

RESEARCH ARTICLE

**PROTECTIVE EFFECT OF *FLACOURTIA INDICA (BURM.F) MERR.*
IN METHOTREXATE INDUCED HEPATOTOXICITY**

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ABSTRACT

The acute toxicity studies of petroleum ether extract of aerial parts of *Flacourtia indica* (Burm.f.) Merr. was done in albino mice. The extract up to a dose of 1750 mg/ kg was tolerated in mice. The extract was further evaluated for hepatoprotective effect in methotrexate ((20mg/kg ip for 1 day) induced hepatotoxicity in rat models. MTX induced hepatotoxicity characterized by significant alterations in marker enzymes for liver function and oxidative stress were observed. The *Flacourtia* extract treatment in a dose of 350mg/kg orally for 5 days significantly improved the level of marker enzymes for liver function and oxidative stress; which was evident in histopathology also where a relative degree of reversal of methotrexate induced necrosis was observed.

KEYWORDS: *Flacourtia indica*, *methotrexate*, *hepatoprotective*, *enzymes*.

INTRODUCTION

Flacourtia indica, a small tree with axillary thorns and often with tufts of branched thorns on the stem has been widely distributed throughout India. It has been used for various ailments in traditional system of medicine. Fruits and aerial parts are used as appetizing and digestive, diuretic, in jaundice and enlarged spleen ^(1, 2). Though the plant has been used in the traditional medical practice in our country, scientific studies to describe its ethno medical values are lacking. . Our study is an attempt to determine the LD50 of the drug and to evaluate the protective effect of the petroleum ether extract of the aerial parts of the plant in methotrexate induced hepatotoxicity.

MATERIALS AND METHODS**A) Plant Study:**

i. *Plant collection and preparation of extract:*
The plant *Flacourtia indica* (Burm.f.) Merr. was identified, collected in bulk from Thiruvananthapuram district of Kerala during the month of June. Washed well to remove the soil and dust. They were cut into small pieces and spread in sheets. It was sun dried for 3 days and one day in a hot air oven at 40°C, powdered and sieved through a No: 10 sieve and the coarse powder was used for extraction.

The coarse powder of the drug was extracted by maceration using petroleum ether (40-60°C). For this, 500 g of the powder material of plant was taken in a 2000ml flat bottomed flask and macerated using petroleum ether (40-60°C) for seven days. It was then filtered and the collected extract was evaporated and dried and the percentage yield was calculated.

ii. Preliminary phyto-chemical screening of the extract: Phyto-chemical investigation of the extract was carried out to confirm the presence of secondary metabolites by various qualitative tests for alkaloids, carbohydrates, proteins and amino acids, steroids, fixed oils and fats, phenolic compounds and tannins, and flavanoids⁽³⁾

B) Animal Study:

i) Animal maintenance and care.

Young male and female albino rats (Wistar strain) weighing 100-200g and 4-6 months of age and adult male albino mice (Swiss strain) weighing 20-25g were used for the study. The animals were fed on standard synthetic pellet and water ad libitum.

The animals were maintained under standard conditions of relative humidity, 12 hours light-dark cycle, adequate ventilation and ambient room temperature. The study protocol was approved by the Institutional Animal Ethics committee, Medical College, Thiruvananthapuram (IAEC No. 04/70/2009/MCT).

ii. Acute toxicity studies⁽⁴⁾

Healthy albino mice of either sex weighing between 20 and 25g, starved overnight were used for acute toxicity studies. The animals were grouped into 5 groups of 6 animals each.

In a previous study, petroleum ether extract of *F.indica* (Burm.f) Merr. in a dose of 1500mg/kg was used to evaluate its hepatoprotective effect⁽⁵⁾. The dose selected in the present study was based on this study. Hence, the dose selected for the acute toxicity study for the 5 different doses were 750mg/kg, 1000mg/kg, 1250mg/kg, 1500mg/kg and 1750mg/kg. The drug was prepared as a suspension in 1% CMC and was given orally. The control group received the vehicle (10ml/kg body weight). After the drug administration, the animals were observed closely for 4 hours and intermittently up to 24 hours. The number of animals which died at the end of 24 hours was noted.

iii. Selection of dose of extract and methotrexate for the hepatoprotective study.

In the acute toxicity study, the extract up to a dose of 1750 mg/kg in albino mice (up to 24 hrs) did not cause any death in animals. Hence, 1/5th of the maximum tolerated dose (1750mg/kg), ie 350mg/kg was used for the protective effect of *Flacourtia indica* in methotrexate induced toxicity.

Methotrexate was obtained from Cadila Pharmaceuticals and was dissolved in normal saline. The extract being insoluble in water, a suspension in 1% CMC was prepared and was used for the study. Methotrexate (MTX) was given as intra-peritoneal injection in a dose of 20mg/kg and the extract was administered orally.

iv. Evaluation of hepatoprotective activity.

Experimental animals were randomly selected and were divided in to 4 groups of 6 animals each.

- 1) **Group 1:** (served as control); they received normal saline for 1 day (1ml/kg ip).

- 2) **Group 2:** (served as model for acute methotrexate toxicity); they received methotrexate (20mg/kg ip) for 1 day..
- 3) **Group 3:** (This group served as treatment group); animals of this group received methotrexate (20 mg/kg ip) for 1 day followed by Flacourtia extract (350mg/kg orally) for next 5 days.
- 4) **Group 4:** (This group was included to evaluate whether the pathology of methotrexate induced toxicity was reversed in a period of 5 days without any treatment); animals of this group received methotrexate (MTX) (20mg/kg ip) for 1 day followed by normal saline 1ml/kg for the next 5 days.

v. Biochemical Investigations.

After the treatment period, blood was collected by retro-orbital bleeding of animals under ether anaesthesia. The serum was separated and was used for the enzyme level estimation of Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP). After this, the animals were sacrificed, liver was isolated, washed with normal saline, weighed and organ to body weight ratio (wet weight) was calculated. One gram of this tissue was homogenized using 4 times its weight of 140Mm phosphate buffer of pH 7.4. It was then centrifuged and the supernatant was used for antioxidant enzyme assays.

Biochemical parameters namely serum ALT, AST and ALP of four different groups were measured to assess the protective activity of the extract using auto analyzer. The photometric determination of ALT, AST and ALP were based on the reference method of International Federation of Clinical Chemistry (IFCC). Standard kits were supplied by Merck Specialities

Private Limited, Mumbai. The level of ALP, AST and ALP levels in the given serum samples were expressed in Units/Litre⁽⁶⁾.

Using the supernatant of the centrifuged homogenate of the liver tissue, the SOD level⁽⁷⁾, Catalase level⁽⁸⁾, MDA level⁽⁹⁾ and GSH levels⁽¹⁰⁾ were determined.

v. Histopathological Examination.

Liver specimens were fixed in 10% formalin, dehydrated with ascending grades of ethyl alcohol, embedded in paraffin wax, sliced in a rotary microtome, stained with haematoxylin/eosin and studied microscopically to detect the changes in hepatic texture and their photomicrographs were taken. Inflammatory lesions of the sample were graded and that of different groups were compared.

vi. Statistical Analysis: The biochemical data were analysed using Tukey method. The experimental data were expressed as mean \pm SEM. Independent sample t-test were carried out for statistical comparison. Values < 0.05 were considered to be statistically significant.

RESULTS

The percentage yield of the petroleum ether extract of Flacourtia indica (Burm.f.) Merr. was found to be 0.93%w/w. The extract showed the presence of flavanoids, phenolic compounds, steroids, and triterpenoids in the preliminary phytochemical screening.

In the acute toxicity study, no mortality was observed on administering the drug up to 1750mg/kg. Hence the LD50 of the extract is above 1750mg/kg body weight in albino mice. A dose of 350mg/kg (ie 1/5th of 1750mg) was selected for the subsequent studies.

Control groups showed an organ to body weight ratio of 0.0312 ± 0.0001 and in methotrexate treated group, the value increased significantly (0.0378 ± 0.0012 , $p < 0.0001$), but it was decreased significantly (0.0327 ± 0.0004 , $p < 0.0001$) in the group treated with *Flacourtia* extract. While, there was no significant difference in the value of untreated group (group 4) as compared to MTX treated group.

The control samples showed an ALT level of 34.50 ± 1.45 U/L, while the MTX toxicity induced groups showed values as 53.57 ± 1.17 U/L and it was statistically significant ($p < 0.0001$). Drug treated group showed a significant decrease in serum ALT values (42.00 ± 0.073 U/L, $p < 0.0001$) as compared MTX toxicity group (group 2). The animals which were left untreated and sacrificed on 6th day did not show significant decrease in the ALT values. Results are shown in the Table-I.

The control samples showed an AST level of 173.67 ± 4.42 U/L, while the MTX toxicity induced group showed values as 250.33 ± 1.12 U/L and it was statistically significant ($p < 0.0001$). Drug treated group showed a significant decrease in serum AST values (203.67 ± 3.33 U/L, $p < 0.0001$) as compared MTX toxicity group (group 2). The animals which were left untreated and sacrificed on 6th day did not show significant decrease in the AST values. (Results shown in the Table-I).

The control samples showed an ALP enzyme level of 296.67 ± 3.01 U/L, while the toxicity induced groups showed values as 330.00 ± 3.13 U/L and it was statistically significant ($p < 0.0001$). Drug treated group showed a significant decrease in serum ALP values (320.5 ± 3.78 U/L, $p < 0.0001$) as compared acute toxicity group (group 2). The animals which were left untreated and sacrificed on 6th day did not show significant decrease in the ALP values.

Table : I
Protective effect of *Flacourtia indica* against methotrexate induced hepatotoxicity in albino rats
Serum ALT, AST and ALP values and Organ to Body Weight Ratio.

Groups	Treatment Dose/route	ALT(U/L) Mean \pm SEM	AST(U/L) Mean \pm SEM	ALP (U/L) Mean \pm SEM	O/BW ratio Mean \pm SEM
1	Control (Normal saline) 1ml/kg,ip	34.50 ± 1.45	173.67 ± 4.42	296.67 ± 3.01	0.0312 ± 0.0001
2	Methotrexate 20mg/kg i.p treated for 1 day	$53.67 \pm 1.17^{\#}$	$250.33 \pm 1.12^{\#}$	$330.00 \pm 3.13^{\#}$	$0.0378 \pm 0.0012^{\#}$
3	Methotrexate 20mg/kg i.p for 1 day + extract 350mg/kg 1% CMC suspension for 5 days	$42.00 \pm 0.73^{**}$	$203.67 \pm 3.33^{**}$	$320.5 \pm 3.78^{**}$	$0.0327 \pm 0.0004^{**}$
4	Methotrexate 20mg/kg i.p for 1 day followed by normal saline 1ml/kg for 5 days	$53.00^{\#}$	$236.5 \pm 2.38^{\#}$	$341.83 \pm 2.63^{\#}$	$0.0373 \pm 0.0006^{\#}$

n=6, $\#p < 0.0001$ as compared to control (Group 1), $^{**}p < 0.0001$ as compared to MTX toxicity group (Group 2).

Acute methotrexate toxicity induced group showed a significant reduction in SOD level($38.64 \pm 1.12, p < 0.0001$) as compared to control animals(55.67 ± 0.9066). The drug treated group significantly prevented the reduction in this SOD level($47.91 \pm 0.395, p < 0.0001$) as compared to acute toxicity group.

Acute methotrexate toxicity induced group showed a significant increase in catalase activity ($3.475 \pm 0.0501, p < 0.0001$) as compared to control animals(2.79 ± 0.02). The drug treated group significantly prevented this elevation in catalase level ($0.0357 \pm 0.008, p < 0.002$) as compared to acute toxicity group.

Acute methotrexate toxicity induced group showed a significant increase in MDA level($2.825 \pm 0.0419, p < 0.0001$) as compared to

control animals(1.4517 ± 0.0344). The drug treated group significantly prevented this elevation in MDA level($2.03 \pm 0.035, p < 0.0001$) as compared to the acute toxicity induced animals

Acute methotrexate toxicity induced group showed a significant decrease in GSH level ($0.0667 \pm 0.003, p < 0.0001$) as compared to control animals (1.9767 ± 0.0058). The drug treated group significantly prevented this decrease in GSH level ($0.16215 \pm 0.0086, p < 0.0001$) as compared to the acute toxicity induced animals. The animals which were left untreated after MTX challenge and sacrificed on the 6th day did not show any reversal of the levels of SOD, Catalase, MDA and GSH as compared to the MTX toxicity group (group 2). The results are shown in table II.

Table-II
Protective effect of *Flacourtia indica* against methotrexate induced hepatotoxicity in albino rats
Liver SOD, Catalase, MDA and GSH levels

Groups	Treatment Dose/route	SOD u/mg of protein	Catalase nmols of H ₂ O ₂ decomposed/mg of protein/mt	MDA nmols/mg of protein	GSH nmols /mg of proteins
1	Control (Normal saline) 1ml/kg; ip	55.67 ± 0.9066	2.7917 ± 0.0288	1.4517 ± 0.0344	1.9767 ± 0.0058
2	Methotrexate treated for 1 day 20mg/kg; ip	$38.6420 \pm 1.1151^{\#}$	$3.475 \pm 0.0472^{\#}$	$2.825 \pm 0.0419^{\#}$	$0.0667 \pm 0.003^{\#}$
3	Methotrexate 20mg/kg for 1 day + drug suspension 350mg/kg orally in 0.5% CMC suspension for 5 days	$47.9183 \pm 0.3953^{***}$	$0.0357 \pm 0.0081^{**}$	$2.03 \pm 0.035^{***}$	$0.16215 \pm 0.0086^{***}$
	Methotrexate for 1 day followed by normal saline for 5 days 20mg/kg methotrexate; ip+1ml/kg normal saline; ip	$42.3338 \pm 0.7193^{\#}$	$3.41 \pm 0.0501^{\#}$	$2.655 \pm 0.0491^{\#}$	$0.0859 \pm 0.0048^{\#}$

n=6, # p=0.0001, as compared to control group (group 1), ***p=0.0001, as compared to MTX toxicity group (group 2), **p=0.002, as compared to MTX toxicity group (group 2),

The histopathological examination of the liver showed that in control groups, normal pathology was evident (Fig 1). In methotrexate toxicity induced

group (Group2) centrilobular necrosis was evident (Fig 2). Also there was moderate to severe sinusoidal congestion (Fig 3), mild to moderate hyalinization of

hepatocytes and mild to moderate cholangiofibrosis. In the liver samples of the animals treated with Flacourtia extract, there was only mild sinusoidal congestion (Fig 4) as compared to the MTX treated

group. While the group which were not treated with Flacourtia extract and left for 5 days showed moderate to high level of sinusoidal congestion and centrilobular inflammation (Fig 5).

Photomicrographs showing histopathological features:

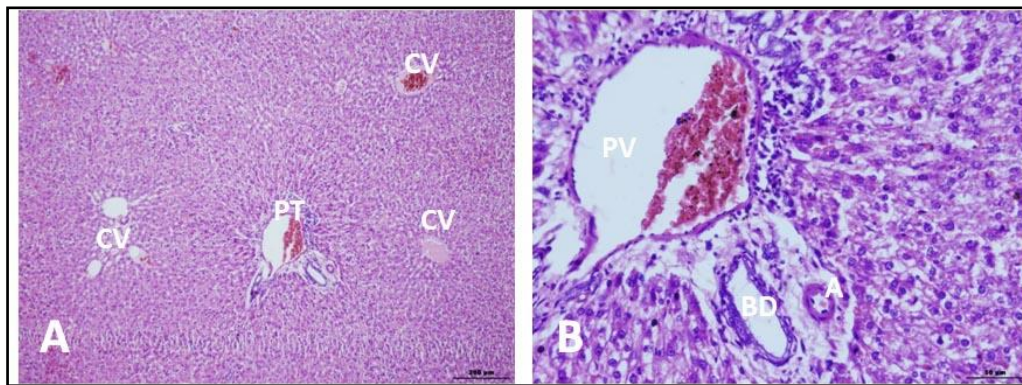


Fig 1: Normal histology of liver tissue (control group) Group-1)

- A) Low magnification (x 100) showing the normal lobular architecture with a few central veins (CV) and a Portal Tract(PT)
- B) Higher magnification(x 400) of a portal tract with artery (A), bile duct(BD) and Portal Vein(PV).

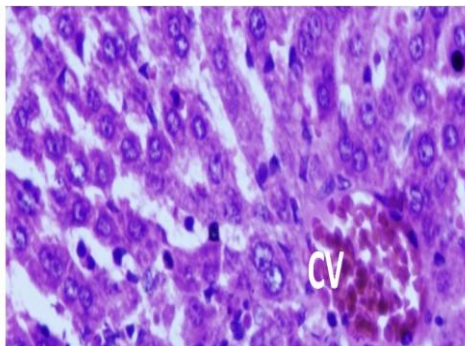


Fig 2: A focus of necrosis near central vein (CV) in methotrexate induced toxicity group(Group-2)(x400)- Animals treated with a single dose of methotrexate (20mg/kg)

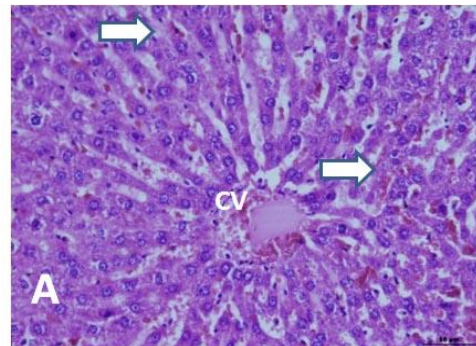


Fig3: Focus of oedema in central vein (CV), Sinusoidal congestion (→) in methotrexate induced toxicity group(Group-2)(x200)-Animals treated with a single dose of methotrexate (20mg/kg)

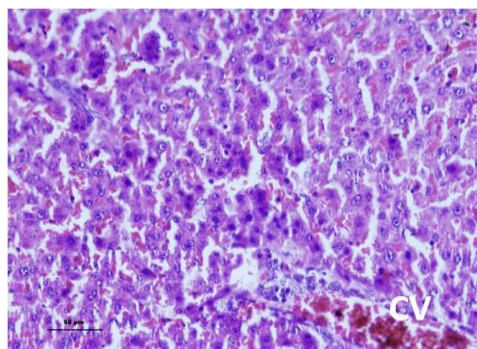


Fig 4: Mild sinusoidal congestion in liver sinusoids (x200) Animals treated with single dose methotrexate(20mg/kg) for 1 day and Flacourtia extract 350mg/kg for next 5 days.(Group-3)

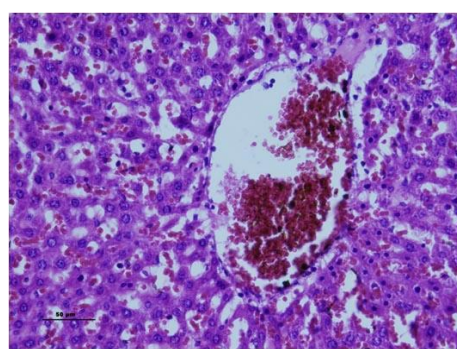


Fig 5 : Moderate congestion in central vein (CV) as well as sinusoids (x200): Animals treated with single dose methotrexate (20mg/kg) for 1 day and normal saline for next 5 days(Group 4).

DISCUSSION:

As far as liver diseases are concerned, traditional system of medicine is highly hope giving¹¹. Our country is blessed with enormous plant varieties about which narrations have been given in traditional literatures but their potential in each condition has yet to be revealed. In traditional medicine, Flacourtia indica is used for jaundice and splenomegaly. But, the effect this drug in other liver pathology like drug induced hepatotoxicity has not yet screened. Protective effect of the plant in methotrexate induced toxicity is the present study.

Previous study on the hepatoprotective effect of Flacourtia indica revealed the high efficacy of petroleum ether extract compared to other extracts the drug. It also showed to be effective at a single dose of 1500mg/kg.(1). Based on this study, the same solvent was used for the extraction and the dose was selected for acute toxicity studies. The extract was tolerated upto 1750g/kg in albino mice.

Among the disease modifying drugs used in rheumatoid arthritis, methotrexate is regarded as first choice drug because of the earlier onset of action and superior efficacy. MTX is also an effective drug in the therapy of cancer. But the high toxic effect of the drug makes constant monitoring of the patient necessary¹². Long term weekly oral methotrexate therapy is also an established therapy in the treatment of severe psoriatic skin and joints. Despite its clinical efficacy, protracted use is limited by liver toxicity. Hence the drug was chosen for the study.

Increase in organ to body weight ratio is an indication of pathological alterations like inflammation and toxicity to the affected organ. This can be revealed in the biochemical parameters and histopathology also. An increase in organ to body weight ratio seen in methotrexate induced toxicity group animals is probably contributed by the increase in liver weight due to hepatocellular hypertrophy, vacuolization, fat accumulation etc. This was further confirmed by the histopathology of liver samples and

biochemistry. A similar increase in organ to body weight ratio involving vacuolization has been reported in liver toxicity¹². Treatment with *Flacourtia indica* extract reversed this effect.

Any toxicity of the liver is reflected in serum biochemistry also. Liver injury is often associated with a rise in serum ALT, AST and ALP levels. In the present study, a significant elevation in serum ALT, AST and ALP levels were observed in methotrexate treated groups (group2) indicating hepatocellular damage. Similar elevation in enzyme levels in MTX induced toxicity were reported earlier¹³. Treatment with *Flacourtia* extract caused a significant reversal of these elevated enzyme levels supporting the hepatoprotective effect of the drug extract.

It is known that oxidative stress are responsible in the tissue damage caused by methotrexate¹⁴. In our study, investigations of the oxidative stress markers in the liver homogenates of various group revealed a significant elevation of MDA and inhibition of SOD, CAT and GSH of MTX treated group. Thus the significant reduction in glutathione levels promoted by MTX could lead to a reduction in effectiveness in the antioxidant enzyme defense system, sensitizing the cells to ROS and the alterations in oxidative stress markers levels resulted and these effects agreed with similar previous studies¹⁵. While these values were reversed by *Flacourtia* extract treatment suggesting a protective effect of this drug in MTX induced oxidative damage.

The biochemical changes were supported by the histopathological observations also. The centrilobular necrosis and the sinusoidal congestion seen in liver histology of MTX treated group suggest lack of oxygenation of the area and also other toxic injury of the tissue. The cytoprotective effect of *Flacourtia* extract was evident in the histology as there was only mild sinusoidal congestion in this

group. These results may be correlated to the cause of these pathogenetic changes namely the oxidative stress markers; while in untreated MTX induced toxicity group, these pathological changes progressed considerably.

In conclusion, the extract of aerial parts of *Flacourtia indica* (Burm.f.) Merr. protected liver tissues probably by its antioxidant and cytoprotective or other unknown properties. The study suggests that the *Flacourtia* extract could be used along with MTX in cancer chemotherapy or other clinical conditions like rheumatoid arthritis, psoriasis etc where long term MTX therapy is indicated and further studies need to be done to confirm the said effectiveness.

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