

In- Vitro Hepatoprotective Action of the Crude Extracts of *Luffa Amara* (Whole Fruit) And *Rheum Emodi* (Rhizomes) In CCl₄ Intoxicated Rat Hepatocytes

ABSTRACT

Aim: The objective of this in-vitro study involves evaluating the protective action of the extracts of *L. amara* (LA) (whole fruits including seeds) and *R. emodi* (RE) (rhizomes) at various concentrations on isolated primary rat hepatocytes.

Method: The pulverised dried whole fruits of *L. amara* (LA) and rhizomes of *R. emodi* (RE) were extracted successively with petroleum ether (PE), ethanol (EE) and distilled water (AE) and vacuum dried. These extracts of LA petroleum ether (PE), ethanolic (EE) and aqueous (AE) extracts and RE obtained were subjected to in vitro studies at doses of 25, 75, 100, and 150 µg/ml and silymarin (250 µg/ml) in CCl₄ (1%) intoxicated primary hepatocytes monolayer cultures the hepatoprotective action of all the extracts of both plants at different doses was carried out using isolated rat hepatocytes which were subjected to CCl₄ intoxication followed by estimating/ measuring the changes in serum biochemical markers – SGPT, SGOT, ALP, Total proteins (TP), total bilirubin (TB), albumin (ALB) and triglycerides (TGL).

Result: Hepatoprotective activity against CCl₄ was demonstrated in the rat primary monolayer hepatocyte culture using MMT assay with the ethanolic extracts of both plants showing more hepatocyte protective action compared to the aqueous and petroleum ether extracts by reducing the elevated serum marker levels. Alcoholic and aqueous extracts were found to express more protective action towards CCl₄ intoxicated isolated primary rat hepatocytes in a dose dependant manner.

Conclusion: based on the result, it is suggested that the extract with the most hepatocyte protective action at a dose of 150µg is LA ethanolic extract (viability=88.24%), followed by LA aqueous extract (viability=84.31%), RE ethanolic extract (viability=88.24%) and RE aqueous extract (viability=88.24%) - which are comparable to the reference silymarin with viability at 92.15%. the petroleum ether extracts of both plants showed least hepatic cell viability with LA pet ether extract at 49.02% and RE pet ether extract at 47.85%

Key words: *Luffa amara* (LA), *Rheum emodi* (RE), pet ether extract, alcoholic extract, aqueous extract. Abbreviations : LAPE –L.amara pet ether extract, LAEE- L.amara ethanolic extract, LA AE- L.amara aqueous extract, REPE- R.emodi pet ether extract, REEE- R.emodi ethanolic extract, REAE- R.emodi aqueous extract.

Introduction:

Liver being a vital organ for detoxification, can be injured or suffer functional impairment leading many health disorders and diseases.^[1] It is documented that acute hepatitis resembles

closely to viral hepatitis with the causative factors being hepatotoxins and confirmations done using clinical, biochemical and histological findings. Further, as a consequence of many allopathic drug therapies, such as antitubercular drugs, chronic liver diseases like chronic hepatitis, cirrhosis, including vascular lesions and fatty liver get precipitated such that they often resemble viral hepatitis. It is imperative for the chemically induced hepatic injury to be severely modifying or damaging to liver functions, as sequential hepatic injury is related to the type hepatotoxins used on specific animal species for consequential liver disturbances clinically resembling acute hepatotoxicity, like acute viral hepatitis.^{[2][3]}

Such hepatotoxicity has been observed with CCl₄ – a most commonly used toxin in experimental studies, as it readily available in pure form, undergoes hepatic metabolism by cytochrome P450 (especially by P450 2E1) causing free trichloromethyl (CCl₃·) radicals generation, consequently resulting in direct or indirect in damage to membrane and intracellular lipids, nucleic acids, and other molecules which further leads to toxic hepatic damage with the severity ranging from necrosis leading to triacylglycerol accumulation, cirrhosis and cancer depending upon its mode of application and dose.^{[4] [5]}

Carbon tetrachloride has been one of the most commonly used toxins in the experimental study of liver disease. In the rat, doses of carbon tetrachloride in the range of several mg/kg body weight produce liver necrosis within one to two hours^[6] Smaller doses in the range of tenths of ml CCl₄/kg body weight repeated several times weekly can produce cirrhosis within a few months.^[7]

Lipophilic solvents such as CCl₄ might cause liver injury by direct physical interactions with cell membranes (Berger et al. 1986). However, the significance of such direct solvent effects is controversial. As allopathic medicines have a cascade of side effects, herbal drugs including have garnered innumerable worldwide attention in treating diverse diseases such as dengue^[8], jaundice^[9], viral diseases such as HIV-1, SARS related coronavirus, respiratory syncytial virus, including hepatocellular damage in chronic hepatitis B and C.^[10]

Luffa acutangula.var.*amara* or *L.amara* (Cucurbitaceae), is also called karvi turai. Being an annual herb, it's found all over India, but more abundantly in the western peninsula. The fruits are very bitter in taste; the plant is used as a carminative, laxative, tonic for intestines, digestible, used to cure the *vata* and *kapha*, biliousness, liver complaints, leukoderma, piles, ascites, tumour and tumorous, useful in bronchitis and asthma and the seed kernals are used to treat dysentery.^{[11] [12]}

It is also used in treating jaundice when taken in the form of very fine powder through nose, while its seeds show emetic, expectorant, and demulcent properties. The ancient literature also revealed that the plant is significantly used as abortifacient and antifungal agent. The reported chemical examination of *Luffa acutangula* showed the presence of carbohydrates, carotene, fat, protein, phytin, amino acids, alanine, arginine, cystine, glutamic acid, glycine, hydroxyproline, leucine, serine, tryptophan, pipecolic acid, flavonoids and saponins.^[13]

Rheum emodi or Indian or Himalayan rhubarb (Polygonaceae) of Indian origin, is considered as purgative, stomachic, and astringent tonic, possesses aperient, emmenagogue and diuretic properties. Root is used as expectorant, appetizer, as powder applied on cuts, wound, and muscular swelling, toothache, tonsillitis and mumps, ulcers.^[14]

It is used to heal skin sores and scabs. While larger doses are used as laxative, small doses are used to treat dysenteric diarrhea^[15]

Chinese medicine uses rhubarb for ulcer remedy, as a bitter, cold, dry herb used to “clear heat” from the liver, stomach and blood, also to expel helminthes and to treat cancer, fever, upper intestinal bleeding (ulcers), and headache.^{12,13} Its also used in of spring tonics or blood cleansing cures.^{14,15}

The pulverised dried whole fruits of *L. amara* (LA) and rhizomes of *R.emodi* (RE) were extracted successively with petroleum ether (PE), ethanol (EE) and distilled water (AE) and vacuum dried. These extracts of LA petroleum ether (PE), ethanolic (EE) and aqueous (AE) extracts and RE obtained were subjected to in vitro studies at doses of 25, 75, 100, and 150 µg/ml and silymarin (250 µg/ml) in CCl₄ (1%) intoxicated primary hepatocytes monolayer cultures

Materials and method:

Preparation of plant extracts:

The whole fruits of *L.amara* and rhizomes of *R.emodi* were identified and collected from the department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh, India. Both plants were subjected to pulverisation and extraction with petroleum ether, ethanol and distilled water using standard methods of soxhlet.^{[16] [17] [18]} The extracts obtained were concentrated and dried under vacuum.^[19]

Animals: Witstar albino rat was obtained from the animal house of Deccan College of Medical Sciences, Hyderabad for this study. The animal was maintained under standard laboratory conditions (12 hrs light: dark cycle, with 60-70 % humidity at $25 \pm 2^\circ\text{C}$) with food and water *ad libitum*. The experiment was conducted after the approval from Institutional Animal Ethics Committee constituted as per CPCSEA guidelines. Protocols for the experiment described below were approved by the ethical board. For this in-vitro study, the albino rat was fasted 18 to 24 hrs with water *ad libitum* prior to the experiment.

Isolation of rat hepatic cells ^{[20] [21] [22] [23]}.

To prepare the primary hepatocyte culture, the albino rat was cleaned with rectified alcohol, anaesthetised, followed by dissecting it to expose and cannulate (needle no.25) the portal vein which is then connected to an infusion set. Sterile equipment and glassware was used throughout the experiment. The needle being tied in place, the inferior vena cava was cut below the renal vein and the liver was immediately perfused with Ca^{2+} Mg^{2+} Hanks Buffer solution (at pH 7.4). when the liver turns white after thorough perfusion, the cannula is removed and the isolated liver is minced in a petri dish having Ca^{2+} Mg^{2+} Hanks Buffer solution (HBSS). This was then transferred to a conical flask (having 10ml of 0.75% collagenase in HBSS), stirred with the aid of a magnetic stirrer at 37°C for 10 min. and centrifuged at 50 rpm for 10min. After aspirating the supernatant, the cells are again suspended in HBSS, followed by washing them twice and counting them using trypan blue dye. The isolated hepatic cells were cultured in sterile culture bottles containing Eagles MEM (having 10% inactivated serum at a density = 0.5×10^9 cell/ L. These bottles were incubated for 24 hrs at 37°C under 5% CO_2 in a humidified incubator after which their viability was tested. The hepatocytes which accumulated at the bottom of the tubes were observed for growth. Cytotoxicity of both plant extracts of *L.amara* and *R. emodi* were assessed using the primary hepatocyte culture obtained.

Collagenase perfusion technique ^[24] was utilised to prepare rat hepatocytes, with the cells undergoing many centrifugations so as to be purified and later inoculated at 0.5×10^8 cell/ L density on collagen coated plates, which in turn are incubated for 24 hrs. This was followed by exposing the medium to CCl_4 (1%) with / without the samples, and sylimarin (250 $\mu\text{g}/\text{ml}$) being taken as the reference standard to be tested for their hepatoprotective activity. ^{[25] [26]}. After 1.5 hrs, the culture medium was collected and tested for hepatic parameters like ALT,

AST, ALP, TG, TB, ALB and TP. Cell viability was estimated by trypan blue dye exclusion method ^[27]

Results:

Preliminary phytochemical tests for LA revealed the presence of the following; LAPE- fixed oils and triterpenes. LAEE & LAEE- flavonoids, glycosides, saponins, sterols, proteins, amino acids and carbohydrates. REPE- triterpenes, REEE- glycosides, alkaloids, tannins, terpenes, saponins and flavonoids. REAE- flavonoids, alkaloids, saponins, terpenes, proteins and carbohydrates.

Treatment of primary rat hepatocytes with CCl₄ resulted in raised **SGPT, SGOT**, ALP, TB levels and lowered ALB, TG, TP (total proteins) levels as a consequence of membrane damage and enzyme release – determined by **serum enzyme levels** which are generally found to be elevated in hepatic stress and damage. The cytoprotective activity of the crude extracts of both plants LA and RE showed significant protection against 1 % CCl₄ intoxication in rat hepatocyte culture (tables 1 &2) with the ethanolic extract of LA showing more protection (P < 0.001) at nearly all doses followed by the aqueous (P < 0.001) and pet ether extracts (P <0.05– 0.01) being variable with dose. Similarly, significant restoration of hepatic parameters was imparted by the alcoholic (P < 0.001) and aqueous (P < 0.001) extracts of RE with petroleum extracts of both plants showing least protective action (P <0.05–0.01). Maximum hepatoprotective effect was seen with LAEE at a dose of 150µg among all extracts and least with 25 µg of all extracts. The percentage viability of cells was found to be more with sylimarin (92.15%) taken as reference, followed by LEEE at 150 µg (88.24%), LAEE 150 µg (84.31 %), REAE 150 µg (82.35 %), REAE 150 µg (80.39 %), while least was observed with CCL₄ (27%).

Table- 1. Effect of the plant extracts of LA on the biochemical parameters of CCl₄ induced toxicity in fresh isolates of rat hepatocytes, n=3 replicates

Groups	Parameters in							% Viable
	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	TG (mg/dl)	TP (g/dl)	TB (mg/dl)	ALB (g/dl)	
Control	15.62±0.017	11.02±0.005	34.05±0.017	161.7±2.028	1.32±0.017	0.21±0.031	2.83±0.013	100
CCl ₄ (1%)	59±0.008 ^Φ	85.98±0.014 ^Φ	96.97±0.029 ^Φ	73.33±2.603 ^Φ	0.36±0.010 ^Φ	0.70±0.008 ^Φ	0.71±0.008 ^Φ	27
CCl ₄ 1% + Syllimarin 250 µg	19.72±0.023	15±0.005	35.90±0.032	156.3±2.404	1.26±0.010	0.26±0.020	2.31±0.010	92.15
LAPE 25µg	53.05±0.046***	48.34±0.006***	78.32±0.020***	103.7±2.848**	0.46±0.033*	0.62±0.010**	0.84±0.007**	38.43
LAPE 75µg	51.66±0.023***	47.46±0.017***	75.03±0.017***	108.3±2.028**	0.52±0.017**	0.60±0.023*	0.87±0.031**	45.10
LAPE 100 µg	49.33±0.0206***	45.68±0.020***	70.70±0.025***	111.3±2.333**	0.60±0.028**	0.57±0.010**	0.91±0.029**	47.06
LAPE 150 µg	48.58±0.014***	44.95±0.008***	67.99±0.024***	117.3±2.603**	0.66±0.012***	0.51±0.017**	0.92±0.042**	47.85
LAEE 25 µg	32.98±0.030***	28.99±0.0202***	50.15±0.017***	137.7±2.186***	0.57±0.012***	0.33±0.024***	1.96±0.026***	50.98
LAEE 75 µg	29.24±0.122***	24±0.003***	45.99±0.018***	142.7±3.930**	0.87±0.014***	0.32±0.026***	1.98±0.020***	60.78
LAEE 100 µg	22.43±0.027***	18.23±0.005***	42.63±0.329***	149.3±2.963***	1.12±0.023***	0.30±0.030***	2.08±0.020***	80.39
LAEE150 µg	21.38±0.036***	17.83±0.020***	38.41±0.020***	152.3±2.603***	1.23±0.012***	0.27±0.018***	2.15±0.020***	88.24
LAAE 25 µg	31.61±0.020***	26.31±0.0202***	53.24±0.017***	137.7±1.202***	0.54±0.010***	0.37±0.003***	1.94±0.048***	50.98
LAAE 75 µg	28.58±0.031***	24.96±0.023***	48.05±0.012***	139.7±2.728***	0.84±0.014***	0.35±0.017***	1.97±0.026***	64.70
LAAE 100 µg	24.31±0.026***	20.88±0.020***	44.25±0.026***	146.3±2.728***	1.04±0.020***	0.33±0.029***	2.04±0.040***	79.61
LAAE 150 µg	22.04±0.018***	18.88±0.017***	22.99±0.032***	148.3±2.028***	1.15±0.006***	0.29±0.010***	2.05±0.040***	84.31

Significant at ^Φ=P < 0.001 compared to control group; ***=P < 0.001, **=P < 0.01, *= P < 0.05 compared to control group

Table- 2. Effect of the plant extracts of RE on the biochemical parameters of CCl₄ induced toxicity in fresh isolates of rat hepatocytes, n=3 replicates

Groups	Parameters							% Viable
	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	TG (mg/dl)	TP (g/dl)	TB (mg/dl)	ALB (g/dl)	
Control	15.62±0.017	11.02±0.005	34.05±0.017	161.7±2.028	1.32±0.017	0.21±0.031	2.83±0.013	100
CCl ₄ (1%)	59±0.008 ^Φ	85.98±0.014 ^Φ	96.97±0.029 ^Φ	73.33±2.603 ^Φ	0.36±0.010 ^Φ	0.70±0.008 ^Φ	0.71±0.008 ^Φ	27
CCl ₄ (1%) + Syllimarin 250 µg	19.72±0.023	15±0.005	35.90±0.032	156.3±2.404	1.26±0.010	0.26±0.020	2.31±0.010	92.15
REPE 25µg	55.23±0.024***	49.37±0.026***	58.96±0.023***	97.67±2.18**	0.43±0.026 ^{bs}	0.63±0.025 ^{bs}	0.82±0.037 ^{bs}	39.22
REPE 75µg	53.15±0.020***	48.42±0.014***	57.88±0.026***	105.7±2.333**	0.48±0.020**	0.62±0.021*	0.85±0.040*	43.13
REPE 100 µg	50.63±0.025***	45.77±0.014***	54.04±0.023***	108.3±6.888*	0.57±0.014**	0.59±0.011*	0.87±0.029*	49.02
REPE 150 µg	49.54±0.026***	44.47±0.026***	52.42±0.031***	113.7±3.383**	0.61±0.017**	0.53±0.003***	0.89±0.026**	47.85
REEE 25 µg	35.05±0.024***	31.97±0.0202***	51.35±0.036***	135.3±2.906***	0.53±0.032*	0.37±0.003***	1.94±0.026***	50.98
REEE 75 µg	30.30±0.026***	26±0.017***	48.18±0.032***	138.0±2.646***	0.81±0.020***	0.33±0.008***	1.96±0.034***	54.90
REEE 100 µg	23.79±0.023***	19.89±0.023***	43.28±0.037***	147.3±4.333***	1.00±0.014***	0.31±0.032***	2.03±0.029***	74.51
REEE150 µg	22.61±0.020***	18.75±0.017***	39.19±0.031***	152.3±1.453***	1.21±0.020***	0.28±0.006***	2.11±0.066***	80.39
REAE 25 µg	31.02±0.016***	27.87±0.0218***	53.08±0.034***	133.0±2.887***	0.50±0.006***	0.38±0.003***	1.91±0.043***	49.02
REAE 75 µg	30.01±0.017***	26.20±0.026***	49.51±0.026***	137.3±1.453***	0.78±0.024***	0.36±0.003***	1.95±0.035***	66.66
REAE 100 µg	24.89±0.017***	22.38±0.017***	44.50±0.035***	144.3±2.028***	0.96±0.029***	0.34±0.003***	2.00±0.023***	80.39
REAE 150 µg	25.34±0.023***	19.51±0.027***	41.06±0.032***	149.0±1.528***	1.20±0.030***	0.30±0.006***	2.01±0.023***[2]	82.35

Significant at ^Φ=P < 0.001 compared to control group; ***=P < 0.001, **=P < 0.01, *= P < 0.05 compared to control group

Fig 1. Control- CCL₄ – Silymarin

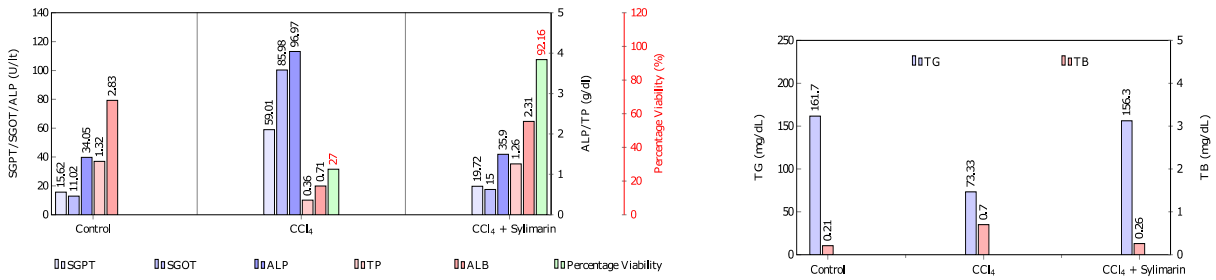


Fig 2. Extracts of L.amara and R.emodi at 25µg

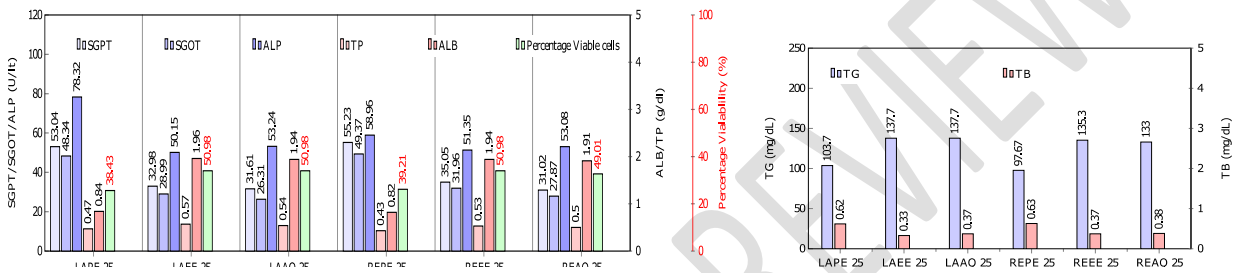


Fig 3. Extracts of L.amara and R.emodi at 75µg

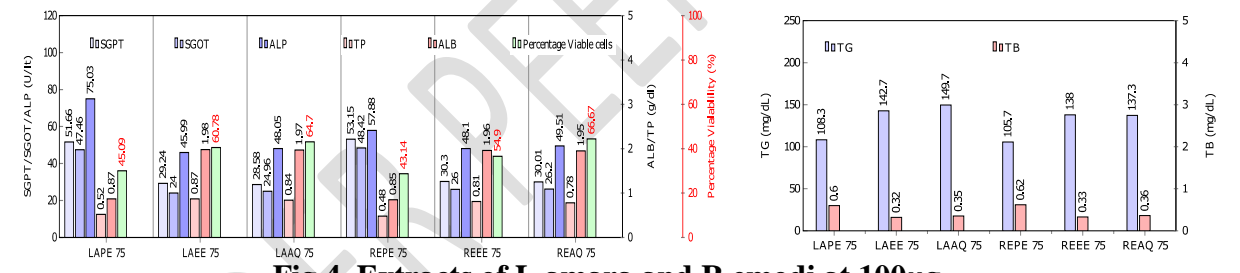


Fig 4. Extracts of L.amara and R.emodi at 100µg

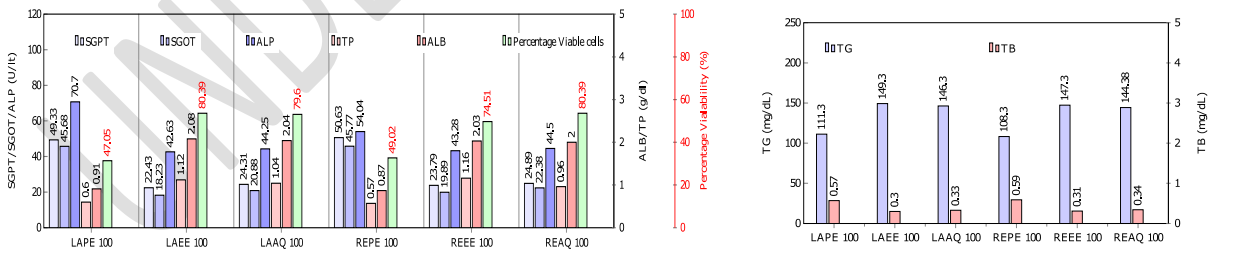
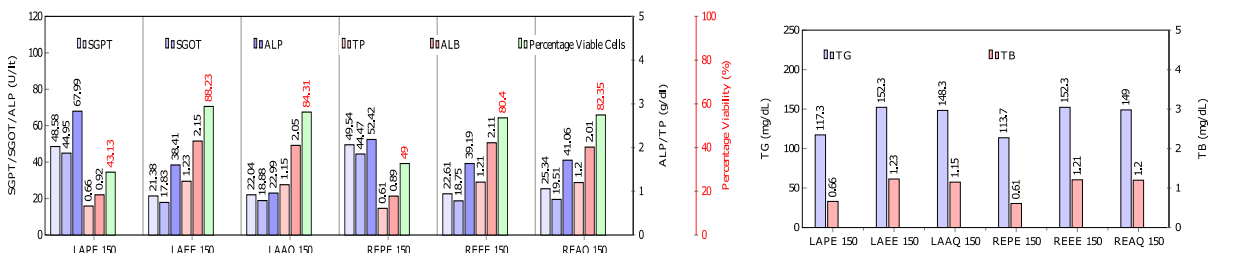


Fig 5. Extracts of L.amara and R.emodi at 150µg



Discussion:

Carbon tetrachloride induced hepatic injury involves the metabolic product trichloromethyl radical formation as a consequence of CCl₄ metabolism by cytochrome P450 enzymes. This further induces free radical formation leading to hepatic cell membrane damage via lipid peroxidation of fatty acid and/ or covalently binding to proteins, DNA or lipids. [28] [29]

Flavonoids are generally considered free radical scavengers. [30]

As invitro hepatoprotective activity has not been reported on the whole fruit extracts of *Luffa amara*, this experiment was aimed to estimate the protectiveness of all the afore mentioned extracts in CCl₄ induced hepatic damage. In this study, CCl₄ hepatotoxin reduced cell viability indicative of hepatocytic plasma membrane damage and subsequent release of liver enzymes. Incubation of the rat hepatocytes for 1.5 hrs with both the whole fruit extracts of *L. amara* and rhizome extracts of *R. emodi* showed effective hepatoprotectiveness in presence of CCl₄ (1%) intoxication comparably at higher doses than lower doses.

Conclusion: Thus, considering the presence of flavonoids in both plant extracts, hypothetical assumption is conclusive of flavonoids being responsible for hepatoprotective action. This study further emphasises the possibility of using this combination of plant extracts for their synergistic use for futuristic safe herbal treatments of hepatic disorders as well as other pathological ailments as claimed by native practitioners of Ayurveda and Unani medicine.

NOTE:

The study highlights the efficacy of "Herbal" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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