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Hepatoprotective Effect of *Alpinia Officinarum* on Hepatic Ischemia/Reperfusion Induced Injury in Rats

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ABSTRACT

To study the hepatoprotective effect of chloroform extract of *Alpinia officinarum* at 200 and 400 mg/kg against hepatic ischemia/reperfusion injury. 24 male wistar rats were divided in to four groups. The normal control group, model control group and extract treated group (at a dose of 200 and 400 mg/kg) were orally fed with 1% DMSO, 1% sodium CMC in saline as vehicle for 21 days followed by ischemia/reperfusion on twenty second day. Blood and liver samples were obtained from all the animals on 22nd day for biochemical analysis of AST, ALT, ALP and LDH and histopathological studies were also performed. The results showed that the ischemia/reperfusion injury causes significant ($p<0.001$) increase in the levels of AST, ALT, ALP and LDH in model control group whereas supplementation with chloroform extract of *Alpinia officinarum* significantly ($p<0.01$) reduced the elevated levels of above parameters. Histopathological analysis showed high degree of congestion and mild necrosis in model control group which were reduced to minimum levels in drug treated groups. *Alpinia officinarum* increased the free radicals scavenging activity in the hepatic I/R injury in rats. Reduced level of liver enzymes and histopathological studies evident that *Alpinia officinarum* possesses beneficial effect on the hepatocytes against hepatic I/R injury.

Keywords: Ischemia-Reperfusion (I/R) injury, *Alpinia officinarum*, AST (Aspartate transaminase), ALT (Alanine transaminase), ALP (Alkaline phosphatase) & LDH (Lactate dehydrogenase).

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INTRODUCTION

Hepatic Ischemia and Reperfusion Injury (I/R) occurs in a number of clinical hepatic surgeries, particularly in liver transplantation and hepatic resection [1, 2], and hepatic steatosis is a primary factor increasing the extent of cellular injury incurred during I/R. The increasing use of steatotic livers for transplantation induces higher graft nonfunction rates, increased retransplantation rates which ultimately increased recipient mortality[3]. Both pro-inflammatory cytokines and reactive oxygen species (ROS) play an important role in liver IRI [4, 5]. ROS generated by Kupffer cells and hepatocytes due to I/R injury lead to direct damage on endothelial cells (ECs) and hepatocytes. The use of antioxidants to reduce ischemia/ reperfusion injury and to improve hepatic recovery from dysfunction is the only better solution. The pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), released by activated Kupffer cells after reperfusion, have dual role: over range expression of TNFα and I L-1β can induce more production of cytokines and granulocyte colony- stimulating factor, which enhance Kupffer cells activation and promote neutrophil infiltration in microcirculation of liver [6, 7], then aggravate hepatic sterile inflammation after ischemia and reperfusion. One the other hand, TNFα and IL-1 are indispensable for liver regeneration [8].

Simultaneously At the same time, cells can spontaneously respond to injury by activating defensive mechanisms of themselves. Among them, hypoxia inducible factor- 1 alpha (HIF-1α) is a one of the key nuclear factor which acts as the oxygen sensor. It plays an important role in the pathogenesis of various hypoxic conditions such as ischemic diseases [9, 10].

The molecular mechanisms underlying I/R are multifactorial, and many investigations have their focus on the intervention of hepatic I/R [11-13].

The use of medicinal herbs for healing disease has been documented in history. According to the world health organization, 80% of the world population uses plant based remedies as their primary form of healthcare [14].

The use of herbal medicines is a science based approach for the treatment and prevention of disease is known as phytotherapy flourishing the quest for significant source of synthetic and herbal drugs. *Alpinia officinarum* (*A. Officinarum*) or lesser galangal is a member of Zingiberaceae family. The ginger like root stock (rhizome) of the plant is widely used in Indonesia and Malaysia as a food flavouring and spice. *Alpinia officinarum* is commonly use as food in Thailand and is considered as neutraceuticals. The rhizome contains volatile oil, an acrid resin, kaempferid, galangin, alpinin and galangol, with an unknown gummy substance and

lignin. *Alpinia Officinarum* proved to be Anti-inflammatory [15] and antioxidant [16]. Galangin a flavonol class of flavonoids present in large quantity in *Alpinia officinarum*. Galangin[17] showed various pharmacological activities such as anti-mutagenic, anti-clastogenic, anti-oxidative, radical scavenging, metabolic enzyme modulating and anticancer activity. Thus we planned this study to evaluate protective effect of galangin present in the plant *Alpinia officinarum* against hepatic ischemia/reperfusion injury.

MATERIALS AND METHODS

Chemicals:

Xylazine (Indian Immunological Ltd., India), ketamine (Neon Laboratories., India), chloroform (SD fine – Chem Ltd., India), n-hexane (SD fine – Chem Ltd., India), DMSO (SD fine – Chem Ltd., India), and spirit (SD fine – Chem Ltd., India). All the chemicals of analytical grade were purchased from local vender from Hyderabad, India. Kits for the determination of AST, ALT, ALP and LDH were purchased from Span Diagnostics Ltd., India.

Source of plant

In the present study, rhizomes of *Alpinia officinarum* hanes were collected from Hyderabad, Andhra Pradesh in the (year march 2012) and was authentified by Dr. V.C. Gupta, Deputy Director (Botany), Central Research Institute for Unani Medicines, Department of Ayush, Hyderabad, Andhra Pradesh, India.

Preparation of plant extract

The rhizomes of *Alpinia officinarum* were separated from the stem, washed thoroughly, and dried in an oven at 50 °C for three days. The dried rhizomes were then grounded to powder. 3.5 kilograms of dried powder was subjected to soxhlet apparatus with n-hexane for 8 hours followed by chloroform for 8 hours. The cycle continued for around two months to extract the total amount of chemical constituents dried rhizome. The total amounts of chloroform and n-hexane used was around four litres each. The extracted solutions were then filtered through a filter paper (Whatman No. 1). The filtrate was then concentrated by evaporation under reduced pressure to afford n-hexane crude extract as dark yellow oil and chloroform crude extract as dark yellow slush.

Preliminary Phytochemical identification in the chloroform extract of *Alpina Offficinarum*

Alpinia Officinarum was subjected for the qualitative analysis by using the standard phytochemical test to evaluate the presence of various phytoconstituents.

Chemical tests for volatile oils:

The presence of volatile oils was tested by the following chemical tests:

Red colour was not developed when extract was treated with alcoholic solution of Sudan III indicating that volatile oils were absent.

Red colour was not developed when extract was treated with tincture of alkana, in turn confirms that the volatile oils were absent.

Chemical tests for flavonoid glycosides:

Ammonia test:

Filter paper dipped in solution of extract was exposed to ammonia vapor. Formation of yellow spot on filter paper indicated the presence of flavonoids.

Vanillin HCl test:

Vanillin HCl was added to the solution of extract, formation of pink colour indicated the presence of flavonoids. Chemical tests for the presence of other glycosides were done such as Borntrager's test, Hemolysis test, and Libermann, Bruchard test to identify Anthraquinone glycosides, Saponin glycosides, and triterpenoid glycosides respectively and found them to be absent.

Chemical tests for resins:

Solubility test:

Extract was insoluble in water, rarely soluble in light petroleum and was completely soluble in alcohol, ether, acetone, fixed oils and volatile oils indicating the presence of resins.

Turbidity test:

Extract was mixed with alcohol and water was added in excess and turbidity was observed.

HCl test:

Extract was dissolved in a mix of few ml of acetone and 3 mL of dilute HCl then solution was heated on water bath for 30 minutes. Pink colour was observed which indicated the presence of resins.

$FeCl_3$:

Greenish blue colour was observed when few drops of $FeCl_3$ were added to the extract. This was the conformation for the presence of resins.

Preparation of dose

All the doses were prepared in 1% DMSO, 1% Sodium CMC in saline.

Acute Oral Toxicity Study

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation for Development (OECD), revised draft guidelines 423, revised from the

Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India [18].

Female Wistar mice weighing between 20 to 25 g were used for acute toxicity study to determine LD_{50} of extract. The animals were fasted overnight prior to acute experimental procedures.

Acute oral toxicity studies were performed for chloroform extracts of *Alpinia officinarum* according to the toxic class method 423 as per OECD guidelines. The dose of 2000 mg/kg was given and animals are observed individually once during first 30 minutes and periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. In all cases, death was not observed within first 24 hours. Additional observations like change in skin and fur, eyes and mucous membranes and also respiration, circulation, autonomic and CNS and somatomotors activity and behaviour pattern were performed. Attention was also given to observation of tremors and convulsions.

In vivo Evaluation of Hepatoprotective Effect

Animals:

Adult Wistar male rats (230-250 g) were obtained from animal house of CMR College of Pharmacy and were housed and divided into 4 groups containing 6 animals each. All the experimental procedures and protocols used in the study were reviewed and approved by Institutional Animal Ethical Committee Ref: 1657/PO/a/12/CPCSEA, CMR college of Pharmacy, Hyderabad.

EXPERIMENTAL DESIGN

Animals and drug treatment protocol:

Male Albino rats of Wistar strain (230-250 g) were divided in four groups, each containing six animals. Normal control group (Group I) received only saline without ischemia reperfusion, whereas animals from model control group (Group II) received only ischemia reperfusion without any treatment. Animals from Group III and Group IV received chloroform extract of *Alpinia officinarum rhizomes*, (200 mg/kg and 400 mg/kg) p.o. once daily for 21 days prior to ischemia reperfusion.

Hepatic ischemia reperfusion:

At the end of 21 st day food was withdrawn and animals were fasted overnight. On the next day, animals were anesthetized by xylazine (10 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.). Ischemia was produced by clamping the hepatic portal triad, using bulldog clamp for 40 min followed by reperfusion for 40 min by unclamping the triad.

Collection of blood and isolation of liver:

Blood samples were obtained through cardiac puncture from all animals prior to be sacrificed for the determination of serum AST, ALT, ALP and LDH followed by isolation of liver preserved in 10% formalin.

Histopathological and Biochemical assays

Serum Aspartate Aminotransferase (AST), Alanine Amino Transferase (ALT), Alkaline Phosphatise (ALP) and Lactate Dehydrogenase (LDH) were measured in serum samples of rats using the kit from Span diagnostics. For histopathological examination, pieces of liver were fixed in 10% formalin and hydrated tissue sections in 5 µm thickness were stained with Hematoxylin and Eosin. The section was observed under light microscope.

Statistical Analysis

The data were expressed as Mean ± SEM. Statistical analysis was done by One way ANOVA followed by Dennett's post analysis using Graph Pad Prism version 5.0, USA. The minimum level of significance was fixed at $p<0.05$. Statistical significance was divided as recommended by Graph Pad Prism software version 5.0.

RESULTS AND DISCUSSION

Phytochemicals present in *Alpinia Officinarum* (AO)

Refer to the Table **1**

Acute Oral Toxicity

The chloroform extract was found to be safe at (MTD) maximum toxic dose>2000 mg/kg as observed according to the OECD 423 guidelines. MTD_{50}> 2000 mg/kg, drug was found to be safe and nontoxic, as no mortality occurred and no behavioural changes were observed. Hence the drug was considered safe at 2000 mg/kg. Thus, the final doses selected were 200 and 400 mg/kg.

Biochemical findings

Effect of *Alpinia officinarum* on levels of AST

Reduction in AST level was observed with 200 & 400 mg/kg of AO (*Alpinia officinarum*) in compare to model control group. A decreased level of 153.5 ± 50 (IU/L) and 119.16 ± 37 (IU/L) was observed. 400 mg/kg dose treated rats showed decrease in AST levels in serum when compared with 200 mg/kg dose fed rats. Model control group showed a significant increase ($p < 0.05$) in AST, when compared to other group indicating that disease is induced as shown in Fig-1. 200 & 400 mg/kg showed significant increase($p < 0.05$) in AST level when compared with normal control group and not significant but decrease AST levels when compared to model

control group indicating that drug is effective in reducing abnormal AST levels.

Effect of *Alpinia officinarum* on ALT level

There was significant increase in the level of ALT in model control group when compared with normal control group on hepatic ischemia injury 231.16 ± 65.5 (IU/L), 200 ± 35 (IU/L) was observed with 200 mg/kg and 400 mg/kg treated animals which was significantly less in compare to model control group 296 ± 72 (IU/L) but was very high in compare to normal control group (43.6 ± 6.5) as shown in table-2. 400 mg/kg dose treated rats showed decrease in AST levels in serum when compared with 200 mg/kg dose fed rats. Model control group showed a significant increase ($P < 0.01$) in ALT, when compared to other normal control group which indicates that ischemia is induced as shown by Fig-2. 200 mg/kg and 400 mg/kg treated group showed significant increase ($P < 0.05$) in ALT when compared with normal control group and not significant but decrease in ALT levels when compared to model control group indicating that drug is effective in reducing abnormal ALT levels.

Effect of *Alpinia officinarum* on levels of ALP and LDH

Animals showed significant rise in the level of ALP in model control group when compared with normal control group. The level of ALP at 200 mg/kg and 400 mg/kg dose was 258.6 ± 56.7 (IU/L) and 202.8 ± 35.7 (IU/L) in compare to control group which was 27.5 ± 4.0 (IU/L) respectively as shown in table-2. The level of LDH at 200 mg/kg and 400 mg/kg dose was 2063.5 ± 501 (IU/L) and 1838 ± 321.98 (IU/L) in compare to control group which was 132 ± 25.8 (IU/L) respectively as shown in table-2. Both the doses have significant increase in LDH and ALP when compared with normal control group animals but shows reduction in compare to model control group.400 mg/kg and 200 mg/kg dose of AO treated animals showed significant decrease in ALP and LDH levels in serum when compared with normal control group animals. Model control group showed a significant increase ($P < 0.001$) in ALP and LDH, when compared to normal control group as shown in Fig-3 and 4. Results indicating that LDH and ALP showing a significant decrease with 200 and 400 mg/kg doses of extract when compared to model control group indicating that extract showing better results in reducing abnormal LDH and ALP levels which are raised due to ischemic disorder.

Histopathological findings

This evaluation showed that there were no pathological changes in liver tissue of group one (Normal control). The liver tissue is within normal limits. Section showing liver with parenchyma with preserved architecture and portal track. There was no necrosis, congestion,

inflammation findings and vascular degradation in normal control group. In model control group there are multiple portal tracks. The central veins are dilated and dilated sinusoids. There is a focal prominence of Kupffer cells, Mild necrosis and inflammation. Sections of the liver show no necrosis in 400 mg/kg treated animals but mild inflammation was observed as in 200 mg/kg treated group, vascular degeneration, sinusoidal dilation, vascular congestion were almost absent when compared to model control group.

This study underlines three points:

(1) The emergence of free oxygen radicals that arise after hepatic I/R and cause injury can be prevented by *Alpinia officinarum.*

(2) The increase in AST, ALT, ALP and LD is an indication of tissue damage in hepatic I/R injury which was significantly reduced after AO treatment.

(3) In the hepatic I/R injury, histopathological examination of liver tissue shows considerably less hepatocytes injury with AO treatment.

A. officinarum Hance shows the strongest antioxidant properties in reducing power, DPPH assay *A. officinarum* Hance may be suggested as a new potential source of natural antioxidant[19].

A rodent mode1 of partial hepatic I/R injury were chosen for several reasons. Partial hepatic I/R models in rats are well described, allowing us to compare our results with those of others. Selective clamping of the hepatic vasculature prevents splanchnic pooling and the breakdown of gut barrier function, allowing the effects of I/R to be examined in isolation. A pre-treatment mode1 was chosen as it may be relevant to the elective hepatic resection or transplant scenario. Furthermore the pre-treatment mode1 would allow us to examine the effects of *Alpinia Officinarum* as a form of stress preconditioning that may confer delayed ischemic tolerance. After completion of Oral acute toxicity as per OECD guideline 423, it was found that MTD was more than 2000 mg/kg and drug was found to be safe. Thus, 200 mg/kg, 400 mg/kg dose were fixed for the study.

Alpinia officinarum rhizome proved to be effective in reducing the extent of hepatic damage given in doses 200 mg/kg and 400 mg/kg body weight by enhancing the endogenous anti-oxidant status in rats. The potential hepatoprotective activity of AO may be due to the presence of therapeutic phytochemicals such as flavones and flavonoids. The hepatoprotective effect of *A. officinarum* is probably related to a counteraction of free radicals by its antioxidant property. The present study clearly demonstrated the hepatic ischemia induced oxidative stress which was evidenced by a significant fall in endogenous anti-oxidant enzyme along with rise in other biochemical parameter levels.

AST, ALT are the most sensitive test for Inflammation indicators of cells. High LDH levels can be measured as a surrogate for tissue breakdown.Raise in ALP indicate that bile duct is blocked in all the groups except in normal control. Increased ALP levels as the synthesis increased due to biliary pressure. Decrease in levels of extract treated groups shows improvement in secretary mechanism of hepatocytes.

Alpinia officinarum rhizome extract not only decreased the level of AST, ALT and ALP but also attenuated increase in lactate dehydrogenase LDH, in comparison with rats of hepatic ischemia/reperfusion control group. Ischemia caused by oxidative stress is a major cause of death and disability worldwide. The present study demonstrated that the plant extracts in experimental rats improved defence system. The data of the present study clearly showed plant extract modulated most of the biochemical parameters were maintained to normal status in ischemic reperfusion in rats.

Inhibition of NF-κB and AP-1 activation

Alpinia officinarum also inhibited the activation of two pivotal redox-sensitive transcription factors, namely nuclear factor κB (NF-κB). AO has been previously reported to inhibit NF-κB binding activity in various cell culture models, supporting these data. Activation of transcription factors, such as NF-κB and AP-1, has been linked to the pathophysiology of I/R injury by activating inflammatory cascades leading to organ damage. Thus, inhibition of these proinflammatory mediators might contribute to a protective effect of *Alpinia officinarum* on ischemia.Hydroxy-3'-methoxyphenyl-1-phenylhept-4-en-3-one (HMP) is a naturally occurring phytochemical found in lesser galangal (*Alpinia officinarum)*. In addition, Western blotting and reverse transcription-polymerase chain reaction analysis demonstrated that HMP decreased Lipopolysaccharides (LPS)-induced inducible nitric-oxide synthetase (iNOS) [20].

Inhibition of iNOS and NF-κB, these mechanisms seem most likely to be essential for the protective effect of AO, which were seen at the beginning of the reperfusion period. Therefore, protection by preadministration of AO might be due to the mechanisms discussed above.

In I/R injury of liver during the reperfusion phase, emerging reactive oxygen radicals activate some mediators and can cause inflammatory response and tissue damage and this was evident with increased level of AST, ALT, ALP and LDH [21].

A histopathological examination detected no pathological changes in group I (Figure – 5[A]). Particularly severe necrosis, moderate inflammation, vascular degeneration, and vascular congestion were seen in group II (Figure- 5[B]). Examination of group III (Figure- 5[C, D) &group IV (Figure- 5[E])) showed that the pathological changes existing in group II had almost

decreased. These results proved that AO has a protective effect on hepatic damage created against I/R injury.

Limitations of the study

Because of time constrain of study protocol, it was not possible to conduct study for longer duration hence hepatic I/R-injured animals were sacrificed just after reperfusion in order to observe the early effects of AO.

Feature perspectives

The present study dealt with the short-term effect of AO treatment on hepatic I/R injury. There is a scope of further studies on the long-term effects of AO which would support our findings and would help to determine the time dependent effect of AO to study its possible ability to reverse I/R induced damage in liver tissue and its effects on levels of antioxidants.

The present study showed that the chloroform extract of *Alpinia officinarum* increased the free radicals scavenging activity in hepatic ischemia reperfusion injury in rats. Significant reduction in level of biochemical parameters like AST, ALT, ALP, LDH and histopathological data proved that *Alpinia officinarum* has beneficial effects on the hepatocytes, against hepatic ischemia reperfusion injury.

Table- 1 Phytochemicals present in *Alpinia Officinarum (*AO)

Phytochemicals in AO	Result
Flavanoids	+++
Volatile oils	+
Anthraquinone Glycosides	–
Saponin Glycosides	–
Resins	+

Table-2 Comparison of all biochemical parameters

GROUPS	AST	ALT	ALP	LDH
Group I	54 ± 2.82	43.6 ± 6.5	27.5 ± 4.0	132 ± 25.8
Group II	197.82 ± 54*	296 ± 72**	431± 64.9***	2547.83 ± 437***
Group III	153.54 ± 38.62*	231.16 ± 65.5*	258.6 ± 56.7**#	2063.5 ± 501**
Group IV	119.16 ± 10.93	200 ± 35	202.8 ± 35.7*##	1838 ± 321.98*

AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

*p<0.05, ** p<0.01, ***p<0.001 when compared to normal control.

#p<0.05, ##p<0.001when compared to model control.

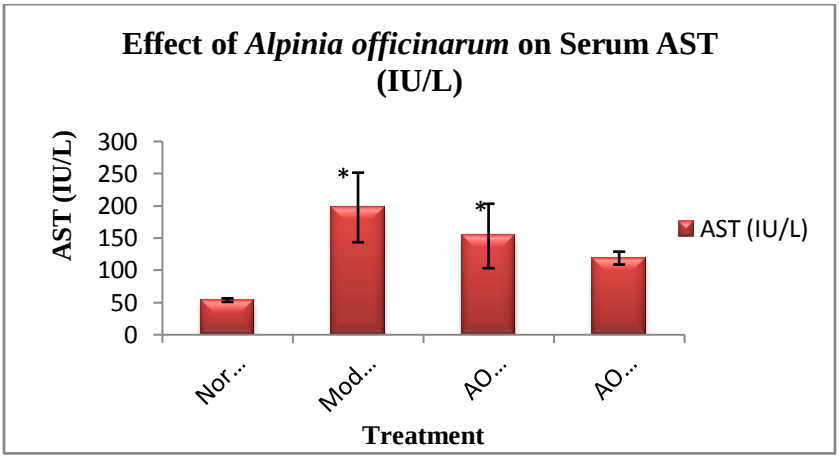


Figure-1 Effect of *Alpinia officinarum* on AST level

***p<0.05, ** p<0.01, ***p<0.001** when compared to normal control

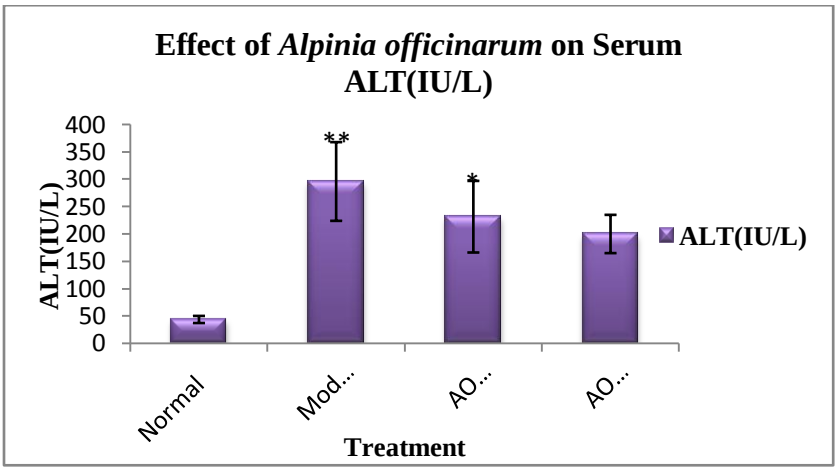


Figure-2 Effect of *Alpinia officinarum* on ALT level

***p<0.05, ** p<0.01, ***p<0.001** when compared to normal contro

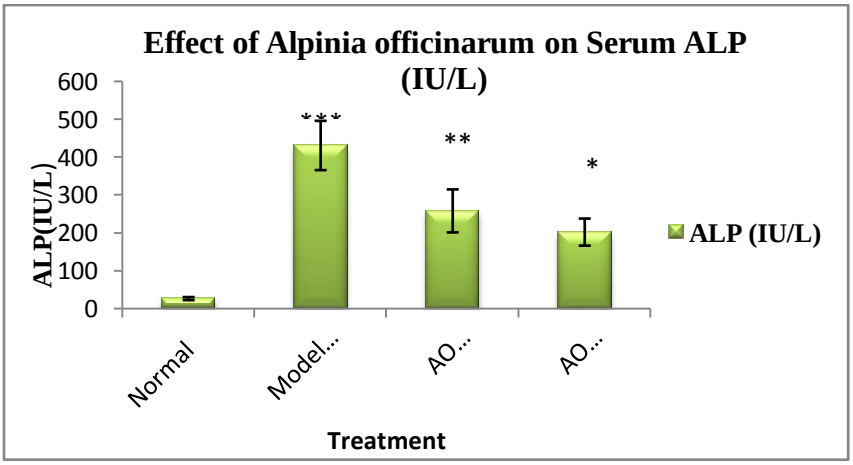


Figure-3 Effect of *Alpinia officinarum* on ALP level

***p<0.05, ** p<0.01, ***p<0.001** when compared to normal control

 # p<0.05, ##p<0.01 when compared to model control group

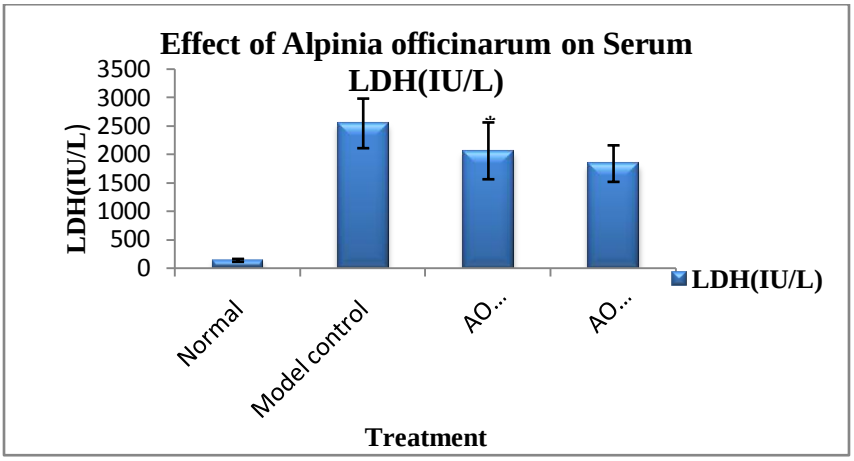
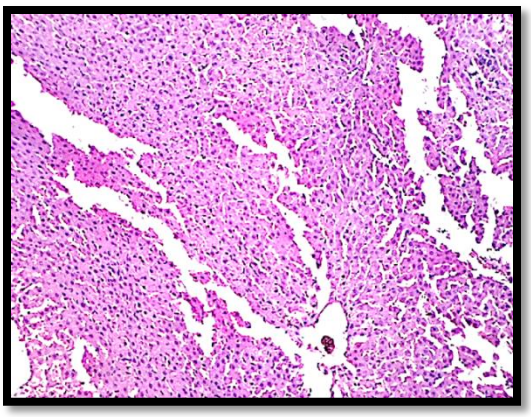
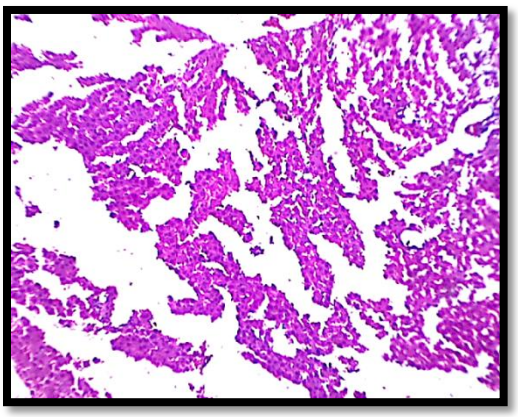


Figure-4 Effect of *Alpinia officinarum* on LDH level

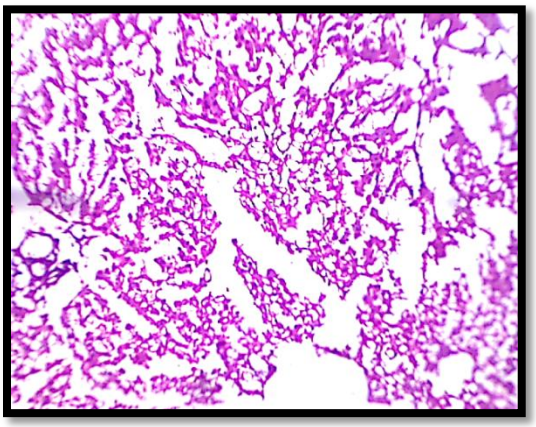
***p<0.05, ** p<0.01, ***p<0.001** when compared to normal control

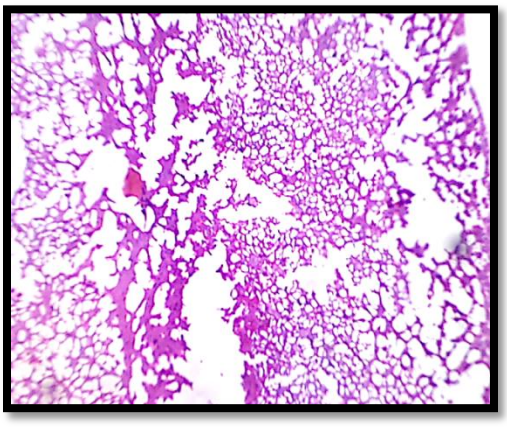


(A) Group I Normal Control

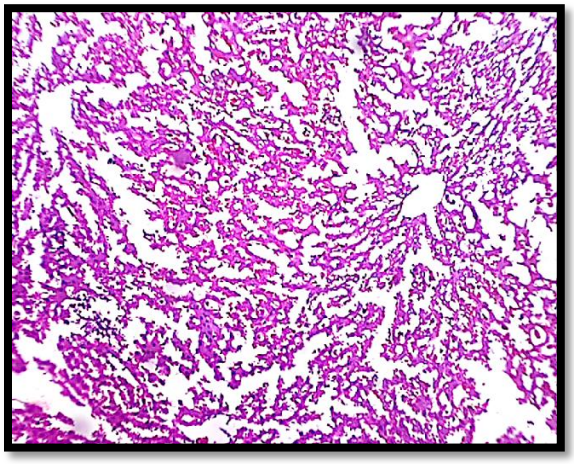


(B) Group II Model Control





(C, D) Group III (200 mg/kg treated)



(E) Group IV (400 mg/kg treated)

Figure 5 The Histopathological Images

CONCLUSION

The present study showed that the chloroform extract of *Alpinia officinarum* increased the free radicals scavenging activity in hepatic ischemia reperfusion injury in rats. Significant reduction in level of biochemical parameters like AST, ALT, ALP, LDH and histopathological data proved that *Alpinia officinarum* has beneficial effects on the hepatocytes, against hepatic ischemia reperfusion injury.

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