Preparation of drug doses:

After extracting the *Rubia cordifolia* root powder, through oral gavage method the drug was delivered as doses of 150 mg/kg for the lower dose and 1500 mg/kg for the higher dose.

2.5 Study design:

Male white albino (Sprague-Dawley) rats were procured from Pharmacology lab Jahangirnagar University, Savar for experiments. The rats were maintained under standard laboratory conditions. The rats were divided into 3 groups, where each group consisted of 6 rats weighed between 70 - 90g. The control group (C1) were given 0.5 ml of water, and the last twelve rats were taken for treated groups: (T1) at low dose (150 mg/kg), and (T2) high dose (1500 mg/kg). All the experimental rats were housed with ad libitum access to food and water and were caged in a (60 ×38 ×20 cm3) plastic cage under a controlled temperature of 21±5˚C with a 12-h light/dark cycle. All the doses were administered once per day by oral gavage for 28 days. All the doses and saline were administered 30-40 minutes before taking the body weight. All the doses were administered

between 10:00 am. and 2:00 pm every day. Initial body weights were recorded, and weight measurement was continued throughout the study period with an interval of four days. After the completion of the tests, each rat was euthanized following the study protocol and prepared for histopathological test (Neelotpol et al., 2024).

2.6 Biochemical Analysis:

To determine the enzymatic activities of the liver in both control and experimental groups, the level of clinical biochemistry was assessed using aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and gamma glutamyl transpeptidase (GGT). The activity of all serum enzymes was assessed utilizing commercially available kits in accordance with the manufacturer's guidelines (Hasan et al., 2017).

2.7 Histopathology:

Three groups of rats were examined in this experiment. The dose selection for the drug was based on acute toxicity test. The rats were put under sedation with the purpose of euthanasia on the 28th day of the trial with an overdose of ketamine (500 mg/kg). Subsequently, euthanasia occurred and was confirmed with the “toe pinch” method. The rat liver was separated from the body, weighed, and kept in 10% neutrally buffered formalin. A few days later, the liver was routinely processed using the Hematoxylin and Eosin (H&E) staining procedure for histological examinations. Eventually, a light microscope (Olympus, CX43, Japan) was used to visualize those tissues. Using an Olympus D22 camera, photomicrographs of the liver portions were obtained (Neelotpol et al., 2024; Chandrashekar et al., 2018).

2.8 Statistical Analysis:

The data analysis was conducted using a one-way ANOVA, a statistical method using portable IBM SPSS statistics version 19. All the values were expressed as Mean±SEM. The significance level indicated by the *p*-values denote how likely it is that the observed differences occurred by chance. A two-tailed *p*-value of <0.05 was considered statistically significant. W4°C Hile the independent t-test was used for comparisons when the data could be shown to be normally distributed.

2.9 Ethical Approval:

To ensure the highest possible standards of animal welfare, all procedures followed the ethical criteria set by the BRAC University Institutional Review Board (IRB)(Ethical clearance no: BBEC, JU/M 2024/ 11 (154)). Alongside, the animals were treated with empathy throughout the experimental period and maximum care was taken. In case of handling rats the internationally accepted guide for the care and use of laboratory animals, published by the US National Institutes of Health (NIH Publication No. 85-23, Revised in 1985) was followed.

Chapter 3

Result & Discussion:

3.1 Literature Review

Upon reviewing the current study on *Rubia cordifolia Linn*.(Manjistha), we have obtained some valid data concerning the liver function associated with the plant's root from several reliable sources. In clinical and preclinical settings, elevated ALT and AST levels are frequently utilized as markers of liver damage (Thakur et al., 2024). A reduction in these enzymes indicates an inhibition of hepatocellular damage (*Clinical Course and Diagnosis of Drug Induced Liver Disease*, 2012). Numerous herbal extracts, such as those derived from Rubia cordifolia (reported for its anti-inflammatory and hepatoprotective attributes), have demonstrated the ability to reduce the release of ALT and AST by lowering oxidative stress or inflammatory pathways associated with hepatocyte injury (Rao et al., 2006). ALP levels are used to assess liver bile duct function and are often elevated in cholestatic liver conditions (*Evaluating Liver Test Abnormalities: Tests of Cholestatic Injury*, n.d.). The decrease in ALP levels in the MNJ-treated groups may suggest an effect on bile secretion or a reduction in cholestasis (Levitt et al., 2022). A reduction in ALP could also reflect a decrease in liver tissue inflammation, which often results from systemic or localized stress. Previous studies on hepatoprotective herbs have shown that such plants can modulate bile flow and reduce the pathological elevation of ALP (Gostyńska et al., 2024). Increased bilirubin levels, as noted in the control group, generally signify hepatic impairment in the metabolism or elimination of bilirubin (Guerra Ruiz et al., 2021). The statistically significant decrease in bilirubin levels in the MNJ-treated groups indicates that the treatment may have positively influenced hepatic detoxification mechanisms, either by improving bilirubin conjugation or excretion (Saleem & Naseer, 2014).

The results section presents key findings on the biochemical and histopathological properties and a slight idea about the toxicity profile of *Rubia cordifolia* Linn.

The following graph (Figure 2) showed the body weight gain of the treated group rats compared with the rats of control group.

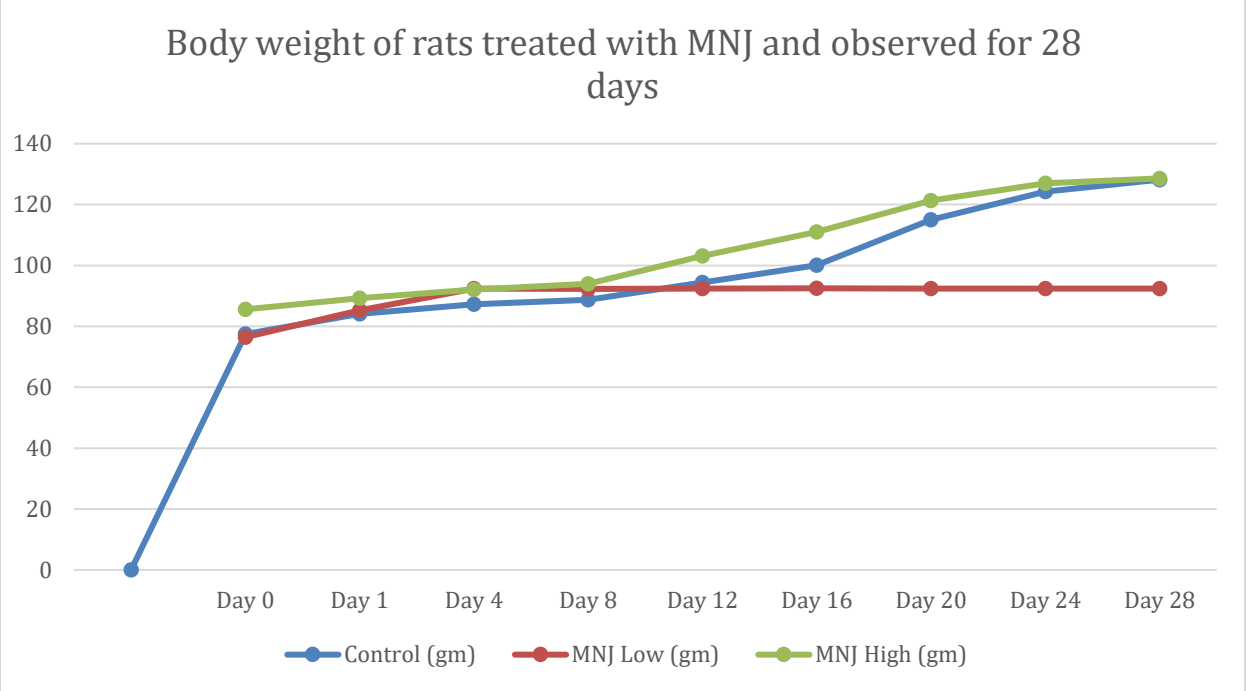


Figure 2: Graphical representation of the body weight of rats treated with Manjistha(MNJ)

Figure 2 summarizes the mean weights (in grams) of subjects across 3 treatment groups over a 28-day period. The control group shows a gradual increase in weight, starting at 75 g on Day 0 and reaching 120.85 g by Day 28. In contrast, both MNJ Low and MNJ High groups demonstrate a more pronounced weight gain, particularly evident from Day 12 onward, with MNJ High reaching 124.2 g by Day 28. Notably, the MNJ High group consistently exhibits higher mean weights compared to the control and MNJ Low groups throughout the study. The data, presented as mean

± SEM, indicate significant differences in weight gain, particularly in the MNJ-treated groups, suggesting enhanced growth or efficacy of the treatment over the control.

In a study conducted by Lodia s. and Kansala L. (2012), The initial body weights of male rats consisting of 6 groups were 25.4±0.43, 26.8±0.51, 25.5±0.28, 25.4±0.45,and 27.2±0.47, 27.4±0.64 g respectively. The alcoholic root extract of *Rubia cordifolia*, given at doses of 50 and 100 mg/kg body weight, exhibited no significant impact on the body weight of mice compared to control animals.

3.2 Biochemical Assay

The following table (Table 1) showed the biochemical changes of liver function tests.

Table 1: Effects of *Rubia cordifolia* root on liver function tests

Parameters	Control	MNJ Low	MNJ High
	Mean (±SEM)		
Bilirubin	0.17 (0.007)	0.15 (0.002)	0.16 (0.008)
ALT	99.86 (7.85)	64.40 (5.47)***	68.40 (5.28)**
AST	173.71 (14.94)	96.00 (6.81)***	102.80 (8.63)***
ALP	257.71 (19.97)	171.40 (12.64)***	171.60 (8.18)***
GGT	17.29 (2.15)	17.00 (1.52)	9.8 (0.66)

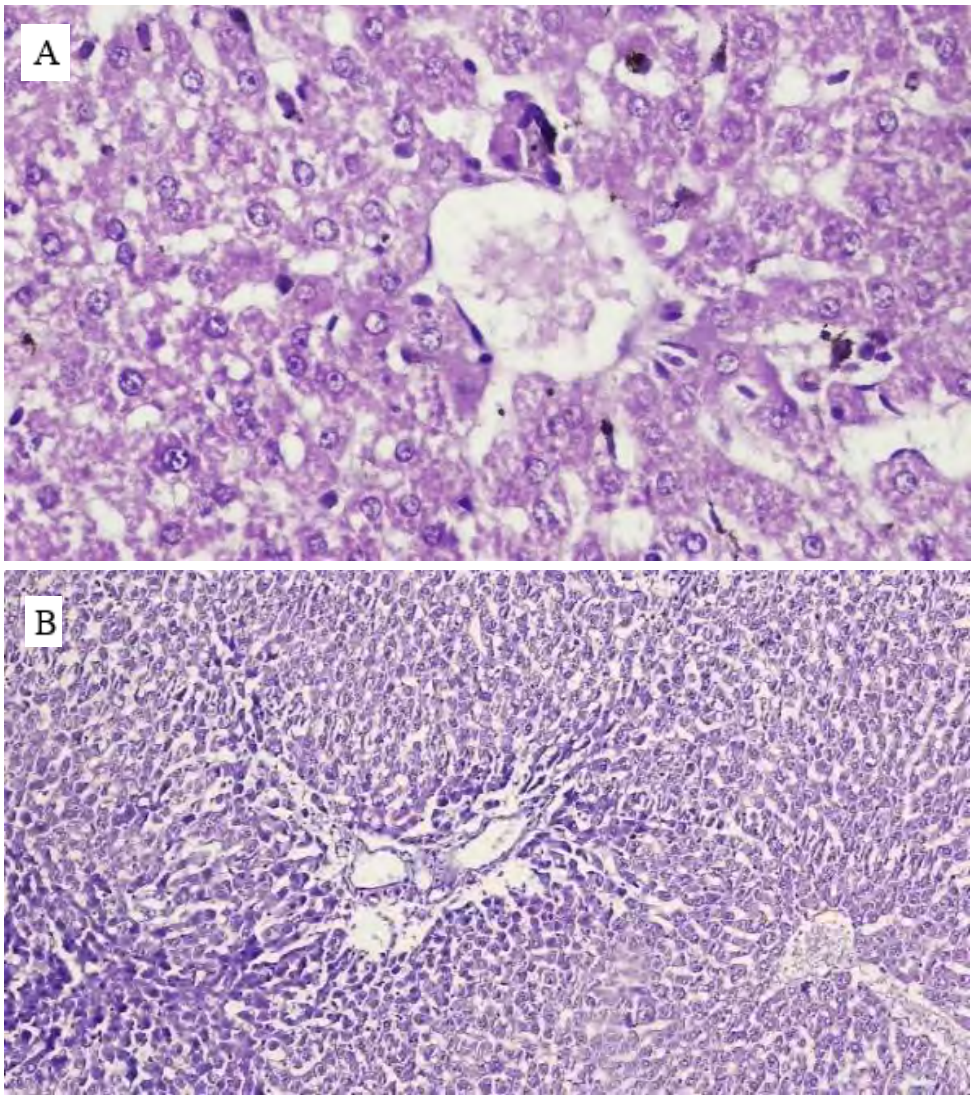
N.B: Data were analysed by one way ANOVA. Values are expressed as Mean±SEM, n=6. P<0.05=*, p<0.01=**, p<0.001=***: MNJ treated groups are significantly different from control group of rats.

Here the obtained data emphasizes that the MNJ treated groups show statistically significant improvements compared to the control group in the measured parameters, with the level of significance indicated by the p-values. The control group shows elevated levels of ALT (99.86), AST (173.71), ALP (257.71), and bilirubin (0.17), indicating potential liver stress.

In comparison, both MNJ treatment groups demonstrate significantly lower enzyme levels; particularly, the MNJ Low group shows a significant reduction in ALT (64.40), AST (96.00), and ALP (171.40), with statistical significance marked by asterisks. The MNJ High group also exhibits reduced enzyme levels, particularly in ALT (68.40) and AST (102.80), suggesting a protective effect on liver function compared to the control. GGT levels remain relatively consistent across groups, with MNJ High showing a marked decrease (9.8), which could indicate enhanced liver health.

3.3 Histopathological Assessment:

The following figure (3) showed the histopathological changes of the rat liver.



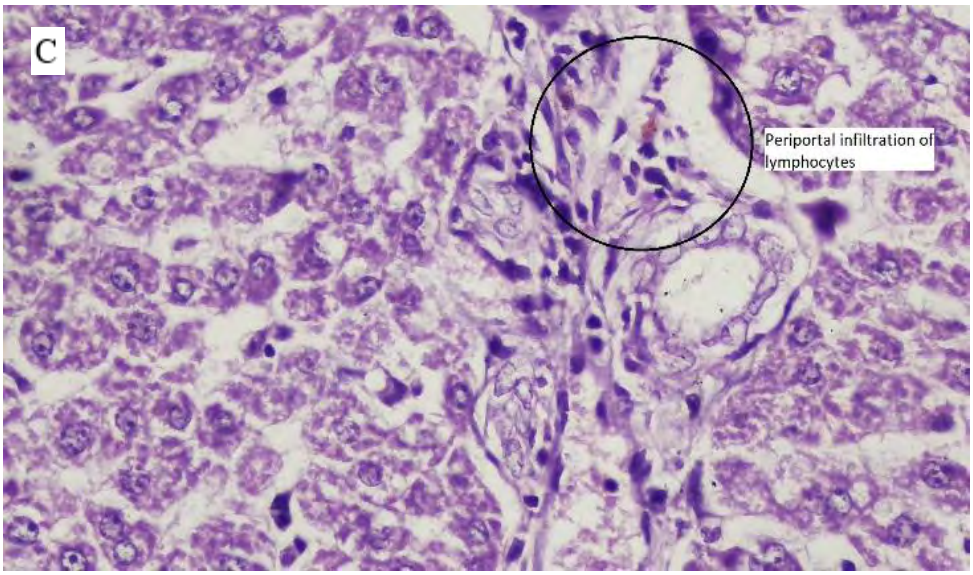


Figure 3. Histopathology of the rat liver (A) Control group (10X) (B) *Rubia cordifolia* 150 mg/kg treated (H and E,10X) (C). *Rubia cordifolia* 1500 mg/kg treated (H and E,40X)

In the histopathology of the rat liver the control group exhibited typical histological features without any abnormal changes, establishing a baseline for comparison. The treated groups provided insightful data into the effects of manjistha on tissue morphology.

The liver of the first treated group, which was given a low dose (150mg/kg) of Manjistha, did not exhibit any notable alterations. The absence of notable changes in histopathology implies that Manjistha at low doses does not have a negative impact on these organs, supporting the idea that it is safe at lower therapeutic concentrations. The lack of significant alterations in the histological framework suggests that the low dosage is well-tolerated and may have positive effects without the chance of any adverse ones.

On the other hand, the second treated group, which was given a larger dose (1500mg/kg) of manjistha, showed some slight changes. The slight lymphocytic infiltration that was observed in the periportal area of the high-dose group in the liver is consistent. Once more, moderate immunological activity rather than substantial liver damage or malfunction is usually linked to this reaction. The liver is an essential organ for metabolism and detoxification, and the fact that it can tolerate such slight alterations shows how resilient the liver is to medicinal substances like manjistha.

According to Siddique et al. (2022) a study regarding CCl4 induced hepatotoxicity in Swiss albino rats showed the presence of inflammatory cells and constricted sinusoids, which indicated apparent hepatocyte enlargement and was the only change from the group that received standard saline treatment but when treated with 100 mg/kg of *Rubia cordifolia* revealed no necrosis and feathery degeneration in the central area and the group treated with 200mg/kg of *Rubia cordifolia* liver sample showed a variety of minor liver damage symptoms which ensured its hepatoprotective activity. In another study a CCl_4 induced rat liver containing the increased levels of SGOT, SGPT, SALP were significantly reduced (50: $P < 0.01$; 100, 200: $P < 0.001$) in the groups treated with 50, 100, and 200 mg/kg of rubiadin (Rao et al., 2005). Overall, the MNJ treatments appear to significantly improve liver function parameters compared to the control group. A study utilizing Swiss albino mice was used to evaluate raw extracts of *R. cordifolia* fruits against acute toxicity. All the findings pointed to the necessity of considering the toxicity profile and the harmful potential that can be caused by using the root extract of *R. cordifolia* on animal liver (Anantharaman et al., 2015).

Overall, the results show that manjistha has a good safety profile, with neither treated group experiencing any serious side effects. Given that the plant has historically been used in traditional and conventional medicine to treat a variety of illnesses, including inflammation and skin diseases, the findings highlight the plant's potential as a therapeutic agent.

Lastly, the mild histological alterations seen at increased dosages imply that manjistha might boost some immune response but that has minor negative effects on the liver. This suggests that manjistha could be a good addition to herbal as well as conventional pharmacotherapy, given that its dosage is carefully regulated to prevent overdosing.

Chapter 4

Conclusion

In conclusion, the qualitative microscopic findings of this investigation emphasize the toxicological potential of manjistha (*Rubia cordifolia Linn*.), highlighting its significant safety profile for clinical applications. These results not only validate the historical significance of Manjistha but also encourage more advanced research to explore its pharmacological properties and mechanisms of action, paving the way for enhanced herbal interventions in healthcare.

4.1 Limitations of the Study

Although this research employed the usage of international standard instruments and protocols, while conducting this research it has come to light that some of the components of this research may need further refinement. The study used only male white albino (Sprague-Dawley) rats, which may not be representative of the responses in other strains or even in female animals, limiting the applicability of the findings is one of them. Another limitation would be that while two dosages were tested, additional doses may be necessary for better understanding the dose-response relationship and the threshold for toxicity.

4.2 Future Prospects

Future research should concentrate on determining the precise mechanism of action by which *Rubia cordifolia* carries out its hepatoprotective benefits. Like any therapeutic drug, more investigation and clinical testing are necessary to completely clarify its safety and efficacy at various dosages for various patient populations. By investigating how it interacts with other medicines and how effective it is in various groups of people, its pharmacological potential can be

explored. Through collaborative studies that combine both clinical trials and in vitro investigations, Significance of *R. cordifolia* in integrative healthcare practices may eventually be expanded and the development of standardized medicinal products will occur.

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