Journal of Bioanalysis & Biomedicine

Prathyusha et al., J Bioanal Biomed 2013, 5:2
DOI: 10.4172/1948-593X.1000076

Research Article Open Access

Hepatoprotective Effect of *Inularacemosa* on Hepatic Ischemia/reperfusion Induced Injury in Rats

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Abstract

Introduction: Hepatic Ischemia-Reperfusion (I/R) injury contributes to organ injury and dysfunction after hepatic surgery and transplantation. I/R induce Kupffer cell activation, leading to the release of pro-inflammatory cytokines that promote injury, increase adhesion molecule expression, and facilitate polymorphonuclear neutrophil injury. *Inularacemosa* contains high concentrations of the flavonol glycosides, which has been shown effect on cardiac function and oxidative stress against isoproterenol – induced myocardial infarction. Historically, the roots were reputed to have Anti-inflammatory and Analgesic effects. The hepatoprotective activity of the drug against hepatic Ischemia/Reperfusion injury has not been reported yet.

Objective: To study the hepatoprotective effect of hydroalcholic extract of *Inularacemosa* at 200 and 400 mg/kg against hepatic ischemia/reperfusion injury.

Methodology: 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 distilled water 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.

Results: The results showed that the ischemia/reperfusion injury causes significant increase in the levels of AST, ALT, ALP and LDH in model control group indicating the cell damage and tissue injury whereas supplementation with hydroalcholic extract of *Inularacemosa* significantly reduced the elevated levels of above parameters. Histopathological analysis showed high degree of congestion and mild necrosis in model control group which was reduced to minimum levels in drug treated groups. *Inularacemosa* increased the free radicals scavenging activity in the early period of hepatic IR injury in rats.

Conclusion: Reduced level of liver enzymes and histopathological studies evident that *Inularacemosa* possesses beneficial effects on the hepatocytes in hepatic I/R injury.

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

Introduction

Reperfusion injury is the tissue damage caused when blood supply returns to the tissue after a period of ischemia [1]. The absence of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. Liver ischemia-reperfusion (I/R) injury is well recognized as a significant cause of morbidity and mortality in 2 principal settings [2]. Firstly, it occurs in major liver resections [3] and transplantation [4,5] where anoxic or ischemic liver injury takes place. Secondly, it happens as a consequence of systemic hypoxia or with conditions that cause low blood flow to the liver resulting in insufficient perfusion. The latter occurs in haemorrhagic, cardiogenic, or septic shock with subsequent fluid resuscitation [6], in cardiovascular surgery with extracorporeal circulation [7] in laparoscopic surgery [8,2] and in abdominal compartment syndromes [9].

In the field of liver transplantation, I/R injury is closely related to the development of primary graft nonfunction (occurs in 5% of grafts) and primary graft dysfunction (occurs in 10-30% of grafts) [10]. Both conditions are associated with high rates of mortality. I/R injury increase the incidence of subsequent graft rejection [11]. Another field where I/R injury affects outcome is hepatic resection or transplantation with steatotic livers. It is reported that 25% of the western population has some degree of hepatic steatosis [12], which is the result of the abnormal accumulation of triacylglycerol within the cytoplasm of hepatocytes, attributed to the effects of alcohol excess, obesity, diabetes, or drugs.

The interest in medicinal plants and phytochemicals has increased for their therapeutic properties in human diseases, including hepatic system. *Inularacemosa* root has been demonstrated to relieve ischemic pain and exhibit cardioprotective effect. Sesiquiterpenes, alantolactone, isoalantolactone, alloalantolactone, and essential oil are the major constituents which accounts for bioactivity of this herb. It also contains several flavanol glycosides, germacranolides and eudesmenes etc. This plant may offer new alternatives to the limited therapeutic options that exist at present in the treatment of liver diseases or their symptoms, and they should be considered for future studies. The potent hepatoprotective activities of the chemically defined molecules

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Received February 08, 2013; **Accepted** April 27, 2013; **Published** April 30, 2013

Citation: Prathyusha M, Indala R, Jagaralmudi A, Ramesh Kumar K (2013) Hepatoprotective Effect of *Inularacemosa* on Hepatic Ischemia/reperfusion Induced Injury in Rats. J Bioanal Biomed 5: 022-027. doi:10.4172/1948-593X.1000076

isolated from natural origins represent an exciting source for effective liver protective agents. On these bases the need for the study exists.

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 study dried roots of *Inularacemosa* were collected from hyderabad (A.P) and were authentified by Dr. V.C. Gupta, Deputy Director (Botany), Central Research Institute for Unani Medicines, Department of Ayush. The dried roots were subjected to size reduction to get uniform coarse powder was further subjected to extraction (Maceration).

Preparation of plant extract

The 2.0 kilo grams of powder was taken and subjected for maceration in n-hexane for two days in closed container with occasional shaking. Later the n-hexane was strained off and marc was pressed and kept with hydroalcholic mixture (Methanol: water in 70:30 ratio) for four days with occasional shaking. The solution was filtered and concentrated till the formation of brown coloured paste. The total amount of n- hexane and Methanol used was about two litres of each.

Phytochemical estimation

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.

Libermann Bruchard test: Alcoholic extract of drug was evaporated to dryness and extracted with $CHCl_3$, add few drops of acetic anhydride followed by conc. H_2SO_4 from side wall of test tube to the $CHCl_3$ extract. Formation of violet to blue coloured ring at the junction of two liquid, indicate the presence of steroid moiety.

Chemical test for terpenoids:

Terpenoids (Salkowski test): 0.2 g of the extract of the on the hexane, ethyl acetate and ethanol extracts of whole plant sample was mixed with 2 ml of chloroform ($CHCl_3$) and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate positive results for the presence of terpenoids.

Preparation of dose

All the doses were prepared in distilled water and the dose was fixed as (100 mg/ml).

Acute oral toxicity study

The acute oral toxicity study was carried out for hydroalcoholic extracts of *Inularacemosa* as per the guidelines set by Organisation 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 [13].

Female C57BL/6J 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.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, autonomic and CNS and somatomotors activity and behaviour pattern were performed.

In vivo evaluation of hepatoprotective effect

Experimental design:

Animals and drug treatment protocol: MaleAlbino 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 hydroalcoholic extract of *Iinularacemosa* roots, (200 mg/kg and 400 mg/kg) p.o. with the help of oral gavage (oral needle) once daily for 21 days prior to ischemia reperfusion. Study were reviewed and approved by Institutional Animal Ethical Committee Ref: 1657/PO/a/12/CPCSEA, CMR college of Pharmacy, Hyderabad.

Hepatic ischemia reperfusion: At the end of 21st 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 *Inularacemosa* (Ir)

Phytochemicals in Ir	Result
Flavanol Glycosides	+++
Essential oils	+
Anthraquinone Glycosides	_
Saponin Glycosides	_
Terpenoids	++

Acute oral toxicity

The hydroalcholic 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 dose selected was 200 mg/kg.

Effect of *Inularacemosa* on levels of AST

Reduction in AST level was observed with 200 & 400 mg/kg of Ir (***Inularacemosa***) in compare to model control group. A decreased level of 148.16 ± 50 (IU/L) and 81.83 ± 37 (IU/L) was observed as shown in (Table 1*)*. 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 Figure 1. 200 & 400 mg/kg showed increase 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 *Inularacemosa* 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. 219.3 ± 38.4 (IU/L), 148 ± 45 (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 ($P\leq0.01$) in ALT, when compared to other normal control group which indicates that ischemia is induced as shown by Figure 2. 200 mg/kg and 400 mg/kg treated group showed significant increase ($P\leq0.05$) 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.

SL. No.	Groups	Mean±SEM(IU/L)
1	Group I (Normal Control)	54±2.82
2	Group II (Model control)	197.83 ± 54***
3	Group III (200 mg/kg)	148.16 ± 50
4	Group IV (400 mg/kg)	81.83 ± 37

*** p<0.05, ** p<0.01, *P<0.001

Table 1. Effect of *Inularacemosa* on AST level.

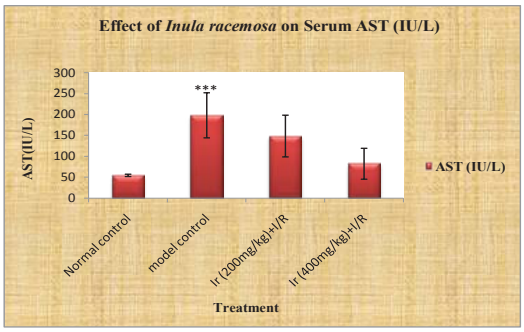

Figure 1: Effect of *Inularacemosa* on AST level.

SL. No.	Groups	Mean ± SEM(IU/L)
1	Group I (Normal Control)	43.6 ± 6.5
2	Group II (Model control)	296 ± 72**
3	Group III (200 mg/kg)	219.3 ± 38.4***
4	Group IV (400 mg/kg)	148 ± 45

*** p<0.05, ** p<0.01, *P<0.001

Table 2. Effect of *Inularacemosa* on ALT level.

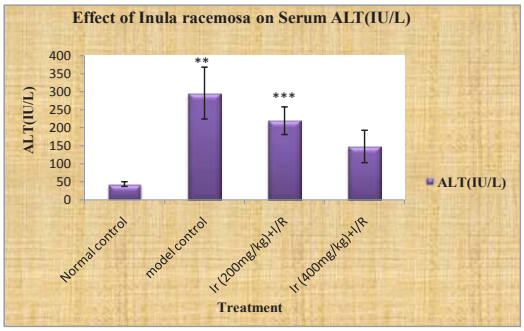

Figure 2: Effect of *Inularacemosa* on ALT level.

Effect of *Inularacemosa* 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 189.3 ± 28.3 (IU/L) and 127 ± 37.05 (IU/L) in compare to control group which was 27.5 ± 4.0 (IU/L) respectively as shown in Table 3. The level of LDH at 200 mg/kg and 400 mg/kg dose was 1702.8 ± 134.47 (IU/L) and 1097 ± 406.45 (IU/L) in compare to control group which was 132 ± 25.8 (IU/L) respectively as shown in Table 4. 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 (Figure 3 and 4).

400 mg/kg and 200 mg/kg dose of Ir 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 Figure 5. 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

Normal Control group: 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 sham group (Figure 6).

Model control group: Sections of liver show the hepatocytes are arranged in cord. 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 (Figure 7).

GROUP III (200 mg/kg treated): Sections of the liver show some necrosis in 200 mg/kg treated animals and mild inflammation was observed, vascular degeneration, sinusoidal dilation, vascular congestion were mild when compared to model control group (Figure 8).

SL. No.	Groups	Mean ± SEM(IU/L)
1	Group I (Normal Control)	27.5 ± 4.0
2	Group II (Model control)	431 ± 64.9*
3	Group III (200 mg/kg)	189.3 ± 28.2***
4	Group IV (400 mg/kg)	127 ± 37.05

***p<0.05, ** p<0.01, *p<0.001

Table 3. Effect of *Inularacemosa* ALP level.

SL. No.	Groups	Mean ± SEM(IU/L)
1	Group I (NormalControl)	132 ± 25.8
2	Group II (Model control)	2547.83 ± 437*
3	Group III (200 mg/kg)	1702.8 ± 134.47**
4	Group IV (400 mg/kg)	1097 ± 406.45

***p<0.05, ** p<0.01, *p<0.001

Table 4: Effect of *Inularacemosa* LDH level.

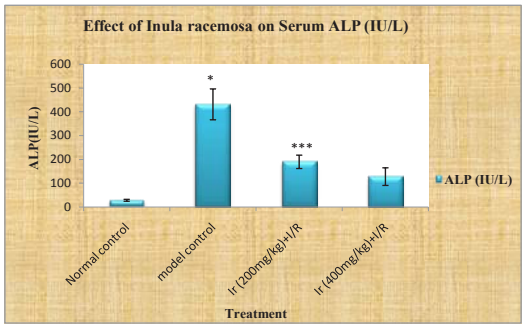

Figure 3: Effect of *Inularacemosa* on ALP level.

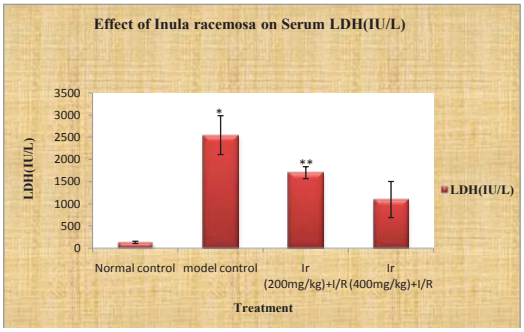

Figure 4: Effect of *Inularacemosa* on LDH level.

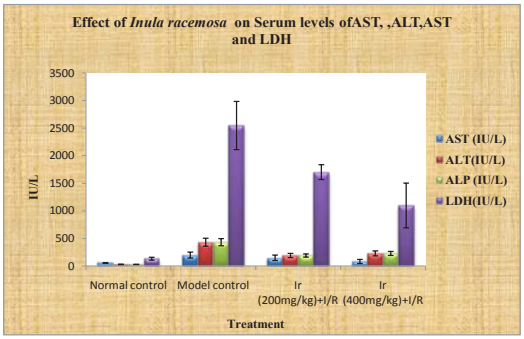

Figure 5: Comparison of AST, ALT, ALP and LDH levels.

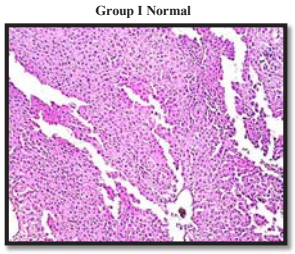

Figure 6: Image of liver slides showing Normal & Model control groups

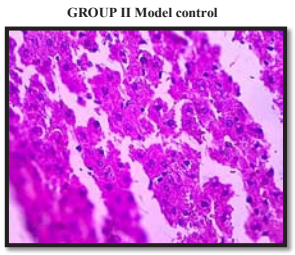

Figure 7: Image of liver slides showing Normal & Model control groups

GROUP IV (400 mg/kg treated): 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 (Figure 9).

Liver pathophysiology consists of many mechanisms that have an impact on liver damage at different levels. These mechanisms include

cellular and molecular interactions, Kupffer cell activation, ROS formation, cytokine and chemokine secretion, vasoconstriction, nitric oxide, imbalance between endothelin and nitric oxide, accumulation of neutrophil leukocytes, alteration in the mitochon- drial permeability, balanced calcium influx into the cell and pH paradox. These complex mechanisms result in cell death, organ dysfunction and even- tually organ loss (Table 5).

Free oxygen radicals are marked mediator's which play a major role in IR injuries of several organs. Research work suggests as antioxidant molecules may provide safety from IR injury IR is a strong antioxidant, breaking up free radicals, it is expected to be protective in hepatic IR injury of rats.

Present study demonstrated the hepatoprotective effect of *I. Racemosa* against ischemia reperfusion injury, as evaluated by attenuation of free radicals improve antioxidant defence as well as lipid peroxidation.

Studies of IR have used different doses and methods of application. No standard dose has been agreed. After completion of Oral acute toxicity as per OECD guideline 423, it was found that MTD found is more than 2000 mg/kg and drug was found to be safe. Thus, final dose selected was 200 mg/kg. We used a total dose of 200 and 400 mg/kg administered orally. We preferred to use dose of IR (before ischemia).

Aim of our research is benefiting from the PE (prophylactic effect) by attempting these trials to reach the definite plasma level of IR.

Detecting the Safety on immediate damage that occurs after reperfusion. This study states that safety of IR was examined after injury of IR. During IR injury to liver in reperfusion phase, emerging reactive oxygen radicals activate some mediators it can cause inflammatory response and tissue damage. Due to this AST, ALT, and LDH activities may increase. AST, ALT, and LDH increases the activities in group 2 of our study support this finding. Study states, it is shown in group 3, ALT, AST, and LDH enzymes, markers of liver parenchymal injury, it have decreased levels in comparison with group 2. Results support the protective effect of IR treatment on IR injury (Figure 5).

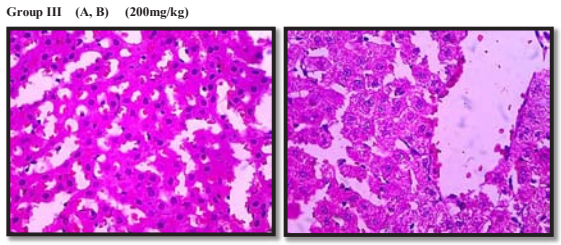

Figure 8: Images of liver slides showing Group III (200 mg/kg).

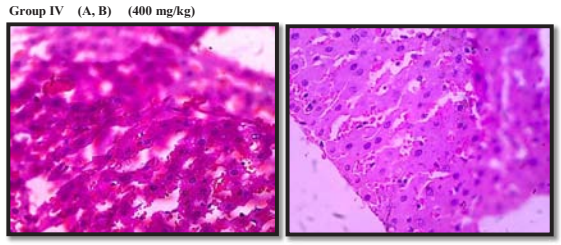

Figure 9: Image of liver slides showing Group IV (400 mg/kg).

GROUPS	AST	ALT	ALP	LDH
Group I	54 ± 2.82	43.6 ± 6.5	27.5 ± 4.0	132 ± 25.8
Group II	197.8 ± 54***	296 ± 72**	431 ± 64.9*	2547.8 ± 37*
Group III	148.16 ± 50	219.3 ± 34.4***	189.3 ± 28.2***	1702.8 ± 134.47**
Group IV	81.83 ± 37	148 ± 45	127 ± 37.05	1097 ± 406.45

***p<0.05, ** p<0.01, *p<0.001

Table 5: Comparison of all parameters.

Oxidative stress occurs particularly in reperfusion after ischemia. Synthesis of proinflammatory cytokines and cell adhesion molecules is activated, and the inflammatory Response is increased by oxidative stress. The antioxidant system has an important role in protection from the damage of oxidative stress.

Histopathological examination detect's no pathological changes in group 1. Severe necrosis, moderate inflammation, vascular congestion and vacuolar degeneration, were seen in group 2. Results of groups 3 & 4 show's decrease in pathologically than Group 2. These results indicate that IR has a protective effect on hepatic damage created with IR.

Study limitation

Because of the study protocol, hepatic IR-injured rats were sacrificed just after reperfusion in order to observe effects of IR. If the rats were alive, we can observe the long-term effects of IR in hepatic IR injury. We expect that further studies on the long-term effects of IR will increase the value of our positive findings.

Conclusion

Decreased level of liver enzymes and histopathological studies proved that hydroalcholic extract of *Inularacemosa* possesses hepatoprotective activity against hepatic Ischemic/reperfusion injury in rats. The results also suggested that the drug has increased antioxidant ability and decreased oxygen free radicals in the early period of hepatic I/R injury.

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