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Hepatoprotective activity of *Luffa acutangula* against CCl_4 and rifampicin induced liver toxicity in rats: A biochemical and histopathological evaluation

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Hepatoprotective activity of hydroalcoholic extract of *Luffa acutangula* (HAELA) against carbon tetrachloride (CCl_4) and rifampicin-induced hepatotoxicity in rats was evaluated and probable mechanism(s) of action has been suggested. Administration of standard drug- silymarin and HAELA showed significant hepatoprotection against CCl_4 and rifampicin induced hepatotoxicity in rats. Hepatoprotective activity of HAELA was due to the decreased levels of serum marker enzymes viz., (AST, ALT, ALP and LDH) and increased total protein including the improvement in histoarchitecture of liver cells of the treated groups as compared to the control group. HAELA also showed significant decrease in *malondialdehyde* (MDA) formation, increased activity of non-enzymatic intracellular antioxidant, glutathione and enzymatic antioxidants, catalase and superoxide dismutase. Results of this study demonstrated that endogenous antioxidants and inhibition of lipid peroxidation of membrane contribute to hepatoprotective activity of HAELA.

Keywords: Antioxidant, Hepatoprotection, *Luffa acutangula*, Serum marker enzymes

The plant *Luffa acutangula* (L.acyvar. *amara* Clarke (Cucurbitaceae), commonly known Ridge Gourd in English and *Kadudodaka* in Marathi, is fairly large climber found in Western, Central and Southern India and regarded as the wild form of cultivated species. In Ayurveda fruits and seeds of *L. acutangula* used to treat jaundice, biliousness, bronchitis and asthma[1]. *L. acutangula* has been shown to possess CNS depressant activity[2], *in vitro* antioxidant activity[3], and larvicidal activity[4]. Phytochemical studies and documented report have indicated that *L. acutangula* contains β-carotenes[5], flavonoids[6], acutosides A-G, oleanane type triterpene saponins[7], acutosides H-I, oleanolic acid saponins[8].

Since hydroalcoholic extract of *L. acutangula* was shown to possess protective potential against the free radicals activity, as evidenced by *in vitro* antioxidant effect[4], the present study has been designed to investigate hepatoprotective activity of hydroalcoholic extract of *L. acutangula* (HAELA) in CCl_4 and rifampicin induced hepatic damage in rats. Efforts were also made to understand the possible mechanism of hepatoprotective activity of HAELA.

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Results of the hepatoprotective activity of HAELA, which can be correlated with its antioxidant activity.

Materials and Methods

Plant material—Fresh fruits of *L. acutangula* were collected from Hindustan Antibiotic colony Pimpri, Pune in the month of December 2007 and were air dried in shade. The plant material was authenticated and deposited at Agharkar Research Institute, Pune, India (Voucher No. Auth 08-005).

Extraction process—Dried fruits of *L. acutangula* was pulverized and extracted with aqueous ethanol (70% v/v; HAELA) for 72 hr by maceration process, which was repeated for 4 times to ensure the complete extraction of chemical constituents from the fruits.

Chemicals—Silymarin (Mircro labs Ltd. Mumbai, India), rifampicin (Lupin Ltd Aurangabad, India), carbon tetrachloride (Qualigens Fine Chemicals, Mumbai, India), thiobarbituric acid (Spectrochem Pvt. Ltd., Mumbai, India), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and epinephrine (Sigma Chemical Co., St Louis, MO, USA), standard reagent and kits (Nirmal Lab, India) for determination of aspartate transaminase (AST), alanine transaminase (ALT), alanine phosphatase (ALP), lactate dehydrogenase (LDH) and total protein (TP) were procured. All other chemicals were obtained from local sources are of analytical grade.

Animals—Wistar rats of either sex (150-200 g) were procured from National Toxicology Center, Pune. Rats, 6 in a group, were housed in clean polypropylene cages under standard conditions of humidity (50 ± 5%), temperature (25 ± 2°C) and light (12 hr light: 12 hr dark cycle) and free access to food and water *ad libitum*. Experiments were conducted after obtaining the approval from Institutional Animal Ethics Committee constituted as per CPCSEA guidelines.

Acute toxicity—Acute toxicity study for HAELA was carried out on mice according to OECD guidelines[9]. Different doses (500, 750, 1000 and 2000 mg/kg) of HAELA were administered orally as a suspension prepared in 1% (w/v) carboxy methyl cellulose (CMC) to all the animals and they were observed for a period of 72 hr for clinical signs, symptoms and mortality.

CCl_4 induced liver injury[10]—Overnight fasted healthy rats were randomly divided into 6 groups, (6 rats/group) and allowed free access to water.

Group I—Normal control, received olive oil (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Group II—CCl_4 control, received 1:1 olive oil and CCl_4 (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Group III—Standard group, received silymarin (200 mg/kg, po) for 10 days, and in addition received 1:1 olive oil and CCl_4 (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Group IV—HAELA 100, received HAELA (100 mg/kg, po) daily for 10 days, and in addition received 1:1 olive oil and CCl_4 (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Group V—HAELA 200, received HAELA (200 mg/kg, po) daily for 10 days, and in addition received 1:1 olive oil and CCl_4 (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Group VI—HAELA 400, received HAELA (400 mg/kg, po) daily for 10 days, and in addition received 1:1 olive oil and CCl_4 (1 ml/kg, ip) daily for 7 days from 4th to 10th day.

Rifampicin induced liver injury[11]—Overnight fasted healthy rats were randomly divided into 6 groups, (6 rats/group) and allowed free access to water.

Group I—Normal control, received CMC (1% w/v; 1ml/kg, po) daily for 30 days.

Group II—Rifampicin control, received rifampicin (100 mg/kg, po) in the form of suspension prepared in 1% CMC daily for 30 days.

Group III—Standard group, received silymarin (200 mg/kg, po) daily for 15 days from 16th to 30th day, and in addition received rifampicin (100 mg/kg, po) in the form of suspension prepared in 1% CMC daily for 30 days.

Group IV—HAELA 100, received HAELA (100 mg/kg, po) daily for 15 days from 16th to 30th day, and in addition received rifampicin (100 mg/kg, po) in the form suspension prepared in 1% CMC daily for 30 days.

Group V—HAELA 200, received HAELA (200 mg/kg, po) daily for 15 days from 16th to 30th day, and in addition received rifampicin (100 mg/kg, po) in the form of suspension prepared in 1 % CMC daily for 30 days.

Group VI—HAELA 400, received HAELA (400 mg/kg, po) daily for 15 days from 16th to 30th day, and in addition received rifampicin (100 mg/kg, po) in the form of suspension prepared in 1% CMC daily for 30 days.

Biochemical studies—Rats were sacrificed 24 h after the administration of last dose of CCl_4, rifampicin and HAELA, and blood samples were collected by cardiac puncture under light ether anesthesia. Serum was separated by centrifugation at 2500 rpm (Remi, USA) below 30°C for 30 min and marker enzymes viz AST, ALT, ALP, LDH and TP[12-15] were determined in serum using reagents and kits (Nirmal Lab, Jalgaon, India).

Liver homogenates (10% w/v) were prepared in ice cold 10 m*M* Tris buffer (*p*H 7.4). Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS)[16] in 10 % liver homogenates by the method of Ohkawa (1979). Amount of MDA formed was quantified by reaction with thiobarbituric acid and used as an index of lipid peroxidation. The results were expressed as nmole of MDA/g of wet tissue using molar extinction coefficient of chromophore (1.56×10^{-5}/M/cm) and 1, 1, 3, 3-tetraethoxyorioabe as standard. Non-enzymatic antioxidant reduced glutathione (GSH)[17] and enzymatic antioxidants-superoxide dismutase (SOD)[18] and catalase (CAT)[19,20] were also assysed in liver homogenates.

Histopathological studies—Liver tissue sections of control and HAELA rats were fixed in (10%; v/v) formalin solution and after dehydration, the sections of liver were embedded in paraffin wax, cut into 4-6 µm thick sections. Histoarchitectural studies were

carried out after staining with haematoxylin-eosin and then observed under light microscope at 400 × magnifications.

Statistical analysis—The results were expressed as mean ± SE and statastically analyzed by one-way ANOVA followed by Tukey-Kramer test. A value of $P<0.05$ was considered to be statistically significant.

Results and Discussion

Extraction process—Percentage yield of HAELA was found to be 3.2% (w/w). Priliminary phytochemical analysis for HAELA indicated the presence of flavonoid (sulphuric acid test and ferric chloride test), presence of tannins (ninhydrin test), presence of saponins (foam test) and presence of steroids (salkowski test).The other physiochemical and analytical profiles are presented in Table 1.

Table 1—Phtochemical and analytical profile of HAELA

Test	Observation
Physical appearance	Brown amorphous powder
Melting Point	Decomposes above 48°C
Solubility	Freely soluble in methanol, ethanol sparingly soluble in water, ether, chloroform
UV EtOH λ Max nm [$E^{1\%}$ 1cm]	273
R_f values (TLC) *of HAELA	0.76

*solvent system: chloroform: methanol(8:2)

Acute toxicity studies—The LD_{50} value by oral route was not determined as no mortality was observed upto 10 g/kg dose level. No mortality was observed during 72 h of observation period.

Biochemcial studies–Both the CCl_4 and rifampicin treatment significantly increased serum AST, ALT, ALP and LDH ($P<0.001$) and decreased TP level ($P<0.001$). Treatment with silymarin (200 mg/kg) and HAELA (100, 200 and 400 mg/kg) prevented the elevation of serum marker enzymes AST, ALT, ALP and LDH and restored TP in both CCl_4 and rifampicin treated rats (Tables 2 and 3).

Both CCl_4 and rifampicin treatment to control groups of rats showed significant ($P<0.001$) increase in MDA formation, decreased GSH levels significantly ($P<0.001$). Treatment with silymarin (200 mg/kg) and HAELA (100, 200 and 400 mg/kg) prevented the decrease in liver GSH due to CCl_4 and rifampicin, and able to restore the liver GSH levels to almost normal ($P<0.001$). In addition, the levels of CAT and SOD in liver homogenates were also decreased significantly ($P<0.001$) with CCl_4 and rifampicin treatment. Treatment with silymarin and HAELA also significantly ($P<0.01$) restored CAT and SOD levels in liver. Furthermore, silymarin (200 mg/kg) and HAELA (100, 200 and 400 mg/kg) treatment significantly ($P<0.001$) decreased the

Table 2—Effect of HAELA on serum marker enzymes and protein in CCl_4 induced liver injury
[Values are mean ± SEM of 6 rats]

Biochemical Parameter	Vehicle (1 ml/kg)	CCl_4 (1 ml/kg)	Silymarin+CCl_4 (200 mg/kg)	Silymarin+CCl_4 (100 mg/kg)	HAELA+CCl_4 (200 mg/kg)	HAELA+CCl_4 (400 mg/kg)
AST (U/ml)	23.80±6.84	$112.60\pm8.80^{a***}$	$27.20\pm4.30^{b***}$	$64.80\pm9.55^{b**}$	$33.20\pm5.47^{b***}$	$34.00\pm4.18^{b***}$
ALT (U/ml)	35.10±2.75	$87.89\pm2.59^{a***}$	$37.50\pm1.47^{b***}$	77.45±3.52	$71.06\pm2.82^{b**}$	$63.39\pm2.47^{b***}$
ALP (KA units/dl)	2.57±0.41	$46.25\pm3.16^{a***}$	38.34±2.16	$31.24\pm4.07^{b**}$	$32.92\pm2.31^{b*}$	$29.81\pm0.97^{b***}$
TP (gm/dl)	10.82±0.74	$6.02\pm0.33^{a***}$	$9.91\pm0.71^{b***}$	6.10±0.40	$8.62\pm0.29^{b*}$	$9.77\pm0.40^{b***}$
LDH (IU/L)	245.71±7.12	$810.97\pm18.52^{a***}$	$344.10\pm31.01^{b***}$	718.12±48.38	$617.02\pm31.10^{b**}$	$344.21\pm29.35^{b***}$

$^{a***}P<0.001$, CCl_4 control compared with vehicle treated group.
P values: $^{b***}<0.001$, $^{b**}<0.01$, $^{b*}<0.05$, (silymarin+CCl_4) and (HAELA+CCl_4) treated groups compared with CCl_4 control group.

Table 3—Effect of HAELA on serum marker enzymes and protein in rifampicin induced liver injury
[Values are mean ± SEM of 6 rats]

Biochemical Parameter	Vehicle (1ml/kg)	Rifampicin (100 mg/kg)	Silymarin+rifampicin (200 mg/kg)	Silymarin+rifampicin (100 mg/kg)	HAELA+rifampicin (200 mg/kg)	HAELA+rifampicin (400 mg/kg)
AST (U/ml)	33.2±5.04	$114\pm12.53^{a***}$	$36.4\pm8.04^{b***}$	$68.2\pm5.87^{b**}$	$47\pm8.69^{b***}$	$21\pm5.14^{b***}$
ALT (U/ml)	15.8±8.13	$108\pm11.19^{a***}$	$22.2\pm9.52^{b***}$	$70\pm8.86^{b**}$	$69\pm5.51^{b**}$	$17.8\pm3.11^{b***}$
ALP (KA units/dl)	3.3±0.73	$30.82\pm2.06^{a***}$	$8.56\pm1.23^{b***}$	$20.62\pm0.94^{b*}$	$18.08\pm1.98^{b**}$	$6.02\pm0.73^{b***}$
TP(gm/dl)	7.88±0.37	$4.93\pm0.13^{a***}$	$6.88\pm0.37^{b***}$	4.59±0.20	$6.88\pm0.23^{b***}$	$7.08\pm0.21^{b***}$
LDH(IU/L)	249.70±4.07	$692.15\pm77.38^{a***}$	$428.47\pm14.29^{b***}$	$461.43\pm15.43^{b**}$	$374.17\pm16.48^{b***}$	$308.41\pm19.06^{b***}$

P values: $^{a***}P<0.001$, Rifampicin control compared with vehicle treated group.
P values: $^{b***}<0.001$, $^{b**}<0.01$, $^{b*}<0.05$, (silymarin+ rifampicin) and (HAELA+rifampicin) treated groups compared with rifampicin control group.

increased concentration of MDA formation in liver due to CCl_4 and rifampicin treatment (Tables 4 and 5).

Histopathological studies—The livers of animals of vehicle treated control group showed normal histology (Fig. 1a). The liver sections of animals treated with CCl_4 and rifampicin showed a moderate degree of centrilobular necrosis with fatty and lymphocytes infiltration. The perilobular zones showed moderate diffused granular degeneration. The sinusoids were moderately obliterated. (Fig. 1b and 2b).

Compared with lesions observed with CCl_4 and rifampicin groups, the lesions noted in the livers of silymarin treated animals were of a much milder degree (Fig. 1c and 2c). Further, a mild degree of centrilobular fatty infiltration and lymphocyte infiltration was observed. Regenerative foci of a mild to moderate degree were also noted in the livers of silymarin treated rats.

The livers of the animals treated with HAELA 100, 200 and 400 mg/kg exhibited mild to moderate degree centrilobular fatty and lymphocyte infiltration, regeneration of hepatocytes with prominent nucleus and very few infiltration of inflammatory cells. (Fig. 1d-1f and 2d-2f).

Carbon tetrachloride (CCl_4), a well known and most commonly used halogenated hydrocarbon belongs to the class of chemical hepatotoxins, possessing wide spectrum of hepatocellular dysfunction (decreased lipid secretion, decreased protein synthesis, loss of glycogen, and finally, hepatocellular necrosis). The toxic action of CCl_4 was found to be due to the formation of active metabolite, the trichloromethyl ($CCl_3^{\bullet}$) radical through metabolic pathways including cytochrome P_{450} in the mixed-function oxidase system of the liver endoplasmic reticulum. The trichloromethyl ($CCl_3^{\bullet}$) radical which readily reacts with oxygen to form a trichloromethylperoxyl radical ($CCl_3O_2^{\bullet}$)[21-22]. This radical forms covalent bond with sulfhydryl groups of several membrane molecules like GSH leading to depletion and causes lipid peroxidation. The lipid peroxidation leads to formation of lipid peroxides, which in turn to yields products like MDA, which is known to cause loss of integrity of cell membranes and damage hepatic tissue[23] and also create imbalance in the redox status of hepatocyte and liver lysosomal instability, which leads to leakage of serum marker enzymes[24-26]. It is possible that hepatocellular damage

Table 4—Effect of HAELA on in CCl_4 induced changes in the activities of liver antioxidant enzymes, malondialdehyde (MDA) and reduced glutathione (GSH) injury

[Values are mean ± SEM of 6 rats]

Liver Antioxidant Enzymes	Vehicle	CCl_4	Silymarin+CCl_4	HAELA+ CCl_4		
	(1 ml/kg)	(1 ml/kg)	(200 mg/kg)	(100 mg/kg)	(200 mg/kg)	(400 mg/kg)
MDA formation (nM of MDA/g of wet liver)	6.99±0.41	25.03±1.21^{a***}	19.16±0.62^{b***}	20.49±0.63^{b**}	18.89±0.42^{b***}	15.94±0.58^{b***}
GSH (µg/g of wet liver)	11.72±2.56	1.83±0.30^{a***}	6.54±1.72^{b***}	4.88±0.79^{b*}	5.33±0.37^{b**}	6.67±0.87^{b***}
CAT(µM of H_2O_2/g of wet tissue/min)	32.44±1.58	19.88±1.90^{a***}	28.53±0.80^{b**}	17.41±1.81	24.32±0.81	28.98±0.83^{b**}
SOD (Units/mg of wet tissue)	33.96±3.52	15.90±2.26^{a**}	28.76±1.24^{b*}	27.88±1.84	30.04±4.71^{b*}	32.16±1.36^{b**}

P values: ^{a***}<0.001, CCl_4 control compared with vehicle treated group.
P values: ^{b***}<0.001, ^{b**}<0.01, ^{b*}<0.05, (silymarin+CCl_4) and (HAELA+CCl_4) treated groups compared with CCl_4 control group.

Table 5—Effect of HAELA on in rifampicin induced changes in the activities of liver antioxidant enzymes, malondialdehyde (MDA) and reduced glutathione (GSH) injury

[Values are mean ± SEM of 6 rats]

Liver Antioxidant Enzymes	Vehicle	Rifampicin	Silymarin+rifampicin	HAELA + rifampicin		
	(1 ml/kg)	(100 mg/kg)	(200 mg/kg)	(100 mg/kg)	(200 mg/kg)	(400 mg/kg)
MDA formation (nM of MDA/g tissue	3.54±1.47	19.32±2.99^{a***}	6.05±1.22^{b***}	12.62±3.45	5.98±1.46^{b***}	4.68±1.01^{b***}
GSH (µg/g of wet liver)	8.60±0.62	2.80±0.84^{a***}	8.0±0.60^{b***}	5.10±0.78	6.87±0.68^{b***}	8.68±0.36^{b***}
CAT (µM of H_2O_2/g of wet tissue/min) SOD	38.83±3.44	15.9±2.26^{a***}	37.04±3.33^{b***}	33.11±1.31^{b**}	38.32±4.67^{b***}	41.95±2.86^{b***}
(Units/mg of wet tissue)	33.21±3.17	15.78±2.18^{a***}	40.46±1.85^{b***}	17.54±3.25	33.91±3.78^{b***}	39.95±1.86^{b***}

P values: ^{a***}<0.001, Rifampicin control compared with vehicle treated group.
P values: ^{b***}<0.001, ^{b**}<0.01, (silymarin+ rifampicin) and (HAELA+rifampicin) treated groups compared with Rifampicin control group

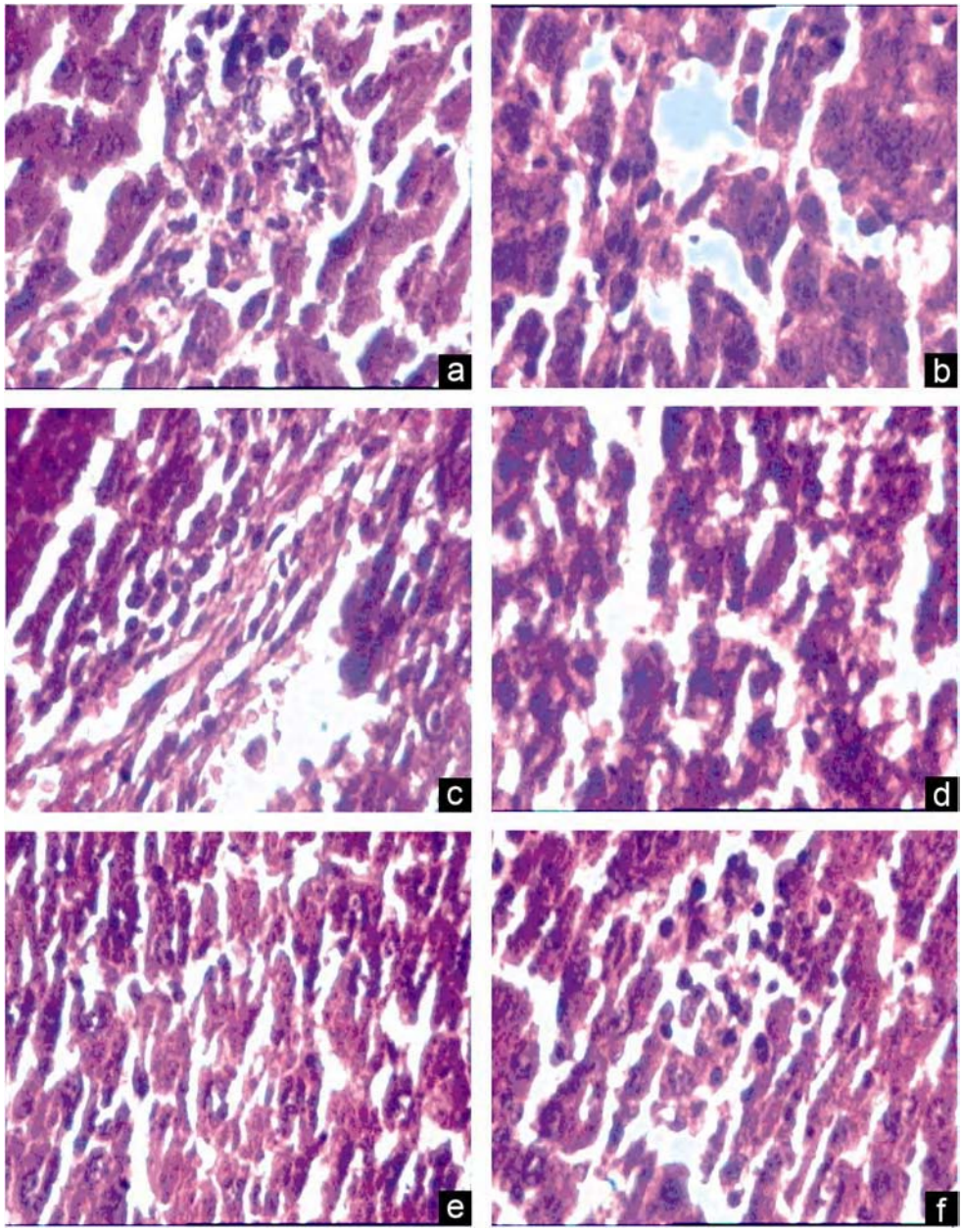

Fig. 1—Effect of HAELA on histopathological examination of rat liver in CCl_4 induced liver toxicity (× 400 H & E). (a) Vehicle control treated group showing normal hepatocytes with prominent nucleus; (b) CCl_4 treated group showing moderate degree of centrilobular necrosis with fatty and lymphocytic infiltration; (c) silymarin (200 mg/kg) + CCl_4 treated group showing milder degree of centrilobular fatty and lymphocytic infiltration and regenerating architecture of hepatocytes with mild necrosis; (d) HAELA (100 mg/kg) + CCl_4 treated group showing moderate degree of centrilobular fatty and leucocytic infiltration with minimal regeneration of hepatocytes; (e) HAELA (200 mg/kg) + CCl_4 treated group showing regeneration of hepatocytes with prominent nucleus and no signs of necrosis or inflammatory infiltrate; (f) HAELA 400 mg/kg + CCl_4 treated group showing regeneration of hepatocytes with prominent nucleus and no necrosis or inflammatory infiltration, close to normal

occurs when the free radicals generation exceeds the cellular radicals scavenging capacity. Assessments of liver toxicity was done by measuring the marker enzymes viz AST, ALT, ALP and LDH, which are originally present in high concentration in the cytoplasm. When there is hepatic injury these enzymes leak into blood stream inconformity with extent of hepatoxicity.

Similarly, the anti-tubercular drugs including rifampicin alone or in combination with other anti-TB drugs have been documented to be hepatotoxic[27-28]. It was evidenced that biotransformation of rifampicin

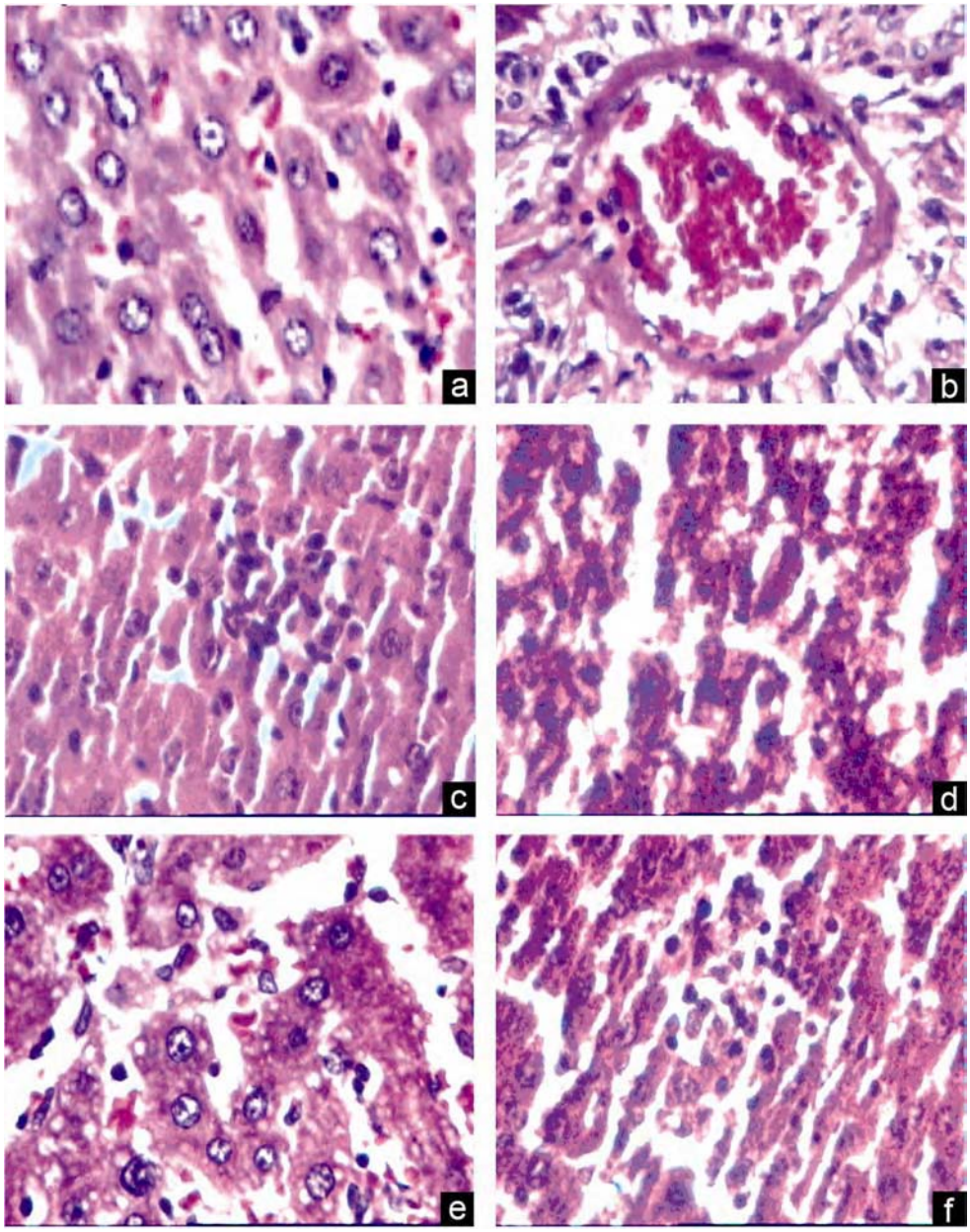

Fig. 2—Effect of HAELA on histopathological examination of rat liver in rifampicin induced liver toxicity (× 400 H & E). (a) vehicle control treated group showing normal hepatocytes having a prominent nucleus; (b) rifampicin control treated group showing portal infiltration along with extensive degeneration and necrosis of hepatocytes; (c) silymarin (200 mg/kg) + rifampicin (100 mg/kg) treated group showing regenerating hepatocytes with no signs of necrosis and inflammation; (d) HAELA (100 mg/kg) + rifampicin (100 mg/kg) treated group showing architecture of hepatocytes with focal areas of necrosis and partly disturbed architecture with portal infiltration; (e) HAELA 200 mg/kg + rifampicin (100 mg/kg) treated group showing regenerating hepatocytes with a prominent nucleus and partly distorted architecture; (f) HAELA 400 mg/kg + rifampicin (100 mg/kg) treated group showing normal architecture of hepatocytes with prominent nucleus. No signs of necrosis and inflammation and are close to normal

into its active metabolite, 25-desacetyl rifampicin reduces the drug metabolizing enzymes and specifically binds to RNA polymerase which inhibits the nucleic acid and protein synthesis responsible for hepatotoxicity[29].

Glutathione (GSH) is one of the most abundant tripeptide non-enzymatic intracellular biological antioxidant[30] present in liver. It is involved in the removal of free radicals such as H_2O_2, superoxide anions and alkoxy radicals, preserving membrane

protein thiols and a substrate for glutathione peroxidase and glutathione reductase.

In the present experiments, CCl_4 and rifampicin treatment increased the activities of GSH related enzymes thereby decreasing the GSH content in liver, whereas treatment with silymarin and HAELA (200 and 400 mg/kg) able to reverse such effects. It may be understood that effect of silymarin and HAELA may be due to an initial reduction in hepatic peroxidative activities, followed by inhibition of the activities of GSH related enzymes. Aforementioned effects with silymarin and HAELA treatment finally results into restoration of GSH content in CCl_4 and rifampicin induced hepatotoxicity.

Superoxide dismutase (SOD) is a key defense enzyme and catalyzes the dismutation of superoxide anions. Catalase (CAT) is a haemeprotein that catalyses the reduction of H_2O_2 and able to prevents the tissue from reactive free oxygen and hydorxy radicals. Decreased in SOD activity can result in the decreased removal of superoxide anions that may inactivate SOD thereby causing an inactivation of H_2O_2 scavenging enzymes. It is quite likely that administration of silymarin and HAELA to CCl_4 and rifampicin treated rats able to prevent effectively the decrease in SOD and CAT activities, which may be directly correlated to scavenging or neutralizing of radicals by silymarin or HAELA resulting in protection of these important defense enzymes.

It is suggested that, saponins in HAELA play an important role as antioxidant for prevention of oxidative hepatic damage. Furthermore, the flavonoids and saponins of HAELA may able to stabilize reactive oxygen species by reacting with them and oxidizes subsequently to more stable and less reactive radicals.

This study demonstrated that, silymarin (200 mg/kg) and HAELA (200 and 400 mg/kg) significantly prevented CCl_4 and rifampicin induced hepatotoxicity in rats.

Histoarchitectural improvement further supported by biochemical changes in liver, reduction in serum marker enzymes and augmentation of endogenous antioxidants in liver, all contributes to its hepatoprotective activity.

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