

***In vitro* Antioxidant and Hepatoprotective Effect of the Whole Plant of *Rungia repens* (L.) Nees, Against CCl₄ Induced Oxidative Stress in Liver Slice Culture Model**

Anagha A. Rajopadhye¹, Anuradha S. Upadhye^{1*}

*Corresponding author:

Dr. Anuradha S. Upadhye,

Scientist,

¹Agharkar Research Institute,

G. G. Agarkar Road,

Pune, India.

Telephone: +020-25654357

Fax: +020- 25651542

A B S T R A C T

Rungia repens (L.) Nees (Family-Acanthaceae) commonly known as 'Pittapapda' or 'Khet papra' is used in the treatment of various diseases in Indian system of Traditional Medicine. The present study aims to investigate antioxidant and hepatoprotective activity of hexane (RPH), ethanol (RPE) and water extract (RPW) of the whole plant of *Rungia repens* by employing photochemiluminescence and spectrophotometric methods. The results showed that RPE and RPW significantly inhibited DPPH, NO and SOD radical in dose dependent manner. The trade of phenol content was as: RPH (44 mg PCE/g) < RPW (154 mg PCE/g) < RPE (209 mg PCE/g). Significant correlation was shown by total phenol content and free radical scavenging activity of all three extracts. The hepatoprotective effect was assayed in CCl₄-induced cytotoxicity in a liver slice culture model. The results revealed that significant depletion was observed in lactate dehydrogenase, lipid peroxidation, antioxidative enzymes SOD, CAT, and GR on administration of the RPE and RPW or ascorbic acid use as standard in the CCl₄- induced cytotoxicity in the liver. Ethanol and water extracts of *Rungia repens* have prevented significant oxidative liver damage.

Keywords:- Extracts of *Rungia repens*; Photochemiluminescence; CCl₄, Oxidative Stress; Liver Slice Culture Model.

Introduction

Reactive oxygen species (ROS) including oxygen-free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies. *In vitro* and *in vivo* studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, therefore preventing the occurrence of hepatic necrosis. Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils [1]. Consequently, the development of an alternative antioxidant from natural origin has

attracted considerable attention, and many researchers have focused on the discovery of new natural antioxidants for quenching biologically harmful radicals [2, 3].

In Indian traditional medicine system, the whole plant of *Rungia repens* (L.) Nees (Family-Acanthaceae) commonly known as 'Pittapapda' is used in jaundice, liver disorder, boils, inflammatory, swellings and ulcers [4-6]. A perusal of the literature showed no reports on *in vitro* antioxidant and hepatoprotective studies on the whole plant of *Rungia repens*. In the present study, work has been emphasized on evaluation of *in vitro*

antioxidant and hepatoprotective study of various extracts of whole plant *Rungia repens*.

Material and Methods

Plant material and Extraction

The herbs were collected from Junner (Maharashtra, India) during post monsoon season of 2008. The plant sample was identified, authenticated and deposited in Agharkar Herbarium of Maharashtra Association of Cultivation Science (AHMA) of Agharkar Research Institute, Pune 411 004 (Voucher Specimen Number: AHMA -24860).

The sample was shade dried, coarsely powdered and stored in an airtight container at $25^{\circ}\text{C} \pm 4^{\circ}\text{C}$. Powdered material (75 g) was extracted successively with hexane, ethanol and water using ASE 100 accelerated solvent extractor (Dionex, Vienna, Austria). Extraction was performed at 100 bar and temperature 60°C for 20 min in the five replicate cycles. The extracts were concentrated under vacuum using rotary evaporator and yields of hexane (RPH), ethanol (RPE) and water extracts (RPW) were 1.05, 5.96 and 9.23 g respectively. The extracts were stored in refrigerator until further use.

Antioxidant activity

Radical-scavenging effect of the extracts in DPPH radicals

DPPH radical-scavenging ability was assessed according to the method Jung et al. [7]. Briefly, to a methanolic solution of DPPH (60 mM, 2 mL), 50 μL of each test extract at different concentrations dissolved in methanol was added. Absorbance measurements commenced immediately at 515 nm. The decrease in absorbance was determined after 70 min when the absorbance stabilized. The absorbance of the DPPH radical without extracts and the control was measured. Ascorbic acid was used as reference antioxidant. The percent inhibition of the DPPH radical in the samples was calculated according to the formula given below:

$$\% \text{ Inhibition} = [(A_{C(0)} - A_{A(t)}) / A_{C(0)}] \times 100$$

Where $A_{C(0)}$ is the absorbance of the control at $t=0$ min and $A_{A(t)}$ is the absorbance in presence of antioxidant at $t=70$ min.

Nitric Oxide Scavenging

Nitric oxide scavenging activity was measured according to method Marocci et al. [8]. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with each test extract of different concentrations and mixture was incubated at 25°C for 30 min. Then 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess' reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine was measured at 546 nm along with control. Ascorbic acid was used as reference antioxidant. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples.

Superoxide Scavenging Activity

Superoxide scavenging activity was carried out by using alkaline DMSO method [9]. Solid potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 h and the solution was filtered immediately before use. Filtrate (200 mL) was added to 2.8 mL of an aqueous solution containing nitroblue tetrazolium (56 mM), EDTA (10 mM) and potassium phosphate buffer (10 mM, pH 7.4). Test extracts (1 mL) at different concentrations in water were added. The absorbance was recorded at 560 nm against a control in which pure DMSO had been added instead of alkaline DMSO.

Trolox equivalent antioxidant capacity (TEAC) assay

The total antioxidant activity was measured using TEAC assay [10] with minor modifications. TEAC value is based on ability of the antioxidant to scavenge blue-green 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS^+) radical cation relative to ABTS^+ scavenging ability of the water soluble vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). ABTS^+ radical cation can be generated by interaction of $\text{ABTS}^{\cdot+}$ (100 μM), H_2O_2 (50 μM) and peroxidase (4.4 unit/mL). To measure antioxidant capacity, 0.25 mL of each extract at different concentrations was mixed with an equal volume of ABTS^+ , H_2O_2 , peroxides and deionized water. Absorbance was monitored at 734 nm for 10 min. The

decrease in absorbance at 734 nm after the addition of the reactant was used to calculate the TEAC value. TEAC value is expressed as milimolar concentration of trolox solution having an antioxidant equivalent to a 1000 ppm solution of the sample under investigation. Higher TEAC value of sample indicates stronger antioxidant ability.

Photochemiluminescence Assay

The method of Photochemiluminescence (PCL) was used for the determination of the integral antioxidative capacity (AC) of lipid-soluble and water-soluble substances in extracts. Apparatus used was Photochem® with standard kit - Antioxidant capacity of water soluble (ACW) and Antioxidant capacity of lipid soluble (ACL) (Analytik jena AG), where luminol plays a double role of photosensitizer as well as the radical detecting agent [11]. Each extract was measured at 10 µg/mL concentrations. A standard curve was plotted and the results were calculated for lipid soluble substance in trolox equivalents (nmol/g) and water soluble substance in ascorbic acid equivalents (nmol/g).

Total phenolic content

Total phenolic content in each extract was determined with folin-ciocalteu reagent [12] using pyrocatechol as a reference standard. 2 mL of 2 % Na₂CO₃ was added to 0.1 mL extract and mixed thoroughly. After 5 min of incubation, 0.1 mL of 50 % folin-ciocalteu reagent was added and allowed to stand for 2 h with intermitted shaking. The absorbance was measured as micrograms of pyrocatechol equivalent (PCE) by using an equation that was obtained from the standard graph.

In vitro Hepatoprotective Activity

Animals

To assess the hepatoprotective activity, adult albino mice (6–8 weeks old) of either sex breed in the animal house of Agharkar Research Institute, Pune- 411 004, were used for the preparation of liver slices. The Institutional Animal Ethics Committee of Agharkar Research Institute 101/1999/CPCSEA, Pune, India approved the experimental protocols used in the present study.

Liver slice culture

Liver slice culture was maintained following the protocol developed by Wormser and Ben [13] and Invitox protocol No. 42 [14]. The mice were dissected open after cervical dislocation, and liver lobes were removed and transferred to pre-warmed Kred's Ringer Hepes (KRH) (2.5 mM Hepes, pH 7.4, 8mM NaCl, 2.85 mM KCl, 2.5 mM CaCl₂, 1.5 mM KH₂PO₄, 1.18 mM MgSO₄, 5 mM β-hydroxy butarate and 4.0 mM glucose). The liver was cut into thin slices using sharp blade. The slices were weighed and the slices weighing between 4 and 6 mg were used for the experiment. Each experimental system contained 20-22 slices weighing together 100-120 mg. These slices were washed with 10 mL KRH medium, every 10 min over a period of 1 h. These were then pre-incubated for 60 min in small plugged beakers containing 2 mL KRH on a shaker water bath at 37°C. At the end of pre-incubation, the medium was replaced by 2 mL of fresh KRH and incubated for 2 h at 37°C.

Experiment Design

The liver slices were further divided into individual cultures for the further respective treatments. Set 1, control, slices incubated in KRH medium; set 2, slices incubated in 15.5 mM CCl₄; set 3, slices incubated in 25 µg/mL RPH; set 4, slices incubated in 25 µg/mL RPE; set 5 slices incubated in 25 µg/mL RPW; set 6, slices incubated in 15.5 mM CCl₄ + different concentration (5, 10, 25, µg/mL) of RPH; set 7, slices incubated in 15.5 mM CCl₄ + different concentration (5, 10, 25, µg/mL) of RPE; set 8, slices incubated in 15.5 mM CCl₄ + different concentration (5, 10, 25, µg/mL) of RPW; set 9, slices incubated in 15.5 mM CCl₄ + 10 mM ascorbic acid; set 10, slices incubated in 10 mM ascorbic acid .

After the respective treatments, all the cultures were incubated in constant temperature water bath at 37°C for 2 h. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg/mL. The culture medium was collected and used for estimation of lactate dehydrogenase (LDH), which was employed as a cytotoxicity marker. The homogenates were

centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants assayed for LDH, catalase, peroxidase and superoxide dismutase. Ascorbic acid (AA) was used as standard.

Measurement of Lactate dehydrogenase activity, Lipid Peroxidation and Antioxidant Enzymes

Lactate dehydrogenase (LDH; EC 1.1.1.27) was estimated [15] and each unit of enzyme was calculated as 1 μ mol of NAD reduced per minute. Enzyme units in the medium and in tissue homogenate were estimated and percent release of enzyme from liver slices was calculated as the ratio of LDH activity found in the supernatant to the total LDH. Lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) [16].

Antioxidant enzymes namely superoxide dismutase, catalase and glutathione reductase were assayed according to standard methods [17 -20]. Protein in the tissue homogenate was estimated according to the method described by Bradford [21].

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation. The results of treatment effects were analyzed using one-way ANOVA test (Graphpad Prism 4) and p values < 0.001 were considered as very significant and p values < 0.05 were considered as significant.

Results And Discussion

Antioxidant Activity

Antioxidant activity was evaluated in terms of scavenging of DPPH, nitric oxide SOD radical and TEAC, photochemiluminescence in vitro systems. The DPPH radical scavenging assay is commonly employed to evaluate the ability of antioxidants to scavenge free radicals. In the present study, RPE and RPW showed higher antioxidant activity which significantly decreased the DPPH radical as compared to standard (Fig. 1). IC₅₀ values of RPH, RPE and RPW were 29.35 ± 2.45 , 21.67 ± 1.89 and 23.49 ± 2.34 μ g/ mL, respectively. IC₅₀ value of positive control ascorbic acid was 4.1 μ g/mL.

Sodium nitroprusside (SNP) is known to decompose in aqueous solution at physiological pH (7.2) producing NO. SNP spontaneously releases nitric oxide (NO) in solution and the amount of NO released can be inferred by using the Griess' reagent. This reagent reacts with nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO, and therefore allows an indirect estimation of the amount of NO released in the solution [8, 22]. RPE and RPW significantly inhibited nitric oxide in dose dependent manner (Fig. 1). IC₅₀ values of RPH, RPE and RPW were 37.53 ± 1.85 , 20.66 ± 1.73 and 24.34 ± 1.67 μ g/mL, respectively. IC₅₀ value of standard ascorbic acid was 4.9 μ g/mL.

Table 1: Free radical scavenging capacity of *R. repens* extracts

Samples	TEAC (mM)	Photochemiluminescence ACW in ascorbic acid equivalent (nmol g ⁻¹)	Photochemiluminescence ACL in trolox equivalent (nmol g ⁻¹)
RPH	1.87 $\pm$ 0.06	2.213	8.342
RPE	4.19 $\pm$ 0.19	4.641	15.686
RPW	3.58 $\pm$ 0.193	3.541	13.690
AA	4.59 $\pm$ 0.23	-	-

Values are mean $\pm$ SEM of three experiments.

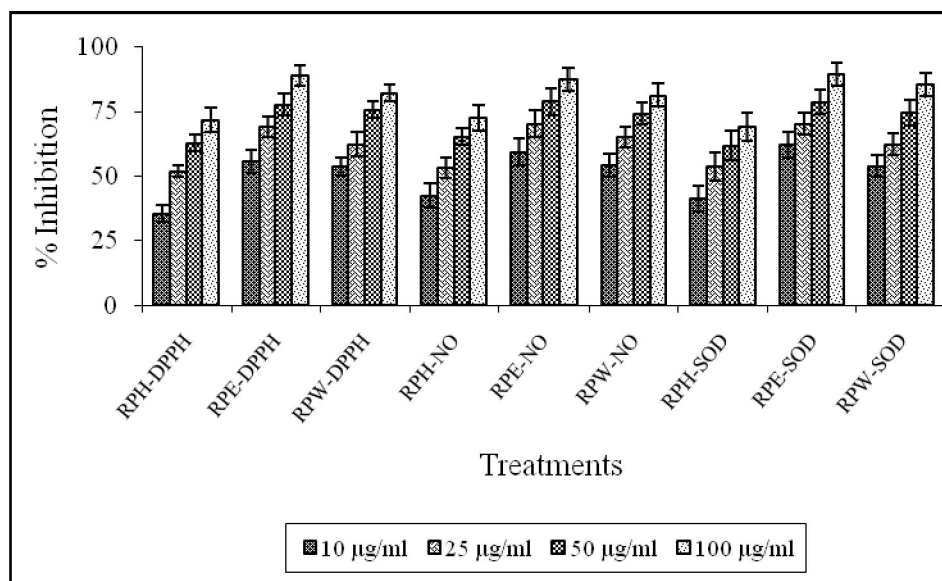


Fig.1: Free radical scavenging capacity of *R. repens* extracts.

Values are mean $\pm$ SEM of three experiments.

Superoxide radical $O_2^{\cdot-}$ is a highly toxic species, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyze the breakdown of $O_2^{\cdot-}$ radical [22]. The potassium superoxide assay was used to measure the superoxide dismutase activity of extracts. RPH showed low superoxide dismutase scavenging activity than RPE and RPW and the activity was dose dependent (Fig. 1). IC_{50} values of RPH, RPE and RPW were 29.74 ± 1.65 , 21.66 ± 2.33 and 24.34 ± 2.87 $\mu\text{g/mL}$, respectively. IC_{50} value of ascorbic acid was 6.3 $\mu\text{g/mL}$.

Peroxyl radicals or other oxidants like potassium persulphate oxidize ABTS to its radical cation, $ABTS^+$. The antioxidant capacities were determined by measuring decrease in the intensity of the blue colour as a result of reaction between the $ABTS^+$ radical [22] and the antioxidant compounds in the sample. The trend of TEAC values was as $RPE > RPW > RPH$ (Table 1).

Photochem® apparatus allowed precise method for the integral antioxidative capacity. Free radicals are

generated in the instrument by means of photosensitizer and detected by their reaction with a chemiluminogenic substance [11]. Calibration curves of standard ascorbic acid and trolox were determined for the calculation of ascorbic acid and trolox equivalents of ACW and ACL. All sample extract had distinctly varied ACW and ACL values. RPE and RPW had high antioxidant capacity than RPH (Table 1).

The key role of phenol compounds is the ability to scavenge free radicals and ROS such as singlet oxygen, superoxide free radical, and hydroxyl radicals. Phenol compounds in the medicinal plants extracts are frequently responsible for the antioxidant status, thus total phenol content has been determined [23]. The trade of phenol content was as: RPH (44 mg PCE/g) < RPW (154 mg PCE/g) < RPE (209 mg PCE/g). A significant correlation was shown by total phenol content and free radical scavenging activities of all extracts. The results exhibited that greater antioxidant activity of RPE and RPW may be due to their highest amount of phenol compounds.

In vitro Hepatoprotective Activity

The liver slice is a microcosm of the intact liver consisting of highly organized cellular community in which different cell types are subject to mutual contact. Such culture offers analysis of hepatotoxic events by measuring the release of LDH into the medium. Therefore, liver slice culture model is an in vitro

technique that offers the advantages of in vivo as it provides desirable complexity of structurally and functionally intact cells [24, 25]. It provides valuable approaches for screening of plant extracts/fractions for their hepatoprotective activity and elucidation of possible mechanism of actions.

Table 2: Effect of extracts in protecting liver cells from CCl₄ induced cytotoxicity by ameliorating oxidative stress

Treatments	LDH Units/100 mg tissue wet wt.	SOD Units/100 mg tissue wet wt.	CAT Units/100 mg tissue wet wt.	GR Units/100 mg tissue wet wt.
Control	7.56±1.16	17.34±1.47	14.00±1.41	0.141±0.012
15.5 mM CCl ₄	43.83±1.63	56.33±2.75	86.5±1.04	0.488±0.008
AA	7.53±1.47	17.5±2.34	14.33±1.75	0.140±0.007
RPH	7.00±2.09	17.43±1.82	14.65±1.37	0.146±0.003
RPE	7.33±1.16	17.23±1.49	14.83±1.22	0.149±0.011
RPW	7.17±1.47	17.45±1.41	14.67±1.67	0.148±0.006
15.5 mM CCl ₄ + RPH 5 µg/mL	37.33±0.81 ^a	46.67±0.81 ^a	43.83±0.75 ^a	0.346±0.055 ^a
15.5 mM CCl ₄ + RPH 10 µg/mL	30.0±0.89 ^a	43.0±0.89 ^a	38.5±0.54 ^a	0.326±0.005 ^a
15.5 mM CCl ₄ + RPH 25µg/mL	26.0±0.89 *	40.17±1.94 *	35.67±0.81 *	0.288±0.008 *
15.5 mM CCl ₄ + RPE 5 µg/mL	27.83±0.75 ^a	29.83±0.75 ^a	39.5±1.37 ^a	0.272±0.008 ^a
15.5 mM CCl ₄ +RPE 10 µg/mL	24.33±2.48 *	24.5±0.54 *	28.24±1.09 *	0.236±0.008 *
15.5 mM CCl ₄ +RPE 25 µg/mL	17.83±1.21 *	18.17±1.16 *	22.74±2.28 *	0.206±0.008 *
15.5 mM CCl ₄ + RPW 5 µg/mL	30.33±1.04 ^a	31.0±0.89 ^a	43.67±1.03 ^a	0.312±0.008 ^a
15.5 mM CCl ₄ +RPW 10 µg/mL	26.5±1.09 *	25.33±1.96*	33.5±1.04 *	0.284±0.005*
15.5 mM CCl ₄ +RPW 25 µg/mL	19.33±1.36 *	19.67±0.81 *	25.17±2.13 *	0.251±0.007 *
15.5 mM CCl ₄ + 50 mM AA	12.67±1.03 *	15.33±1.63 *	19.83±1.83 *	0.189±0.018 *

Cytotoxicity was assessed in terms of % lactate dehydrogenase (LDH) released, and the response to oxidative stress was measured in terms of antioxidant enzymes SOD, superoxide dismutase; CAT, Catalase; GR, Glutathione reductase activity. Ascorbic acid was used as a standard. Values represent means of at least three experiments and their standard deviation.

^a Significant differ compared with respective CCl₄ treated group, $p < 0.05$

*Significant differ compared with respective CCl₄ treated group, $p < 0.001$

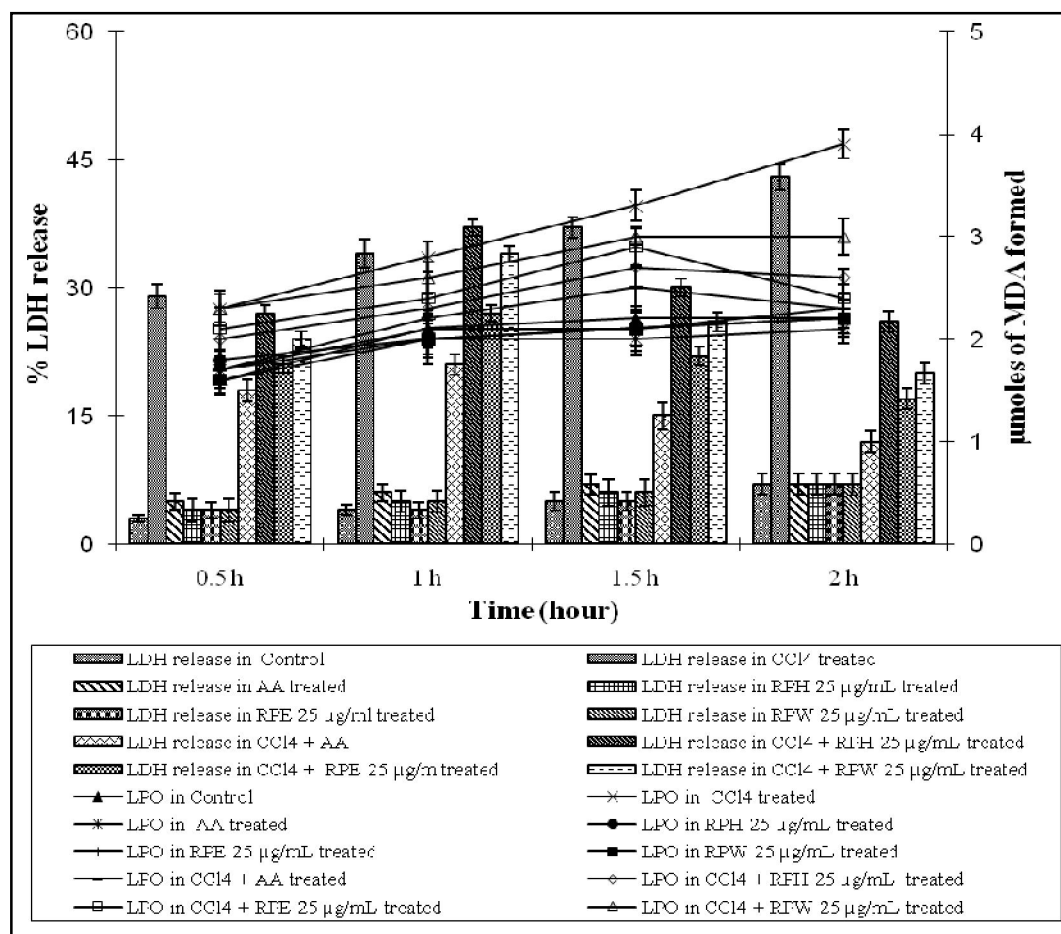


Fig. 2: Percentage release of LDH and extend of lipid peroxidation in liver slice culture in CCl_4 induced cytotoxicity. Values are mean of three experiments; CCl_4 – Carbon tetrachloride; AA, Standard ascorbic acid, at 50 mM concentration; LPO, Lipid peroxidation.

Oxidative stress was induced by adding cytotoxic CCl_4 to the liver slice culture. Release of LDH in the liver slice culture medium was used as cytotoxicity marker. CCl_4 was highly toxic to the treated cells which increased LDH concentration in the medium as compare to control. The RPH, RPE and RPW were found to be non-toxic at dose 25 $\mu\text{g/mL}$. RPH (7.00 ± 2.09 units/100 mg tissue wet wt.), RPE (7.33 ± 1.16 units/100 mg tissue wet wt.) and RPW (7.17 ± 1.47 units/100 mg tissue wet wt.) treated liver slices showed releases of LDH percentage was found to be similar to that of control untreated slices (7.56 ± 1.16 units/100 mg tissue wet wt.). All extracts at dose of 25 $\mu\text{g/mL}$ were used in all further experiments. The LDH release in the culture system treated with CCl_4 was 6 times more (43.56 ± 1.53 units/100 mg

tissue wet wt.) as compared to control. The amount of LDH release in medium reduced very significantly ($p < 0.001$) after addition of RPE and RPW than RPH ($p < 0.05$) along with CCl_4 cytotoxicant. The activity was comparable with ascorbic acid used as standard (Table 2.)

CCl_4 is known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation in liver tissue. The lipid peroxidation levels in the liver slice culture medium were assessed by TBARS assay. Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances and was expressed as μmol of malondialdehyde formed/100 mg tissue. The amount of lipid peroxidation increased folds in CCl_4 (Fig. 2) treated liver cells

compared to respective control. The extent of lipid peroxidation was reduced to near control levels very significantly ($p < 0.001$) when liver cells were treated either with RPE and RPW along with CCl_4 (Fig. 2).

Time course of lipid peroxidation was assessed in the presence of cytotoxic agent alone and together with different extracts. CCl_4 treated cells showed increase in lipid peroxidation paralleled with the increase in LDH release by the cells. However, in presence of RPE and RPW along with cytotoxic agent the lipid peroxidation, like the LDH release, returned to the control levels which was very significant ($p < 0.001$) (Fig. 2). Since lipid peroxidation is caused by free radicals, all extracts appears to reduce the amount of free radicals substantially.

CCl_4 induces oxidative stress in the cells by generation of ROS. Antioxidant enzymes (AOEs) SOD, CAT, and GR protect cells from oxidative stress of highly reactive free radicals. Oxidative SOD and CAT are known enzymes to prevent damage by directly scavenging the harmful active oxygen species. GR plays a role in recycling the oxidized glutathione to reduced glutathione, which acts as an antioxidant [25]. Activities of all three AOEs were checked in liver slice culture treated with CCl_4 alone or CCl_4 and extracts. The activities of SOD, CAT, and GR were increased in the liver tissue treated with CCl_4 . The liver tissue treated with RPE and RPW along with CCl_4 showed very significant ($p < 0.001$) reduced antioxidant enzymes activities when added along with the toxicants to the culture (Table 2).

The results in this study revealed that significant depletion was observed in the lipid peroxidation, antioxidative enzymes SOD, CAT, and GR on the administration of the RPE and RPW or ascorbic acid in the CCl_4 induced toxicity in the liver. Thus, this would indicate that the hexane and ethanol extract of galls on *Rungia repens* has antioxidant and hepatoprotective potentials.

In conclusion, the results of present study suggest that the RPE and RPW could prevent oxidative liver damage. Further comprehensive pharmacological

investigations will be needed to validate the ethnobotanical claims and elucidate the mechanism of hepatoprotective activity.

Acknowledgments

The authors are greatly thankful to the Director, Agharkar Research Institute, Pune, India for providing facilities and encouragement throughout the work.

References

1. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radical metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interaction*. 2006;160:1-40.
2. Ajila CM, Naidu KA, Bhat UJS, Rao P. Bioactive compounds and antioxidant potential of mango peel extract. *Food Chem*. 2007;105:982-988.
3. Mahdy KA, Abd-El-Shaheed A, Khadr ME, El-Shamy KAI. Antioxidant status and lipid peroxidation activity in evaluating hepatocellular damage in children. *East Medit Healt J*. 2009;15:842-852.
4. Desai VG. Aushadhi Sangraha. Bombay, India: Shri Gajanan Book Depo; 1975.
5. Kirtikar KP, Basu BD. Indian Medicinal plants, Vol II. Dehera Dun: Beshen Singh Mahendra Pal Singh; 1984.
6. Sivarajan VV, Balachandra I. Ayurvedic Plants and Their Plant Sources. New Delhi: Oxford and IBH publishing Co. Ltd.; 1996.
7. Jung, K-A, Song T-C, Han D, Kim I-H, Kim Y-E C-H. Lee, Cardiovascular Protective Properties of Kiwifruit Extracts in vitro. *Biol Pharm Bull*. 2005;28:782-1785.
8. Marcocci L, Maguire JJ, Droy-Lefaix MT, Parker L. The nitric oxide-scavenging properties of Ginkgo biloba extract EGB 761. *Biochem Biophys Res Comm*. 1994;201:748-755.
9. Henry LEA, Halliwell B, Hall DO.. The Superoxide Dismutase activity of various photosynthetic organisms measured by a new and rapid assay technique. *FEBS Lett*. 1976;66:303-306.

10. Miller NJ, Diplock AT, Rice-Evans CA. Evaluation of the total antioxidant as a marker of the deterioration of apple juice on storage. *J Agric Food Chem.*1995;43:794-1801.
11. Govindrajan R, Vijayakumar M, Rao CV, Shirwaikar A, Rawat AKS, Mehrotra S, Pushpangadan P. Antioxidant Potential of *Anogeissus latifolia*. *Biol Pharm Bull.* 2004;27:1266-1269.
12. Slinkard K, Singleton VL. Total phenol analysis: Automation and comparison with manual methods. *Am J Enol Vitic.* 1977;28:49-55.
13. Wormser U, Ben ZS. The liver slice system-an in vitro acute toxicity test for assessment of hepatotoxins and their antidotes. *Toxicol In Vitro.* 1990;4:449-451.
14. Invitox Protocol No. 42. Liver slice hepatotoxicity screening system. The ERGATT/FRAME Data Bank of in vitro techniques in toxicology. England: INVITTOX; 1992.
15. Renner K, Amberger A, Konwalinka G, Kofler R, Gnaiger E. Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. *Biochem Biophys Acta* 2003;1642:115-123.
16. McMillan DC, Charles BJ, Jollow DJ. Role of lipid peroxidation in dapsone-induced hemolytic anemia. *J Pharmacol Exp Ther.* 1998;287:868-876.
17. Marklund S, Marklund G. Involvement of superoxide anion radical in auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem.* 1974;47:469-464.
18. Aebi, H.E. Catalase. In: Bergmeyer HU. *Methods of Enzymatic Analysis*, Vol. 3, ed. GmbH, Weinheim: Verlagchemie; 1983;p. 277-282.
19. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959;82:70-77.
20. Bulaj GT, Kortemme T, Goldenberg DP. Ionization-reactivity relationships for cysteine thiols in polypeptides. *Biochem.* 1998;37:8965-8972.
21. Bradford MM. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal Biochem.* 1976;72:248-254.
22. Magalhaes LM, Segundo MA, Reis S, Lima LMC. Methodological aspects about in vitro evaluation of antioxidant properties. *Anal Chim Acta.* 2008;61:1-19.
23. Hall CA, Cuppett SL. Structure-activities of natural antioxidants. In.; Aruoma OI, Cuppett SL. *Antioxidant Methodology: In vivo and in vitro Concepts.* eds. IL: AOCS Press Champaign; 1997;p. 141-172.
24. Frazier JM. In vitro toxicity testing: Application to Safety evaluation. ed. New York: Marcel Dekker, Inc; 1992;p. 45.
25. Naik RS, Mujumdar AM, Ghaskadbi SS. Protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture in vitro. *J Ethnopharmacol.* 2004;95:31-37.