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Activity of Picroliv Isolated from *Picrorhiza kurrooa* against Thioacetamide and *Entamoeba histolytica* Induced Liver Damage

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Abstract: *Picroliv, an iridoid glycoside mixture, isolated from root and rhizome of Picrorhiza kurrooa known to possess significant hepatoprotective activity. Present study demonstrated the hepatoprotective potential of picroliv against thioacetamide and Entamoeba histolytica induced hepatic damage in rats, mastomys, and gerbils. Significant increase in the serum levels of hapatospecific markers was found due to administration of hepatotoxic agent, thioacetamide (200mg/kg b. wt.), indicating wide spread liver damage in the test models which was further elevated after E. histolytica infection. Pinpoint amoebic liver abscesses were found to develop only in gerbils. Administration of picroliv (12 mg/kg b. wt.) resulted in significant recovery of altered serum levels of albumin, protein, triglycerides and cholesterol in all animal models challenged with thioacetamide and E. histolytica. Picroliv also antagonized the changes in the enzyme levels of glutamate oxaloacetate, glutamate pyruvate transferase and alkaline phosphatase in serum. Significant recovery obtained in serum enzyme levels in all animal models and against amoebic liver abscess in gerbils on treatment with picroliv indicated that picroliv possesses therapeutic activity against E. histolytica induced hepatic damage. Further study suggests that Picroliv was more potent than silymarin, a standard hepatoprotective agent.*
Key words: *Entamoeba histolytica, Hepatic amoebiasis, Hepatoprotective, Picroliv, Thioacetamide*

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I. INTRODUCTION

Picrorhiza kurrooa, belonging to family Scrophulariaceae commonly known as Kutki, forms an ingredient of many Indian herbal preparations used for the treatment of liver ailments [1, 2]. It is a small perennial herb growing in the hilly parts of India particularly in Himalayas between 3000 and 5000 meters [3]. Picroliv is a standardized preparation containing of iridoid glycosides: picroside I and kutkoside (1: 1.5, w/w), obtained from ethanolic extract of root and rhizomes of *P. kurrooa* [4]. It has shown marked hepatoprotective activity against several hepatotoxic agents such as aflatoxin B1 [5], oxytetracyclin [6], thioacetamide [7-9], and infections of *Plasmodium berghi* [10], *Leishmania donovani* [11] and *Entamoeba histolytica* [12].

Thioacetamide (TAA) originally used as a fungicide, is a hepatoxin, which induces liver injury occasionally resulting in cirrhosis [13]. TAA represents a second widely used chemical for the induction of experimental liver fibrosis, but can also be employed for the development of acute liver failure and liver tumors in animal models [14]. TAA-induced fulminant hepatic failure has been observed by researchers while studying the detrimental effects of nitric oxide inhibition on hepatic encephalopathy [15, 16]. Reports indicate that toxic metabolites of TAA provoke liver necrosis [17]. Laboratory data further suggests that it has potential efficacy in inducing hepatic failure in rodents [18, 19]. It is noted that these effects are due to its high specificity for the liver, its region specificity for the perivenous area, and the short window of time between its necrogenic effects and liver failure [20,21].

Entamoeba histolytica, the causative agent of human amoebiasis, infects primarily the lumen of the large bowel but also establishes extra intestinal infections at various sites of which the highly encountered is infection of the liver popularly known as hepatic amoebiasis [22]. Hepatic amoebiasis found worldwide, with a high incidence in India, tropical regions of Africa, Mexico and other areas of Central America [23]. It has been reported that reaction to toxic substances may enhance hepatomegaly associated with amoebic infection of the liver [12, 24, 25]. Nevertheless, picroliv has been shown to have a marked hepatoprotective activity against many hepatotoxic compounds its effect against *E. histolytica* induced liver damage is not much studied. This study aims to evaluate the hepatoprotective activity of picroliv against *E. histolytica* infection in liver exposed to TAA toxicity in three different animal models. The activity was compared with silymarin (a standard hepatoprotective compound) and Liv 52 (a well known hepatoprotective drug).

II. MATERIALS AND METHODS

2.1. Animals: Albino rats (Druckray strain), mastomys, and gerbils of either sex, weighing 25-30 grams, bred in animal house of Central Drug Research Institute were used in this study. The animals were fed on standard pellet diet and had free access to water.

***2.2.* Amoebae:** *E. histolytica*, isolated from fecal sample of a patient suffering from acute symptoms, containing haematophagus trophozoites and cysts, maintained in Robinson's medium was used in this study [26].

2.3. Amoebic inoculums: Healthy motile trophozoites from 48 hours old cultures, containing flourishing growth of amoebae, were pooled by low speed centrifugation (14g). The sediment was suspended in fresh overlay ensuring the viability of the trophozoites. Amoebae were counted in a haemocytometer and the number adjusted to approximately 50×10^3 trophozoites in about 0.02 - 0.03 ml.

2.4. Chemicals: TAA purchased from Sigma chemicals Company (St., Lovis, MO, USA). Picroliv supplied by the Pharmaceutical Division of the Institute, silymarin (Aldrich) and Liv 52 (Himalaya) were employed in this study. The rest of the chemicals used in the study were of analytical grade of purity.

2.5. Experimental design:

2.5.1. Hepatotoxicity induction by TAA: Liver damage by was induced through intra peritoneal infection at a dose of 200 mg/kg body weight [4] for two, three and four consecutive days. Best results were obtained with the two consecutive day's treatment as compared to three days dose regimen. In further experiments the two days treatment schedule with TAA was maintained.

Animals were given intraperitoneal (subcutaneous) injections of TAA (200 mg/kg b. wt.) for two consecutive days. A total of 36 animals were used for experiments with each model. Group I consisted of 6 animals which served as normal controls, the remaining 30 animals received treatment with TAA. After TAA treatment was completed, 6 animals were isolated for group II which served as hepatotoxic treated control. The remaining 24 animals were inoculated with *E. histolytica* trophozoites, of which 6 animals were isolated for group III (hepatotoxic and amoebae treated control). The remaining 18 animals, group IV, were used for testing hepatoprotective agents picroliv (Group IVa), silymarin (Group IVb) and Liv 52 (Group IVc), 6 animals in each group.

2.5.2. Production of Hepatic amoebiasis: All the animal models mastomys, gerbils and rats were inoculated with trophozoites of *E. histolytica* and the degree of infection graded by the method of Dutta [27]. In brief, the method consisted of making a small incision below the xiphisternum towards the left from the midline. The liver is easily visible through the small opening. The amoebic inoculum was inoculated into the peritoneal cavity through a 26 gauge needle near the left lobe. The incision was carefully sutured and the wound dabbed with 0.2% gentian violet solution and boric acid powder. The procedure was carried out in properly anesthetized animals.

2.5.3. Hepatoprotective treatment: Hepatoprotective agents picroliv, silymanin and Liv 52 were given orally at daily doses of 12 mg/kg b.wt. with the help of feeding canula. Pilot experiments were conducted with 3, 6, 12 and 20 mg/kg doses of the hepatoprotective agents (data not shown). 12 mg/kg dose offered better protection than 3 and 6 mg/kg while 20 mg/kg did not give a significantly higher degree of protection than 12 mg/kg. Hence, the studies were conducted with dose 12 mg/kg b.wt. of the each hepatoprotective agents. Treatment was started 48 hours after intrahepatic inoculation of amoebae and continued for 4 consecutive days. All the animals were sacrificed 7 days after they were infected with amoebae.

2.5.3. Collection of blood samples: At the end of the experimental schedule, blood was collected from the retro-orbital plexus before sacrificing animals with excess ether anesthesia. Serum recovered was stored for enzyme assays.

2.6. Biochemical assays: The activity of two transaminases in serum *viz.* glutamic oxaloacetate transaminase (SGOT) and glutamic pyruvate transaminase (SGPT) was assayed by the method of Reitman and Frankel [28]. Levels of cholesterol, triglycerides and alkaline phosphatase (ALP) in serum were determined by standard procedures [29]. Serum albumin was determined according to Hirosho et al [30] using bromocresol green. Serum protein estimated by the method of Lowery et al [31].

2.7. Statistical analysis: Data analysis was done by using Student's t test, value of significance $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

III. RESULTS

3.1. Rats: TAA produced liver damage and an elevation of serum enzymes viz. GOT (1.2 fold), GPT (2.3 fold) and ALP (1.5 fold). A significant rise in levels of protein (12%), cholesterol (39%) and triglycerides (57%) were also noted after TAA treatment while albumin level remained unchanged. In TAA treated and *E. histolytica* infected animals (group III) the levels of triglycerides (26%), ALP (26%), GOT (35%) and GPT (47%) showed further elevation. However, no significant change in levels of protein and cholesterol were noted. These elevated

levels of hepatic markers in serum were significantly lowered by hepatoprotective agents at dose 12 mg/kg b. wt. Animals receiving picroliv (12 mg/kg b. wt.) showed protection in altered serum levels of protein (35%), cholesterol (66%), triglycerides (85%), ALP (87%), GOT (79%) and GPT (51%). Silymarin and Liv 52 exhibited similar but less pronounced pattern of protection as compared to picroliv (Table 1).

3.2. Mastomys: Levels of serum cholesterol (1.9 fold) and triglycerides (1.4 fold) were significantly elevated after TAA treatment accompanied with an increase in activities of marker enzymes: ALP (2.4 fold), GOT (1.6 fold) and GPT (2.0 fold). However, the levels of protein and albumin remained unaffected. In group III (animals treated with TAA and infected with *E. histolytica*) the levels of serum cholesterol (23%) triglycerides (19%), ALP (13%), GOT (102%) and GPT (30%) showed a further rise. However no significant change in albumin and protein levels was noted. Treatment with hepatoprotective agents (at dose level 12 mg/kg b. wt.) significantly lowered the elevated levels of the above hepatic markers in serum. The pattern was similar to that of rats. Animals treated with picroliv showed decrease in serum cholesterol (25%), triglycerides (22%), ALP (13%), GOT (69%) and GPT (32%). Liv 52 and silymarin exhibited similar pattern of protection but the effect was comparatively less prominent than that of picroliv (Table 2).

3.3. Gerbils: The levels of serum albumin (1.1 fold), protein (1.4 fold), cholesterol (1.4 fold), triglycerides (2.24 fold), ALP (2 fold), GOT (2.4 fold), and GPT (1.7 fold) were significantly raised by the administration of TAA. In animals exposed to TAA and *E. histolytica* (group III) most of the serum parameters were further elevated with the exception of albumin. The elevated levels of all the markers were reverted to near normal in animals treated with hepatoprotective agents. Protection exhibited by picroliv was 45% in albumin, 32% in protein, 28% in cholesterol, 14% in triglycerides level. The activity levels of the serum ALP (56%), GOT (38%) and GPT (42%) were also lowered by picroliv. Activity of picroliv was compared to known hepatoprotectives Liv 52 and silymarin that exhibited a lesser effect with the same dose regimen. The results are summarized in Table 3.

It was also observed that group III animals (exposed to TAA and *E. histolytica*) developed pinpoint amoebic liver abscesses, which showed motile amoebae in the smears prepared from the infected portion of liver. Positive cultures were obtained when part of the infected liver was inoculated into Robinson's medium along with sterile rice starch.

IV. DISCUSSION

TAA consistently produced hepatic necrosis in rats when administered at a dose of 200mg/kg b. wt. subcutaneously [4]. Necrotic effects by TAA may be affected by membrane injury. Castro et al [32] suggested that a metabolite of TAA (perhaps S-oxide) formed by the action of the amine oxidase of Ziegler is responsible for hepatic membrane injury. Visen et al [4] reported that TAA reduces the viability of hepatic cells with enhanced formation of reactive oxygen and lipid peroxides altering integrity of liver cell membrane and membrane fluidity. It causes apoptosis in liver and results in decrease of antioxidants and formation of lipid hydroperoxides along with a dramatic increase of GOT in plasma [33]. In the present study, further increase in the serum biochemical parameters after *E. histolytica* infection (Table 1, 2 and 3) supports the view of damage to liver cell membrane resulting in leakage of enzymes from cells due to altered permeability of membranes and membrane fluidity [34]. In this study, only gerbils were found to develop pinpoint amoebic abscess of the liver which is in confirmation with previous reports [35, 36]. The toxic effect of TAA might have played a role in creating a microatmosphere for survival and colonization of *E. histolytica* trophozoites in the liver tissue resulting in abscess.

Picroliv, at a dose of 12 mg/kg b. wt., provided significant protection of the elevated levels of serum parameters due to TAA administration followed by *E. histolytica*. Pilot experiments were conducted with 3,6,12 and 20mg/kg doses of hepatoprotective agents. It was observed that 12 mg/kg dose offered better protection than 3 and 6 mg/kg while 20 mg/kg did not give a significantly better protection which is in confirmation with our earlier findings [12]. Hence in the present study, doses of 12 mg/kg b. wt. of the hepatoprotective agents have been used. Picroliv given orally to rats at 12 mg/kg for seven days helped in better management of hemorrhagic resuscitation injury [37] and induced hepatic ischemia-reperfusion injury *in-vivo* by improved hepatocytes glycogen preservation and reduced apoptosis [38]. Picroliv a potent antioxidant inhibits lipid peroxidation and nitric acid release that occurs after hepatocellular injury and alters the activity of glutathione reductase in a favorable manner [37, 39, 40]. An alteration in the levels of serum GOT and GPT towards the respective normal values is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage [12]. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes [41]. Hepatocytes damaged by exposure to galactoseamine, TAA and carbon tetrachloride when incubated with *P. kurrooa* constituents exhibited concentration dependent restorative effect [9]. Biochemical changes in rats induced by aflatoxin B1 poisoning were significantly prevented by oral administration of *P. kurrooa extract* [5]. Picroliv induced recovery might be following restoration of liver cell membrane permeability including repair of injured liver cells and increase of protein and nucleic acid synthesis [42]. Restoring to normal values of altered enzyme levels

by picroliv following hepatic damage by hepatotoxic agent together with *Plasmodium berighei* has also been reported [10]. However, prior to this, the protective activity of picroliv on TAA induced liver damage associated with *E. histolytica* infection has not been investigated.

V. CONCLUSION

In conclusion picroliv demonstrated significant and superior hepatoprotective activity than the known hepatoprotectives both silymarine and Liv52 (table 1-3). Picroliv has a very high LD_{50} > 2500mg/kg p.o. in mouse [43] which is much higher than silymarin [4]. In our study its protective activity against TAA induced liver damage at 12mg/kg dose indicates that it has a high safety margin and a better therapeutic index. The findings of this study suggest that picroliv has the potential to be developed as a protective agent against hepatic disorders due to *E. histolytica*.

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Table 1: Protective effects of picroliv on liver enzymes in hepatic damage induced by TAA and *E. histolytica* in rats

Serum parameter	**Group-I** (untreated)	**Group-II** (TAA treated)	**Group-III** (TAA+ amoeba)	**Group -IV**		
				IVa- picroliv (12mg/kg)+TAA+ amoeba	**IVb-** Liv 52 (12mg/kg)+TAA+ amoeba	**IVc-** silymarin (12mg/kg)+TAA+ amoeba
Albumin (g/dl)	2.43±0.102	2.45±0.1^{NS}	2.42±0.02^{NS}	2.47±0.3	2.42±0.01	2.49±0.03
Protein (g/dl)	5.02±0.169	5.60±0.13*	5.89±0.21^{NS}	5.58±0.11 (35%)	5.63±0.9 (29%)	5.67±0.12 (29%)
Cholesterol (mg/dl)	139.93±2.56	194.63±6.06***	200.8±3.94^{NS}	160.54±9.82 (67%)	189.15±7.23 (20%)	190.28±5.79 (18%)
Triglyceride (mg/dl)	85.30±1.16	134.29±5.8***	169.38±3.9**	97.98±2.29 (85%)	112.82±4.7 (67%)	125.20±8.7 (53%)
Alkaline phosphatese (U/I)	257.85±11.27	398.82±7.83***	501.79±17.21***	289.39±9.82 (87%)	313.40±8.2 (77%)	338.93±9.9 (67%)
GOT^{b} (U/I)	52.63±1.01	153.89±3.49***	207.34±6.82***	84.19±7.8 (80%)	109.27±5.7 (63%)	120.27±6.9 (56%)
GPT^{b} (U/I)	41.58±2.27	94.60±3.08***	139.29±5.3***	89.27±8.9 (51%)	90.97±3.3 (49%)	107.82±11.7 (32%)

$p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***$p \geq 0.05^{NS}$

Group II compared to Group I, Group III compared to Group II, Group IV compared to Group III,

Enzyme unit expressed: ^{a} μ moles of p-nitrophenol released/min/l serum; ^{b} μ moles of pyruvate formed/min/l serum

Figures in parenthesis indicate percent hepatoprotective effect

Table 2: Protective effects of picroliv on liver enzymes in hepatic damage induced by TAA and *E. histolytica* in mastomys

Serum parameter	**Group-I** (untreated)	**Group-II** (TAA)	**Group-III** (TAA+ amoeba)	**Group -IV**		
				IVa-picroliv (12mg/kg)+TAA+amoeba	**IVb-**Liv 52 (12mg/kg)+TAA+amoeba	**IVc-**silymarin (12mg/kg)+TAA+amoeba
Albumin (g/dl)	3.73±0.12	3.90±0.20^{NS}	3.83±2.9^{NS}	4.23±.02	4.10±0.30	4.12±0.01
Protein (g/dl)	6.85±0.25	7.40±0.3^{NS}	7.80±0.01^{NS}	7.72±0.30	7.80±0.21	7.93±0.02
Cholesterol (mg/dl)	177.86±14.9	329.73±9.28***	407.00±13.3**	350.69±7.32 (25%)	385.34±12.92 (10%)	382.01±7.83 (11%)
Triglycerides (mg/dl)	163.81±5.16	229.71±4.23***	273.92±8.27**	249.66±5.21 (22%)	257.31±6.22 (15%)	260.02±3.21 (12.5%)
Alkaline phosphatase (U/I)	203.00±6.23	509.34±11.99***	575.70±8.92**	525.71±10.23 (13%)	560.51±7.30 (4%)	562.90±3.40 (3%)
GOT^{b} (U/I)	29.05±2.26	48.73±2.21***	98.82±6.10***	50.90±3.72 (69%)	84.72±3.24 (20%)	78.91±4.60 (27%)
GPT^{b} (U/I)	44.42±1.22	92.30±2.48***	120.50±4.37**	97.89±9.32 (32%)	96.08±3.21 (32%)	106.13±2.32 (19%)

$p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \geq 0.05^{NS}$

Group II compared to Group I, Group III compared to Group II, Group IV compared to Group III

Enzyme unit expressed: [a] µ moles of p-nitrophenol released/min/l serum, [b] µ moles of pyruvate formed/min/l serum

Figures in parenthesis indicate percent hepatoprotective effect

Table 3: Protective effects of picroliv on liver enzymes in hepatic damage induced by TAA and *E. histolytica* in gerbils

Serum parameter	**Group-I** (untreated)	**Group-II** (TAA treated)	**Group-III** (TAA+amoeba)	**Group-IV**		
				IVa-picroliv (12mg/kg)+TAA + amoeba	**IVb**-Liv 52 (12mg/kg)+TA A+ amoeba	**IVc**-silymarin (12mg/kg)+TA A+ amoeba
Albumin (g/dl)	3.81±0.26	4.30±0.16**	4.72 ±0.03 [NS]	4.30±0.09 (44%)	4.73±0.10	4.72±0.5
Protein (g/dl)	5.11±0.10	6.90±0.48*	8.22±0.09*	7.20±0.30 (32%)	7.91±0.26 (10%)	7.62±0.34 (19%)
Cholesterol (mg/dl)	175.11±3.94	255.31±7.09**	324.33±21.20**	282.20±17.80 (28%)	276.30±8.31 (32%)	300.91±13.92 (16%)
Triglyceride (mg/dl)	162.45±3.45	364.42±4.3***	450.42±8.09**	408.91±11.73 (14%)	413.21±10.50 (13%)	428.72±9.30 (8%)
Alkaline phosphatase (U/I)	191.45±3.45	384.90±12.11***	419.60±16.52*	291.45±9.31 (56%)	365.23±15.22 (24%)	385.33±7.91 (11%)
GOT[a] (U/I)	24.26±1.88	57.51±4.90***	79.21±6.21**	58.30±4.82 (38%)	60.22±3.13 (20%)	66.31±0.70 (24%)
GPT[b] (U/I)	49.6±2.00	88.52±1.71***	124.10±1.80***	93.70±2.90 (42%)	100.81±9.91 (31%)	107±7.22 (23%)

$p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***$p \geq 0.05^{NS}$

Group II compared to Group I, Group III compared to Group II, Group IV compared to Group III

Enzyme unit expressed: [a] µ moles of p-nitrophenol released/min/l serum, [b] µ moles o f pyruvate formed/min/l serum

Figures in parenthesis indicate percent hepatoprotective effect