Synergistic Effects of Geraniin and Rutin in the Antioxidant Properties of Major Lignans in *Phyllanthus amarus*.

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Summary

Although the role of *Phyllanthus amarus* – lignans (PAL) phyllanthin and hypophyllathin, as anti-heapatotoxic substance is well documented, the contribution if any of the gallotanoids, e.g. geraniin, and flavonoids, rutin, co-occurring in that plant has been evaluated before. Hence this study was undertaken. Because, in the milieu of the extract of *P.amarus* the gallotanoids and flavonoids occupy a significant position and, therefore, can modulate its bioactives.

Introduction

Liver is the main organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation[1]. During the course of aerobic metabolic reactions, considerable amounts of Reactive Oxygen Species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) regenerated[2], which undergo a variety of chain reactions and produce free radicals such as OH*. These hydrogen species attack polyunsaturated fatty acids and hereby initiate the process of lipid peroxidation resulting in degradation and inactivation of various important biomolecules[3].

Herbs have recently attracted attention as health beneficial foods and as source materials for drug development. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases including liver disease[4], ischemia, perfusion injury, atherosclerosis, acute hypertension, hemorrhagic shock, diabetes mellitus and cancer with relatively little knowledge regarding their modes of action[5].

Materials and Methods

Animals:

Wistar albino rats of either sex about 20 weeks of age with an average body weight of 200 ±30 g were used for the study. The animals were obtained from Indian Institute of Chemical Biology (IICB), Kolkata, India. They were housed in standard environmental conditions of temperature, humidity and under clear and dark cycles of 12-h.The mice were fed standard laboratory diet (Hindusthan Liver Limited, India) and were given water *ad libitum.*

Chemicals and Drugs used:

Silymarin was purchased from Microlabs (Hosur, Tamilnadu, India), 1-Chloro-2, 4-dinitrobenzene CDNB, bovine serum albumin (Sigma chemical St. Louis, MO, USA), thiobarbituric acid, nitrobluetetrazolium chloride (NBT) (Loba Chemie, Mumbai, India), 5, 5'-dithio bis-2-nitrobenzoic acid (DTNB), carbon tetrachloride, (SICCO research laboratory, Mumbai). All other chemicals and solvent were of analytical grade and commercially available.

Experimental protocol:

Fifty four (54) Wister albino rats of either sex weighing between 200 to 230 gm were used for the present study. The animals were divided into nine groups each consists of six animals having 3 male and 3 female. The animals were housed in plypropylene cages and maintained 24± 2 ^{o} C under 12 hour light dark cycle were fed water *ad-libitum* with standard pellet diet and free access to water. They were initially acclimatized for seven days in the experimental environment prior to study.

Here I have selected four bioactive principles of PA namely Phyllanthin, Hypophyllantin, Rutin and Geraniin. Primarily the individual potentiality of these molecules was observed in different doses for the screening of best combinations and proportion. It is established that when Phyllanthin and Hypophyllanthin is combined with Geraniin and Rutin in proportion of 1:1:0.5:0.5 showed the best result. These initial findings helped for selecting the dose of this present experiment.

Group I was kept as control received distill water (DW) (1ml/kg p.o.), while Group II was received DW consecutively for 9 days and on 9th day received CCl_4 (1ml/kg p.o.). Phyllanthin, Hypophyllanthin and Geranin (1:1:0.5) combination was received by Group III at dose rate of 50 mg/kg/day orally consecutively for 9 days and on 9th day received CCl_4(1ml/kg p.o.). Group IV which received CCl_4 (1ml/kg p.o.) on 9th day was challenged daily orally Phyllanthin, Hypophyllantin and Rutin combination orally (1:1:0.5) at 50 mg/kg for 9 days. Group V and Group VI received combinations (1:1) of Geranin,Rutin and Phyllanthin ,Hypophyllanthin daily for 9 days at 50 mg/kg were treated with CCl_4(1ml/kg p.o.) on 9th day of experiment. Whilst Group VII, VIII and IX received Geranin, Rutin and Siyamarin daily for 9 days at 50 mg/kg orally respectively administered CCl_4 (1ml/kg p.o.) on 9th day . Food was withdrawn on 9th day before administration of CCl_4 to enhance the acute liver damage in all the treated groups.

Table.1. Groups of Experimental animals:

Groups	Treatment	Doses
Group I	Normal animals	Normal saline (0.9 %)
Group II	CCl_4 Treated	(1ml/kg)
Group III	CCl_4 +P+HP+G (1:1:0.5)	(50 mg/kg)
Group IV	CCl_4+P+HP+R (1:1)	(50 mg/kg)
Group V	CCl_4+G+R (1:1)	(50 mg/kg)
Group VI	CCl_4 +P+HP (1:1)	(50 mg/kg)
Group VII	CCl_4+G	(50 mg/kg)
Group VIII	CCl_4+R	(50 mg/kg)
Group IX	CCl_4+Silymarin	(25mg/kg)

Phyllanthin-P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

Biochemical estimations:

Serum glutamic oxaloacetic and glutamic pyruvic transaminase activities[6] and alkaline phosphatase[7] were determined. The total protein concentration and bilirubin were measured by the method of Lowry *et al* [8] and Oser [9] respectively.

Collection of samples:

Blood: The animals were anaesthetized by light ether anesthesia and the blood was withdrawn by making intracardic puncture to the rats at 12th day of experiment. Blood was allowed to coagulate for 30 minutes and serum was harvested by centrifugation at 3000 rpm. The serum was used to estimate SGPT, SGOT, ALP and total bilirubin.

Liver: All the groups of animals were sacrificed after collection of blood and liver was separated, washed in normal saline and soaked in filter paper. Then homogenization of liver tissues were performed on 10% 0.15 M tris-HCl buffer (pH 7.4) and finally centrifuge at 3000 rpm at 4^{o} C for 1h.The supernatant was collected and used for estimation of lipid peroxidation, glutathione and Catalase.

In vivo antioxidant status:

After sacrificed the experimental animals, the liver was removed and washed in ice cold phosphate buffered saline, blotted dry and weighed. The 25 % w/v of liver homogenate was prepared by standard protocol. The supernatant obtained was used for the determination of lipid peroxidation[10] (LPO) and endogenous antioxidant such as reduced glutathione[11] (GSH), and catalase (CAT) [12].

Statistical analysis:

Experimental results were expressed as mean ± SEM analysis of variance was performed by one way ANOVA procedures (SPSS 10.0 for Windows). Significant differences between means were determined by Dunnett's post hoc test. $p<0.05$ implies statistically significance.

Results

In the experiment I, no effect of the drug was observed upto 800 mg.kg body weight. At 1600 mg, there were some behavioral changes like dizziness, rubbing on the wall of cages, drowsiness were noticed which were more prominent at 3200 mg/kg body wt.

In experiment II, the evaluation of enzymes level in CCl_4 treated group were measured (Table.2, Fig.1).The combination of (Phyllanthin - Hypophyllanthin - Geraniin) at the dose of 50mg/kg (p.o) and silymarin (100mg/kg, p.o) produced a significant reduction in serum marker enzymes (P<0.05).Combination of (Phyllanthin - Hypophyllanthin - Rutin; 50mg/kg) also produced a significant reduction in ALT, AST, ALP and serum billirubin (Fig.2.) when compared to CCl_4 treated group. Other groups i.e. (Geraniin - Rutin; 50mg/kg) and (Phyllanthin -Hypophyllanthin; (Phyllanthin - Hypophyllanthin - Geraniin)) also controlled the serum enzymes marker but Rutin (50mg/kg) and Geraniin (50mg/kg) separately did not response in that extant when compared to other treated groups.

The effect of active principle of PA on rat liver lipid peroxidation, glutathione and antioxidant enzyme (catalase) levels (Table-3, Fig.3.) were significantly (P<0.05) changed. The LPO levels were increased and the glutathione level as well as catalase activity were decreased markedly in CCl_4 intoxicated rats when compared with those of the animals in normal control group. Rats treated with the combination of (Phyllanthin - Hypophyllanthin – Geraniin; 50mg/kg) decreased the elevated levels and restricted the alerted glutathione levels and catalase activity towards the normal levels. The results are well comparable with standard drug (silymarin) treated group. The combination of (Phyllanthin –Hypophyllanthin-Rutin; 50mg/kg) also restored partially the normal level of LPO and glutathione but the other groups did not bring back.

Table.2. Effect of *Phyllanthus amarus* bioactives and Silymarin on serum Biochemical parameters in CCl_4 intoxicated rats

(Each value represents the mean ±SEM, six rats in each group)

All values represent the mean ±SEM. P values calculated by ANOVA followed by

Biochemical parameters	**SGOT (IU/L)**	**SGPT (IU/L)**	**ALP (IU/L)**	**Total bilirubin (mg/dl)**	**Total protein (mg/dl)**
Normal animals	70.08 ± 1.26	62.27 ± 0.52	20.7 ± 0.21	0.8 ± 0.001	7.06 ± 0.07
CCl_4 Treated	158.25 ± 0.52	145.6 ± 0.89	70.4 ± 0.97	4.3 ± 0.08	3.60 ± 0.11
CCl_4 +P+HP+G (1:1:0.5) (50 mg/kg)	82.18 ± 0.85	67.13 ± 0.46	25.14± 0.82	1.2 ± 0.08	6.23 ± 0.08
CCl_4+P+HP+R (1:1)(50 mg/kg)	86.76 ± 1.50	73.56 ± 1.60	30.17± 1.51	1.60 ± 0.08	5.53 ± 0.07
CCl_4+G+R (1:1) (50 mg/kg)	89.61 ± 1.82	80.26 ± 1.59	36.57 ± 1.38	1.8 ± 0.03	5.36 ± 0.07
CCl_4 +P+HP (1:1) (50 mg/kg)	95.78 ± 2.35	86.92 ± 2.04	41.29 ± 1.92	2.3 ± 0.07	5.12 ± 0.06
CCl_4+G (50 mg/kg	101.64 ± 3.02	91.18 ± 2.27	42.73 ± 1.75	2.6 ± 0.05	4.76 ± 0.06
CCl_4+R (50 mg/kg)	128.03 ± 0.27	110.96 ± 0.73	45.7 ± 0.18	2.8 ± 0.03	4.38 ± 0.07
CCl_4+Silymarin (25 mg/kg	80.32 ± 0.74	64.56 ± 0.22	28.4 ± 0.62	1.3 ± 0.07	6.03 ± 0.07

Dunnett's post hoc test of significance.p< 0.05implies significance.
Phyllanthin-P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

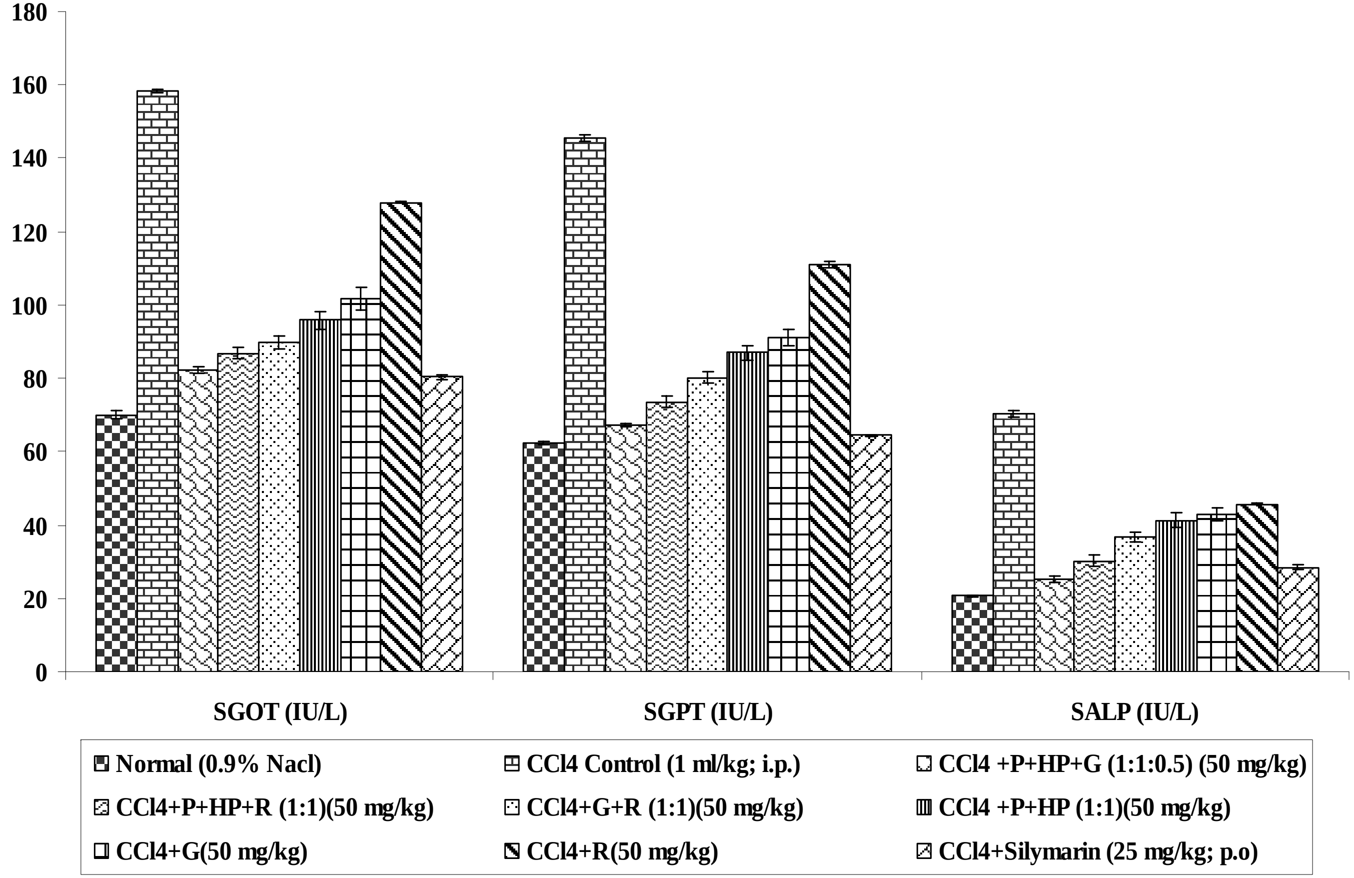
Table.3. Effect of *Phyllanthus amarus* and Silymarin on Biomarker Enzymes and Lipid Peroxidation in carbontetrachloride intoxicated Rats (Values are mean± SEM, 6 rats in each group)

All values represent the mean ±SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance $p < 0.05$ implies significance

Treatment	**Lipid peroxides (µM of MDA/mg of protein)**	**GSH (µg /mg of protein)**	**Catalase (µM of H_2O_2 consumed /min/mg protein)**
Normal animals	114.12± 4.27	305.78 ± 15.64	30.15 ± 0.75
CCl_4 Treated	465.95 ± 10.9	229.25 ± 4.9	14.32 ± 0.59
CCl_4 +P+HP+G (1:1:0.5) (50 mg/kg)	136.29 ± 7.8	325.58 ± 18.2	26.12 ± 0.9
CCl_4+P+HP+R (1:1)(50 mg/kg)	142.75 ± 10.5	302.61 ± 12.7	25.27 ± 1.3
CCl_4+G+R (1:1) (50 mg/kg)	175.26 ± 24.2	294.66 ± 14.1	22.83 ± 2.2
CCl_4 +P+HP (1:1) (50 mg/kg)	237.28 ± 19.4	284.92 ± 26.31	20.05 ±3.4
CCl_4+G (50 mg/kg	256.38 ± 20.14	275.28 ± 13.5	20.2 ± 2.1
CCl_4+R (50 mg/kg)	289.40 ± 27.3	268.63 ± 10.02	19.5 ± 1.7
CCl_4+Silymarin (25 mg/kg	128.40 ± 6.3	333.09 ± 35.5	28.04 ± 1.3

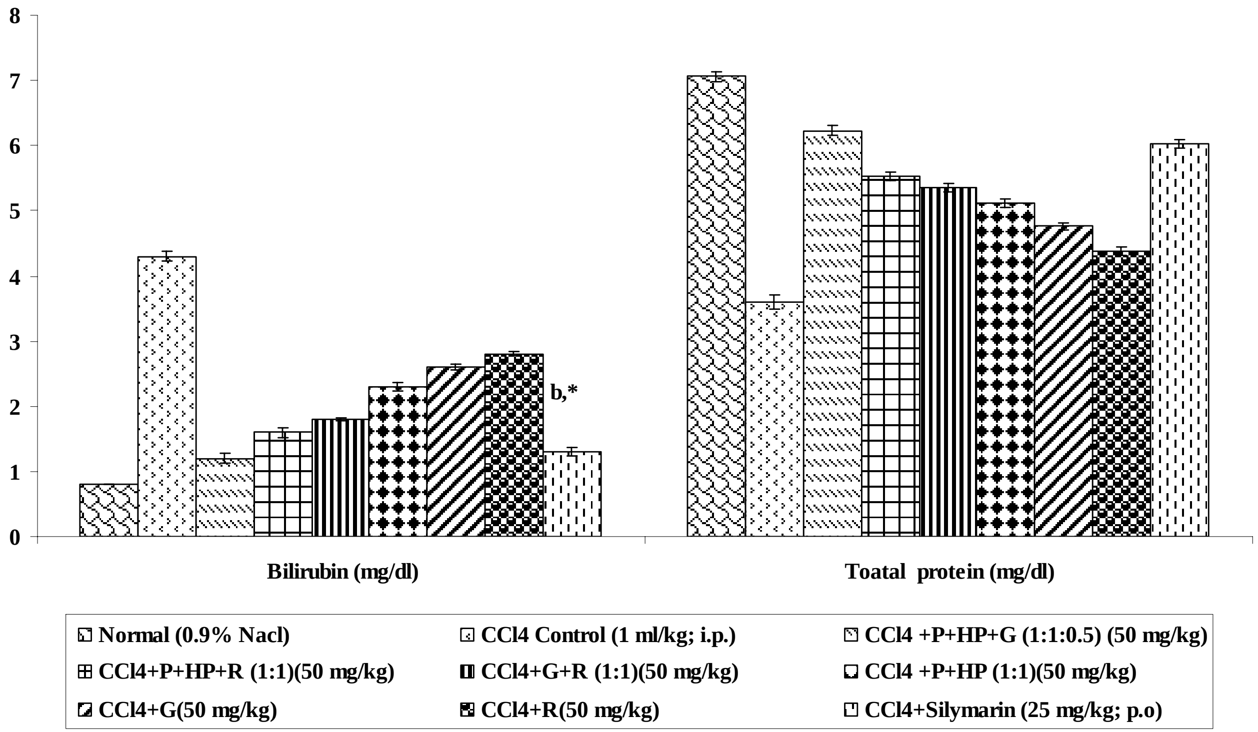
Phyllanthin-P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

Fig.1. Effect of *Phyllanthus amarus* and Silymarin on SGOPT, SGPT and SALP in CCl_4 intoxicated rats



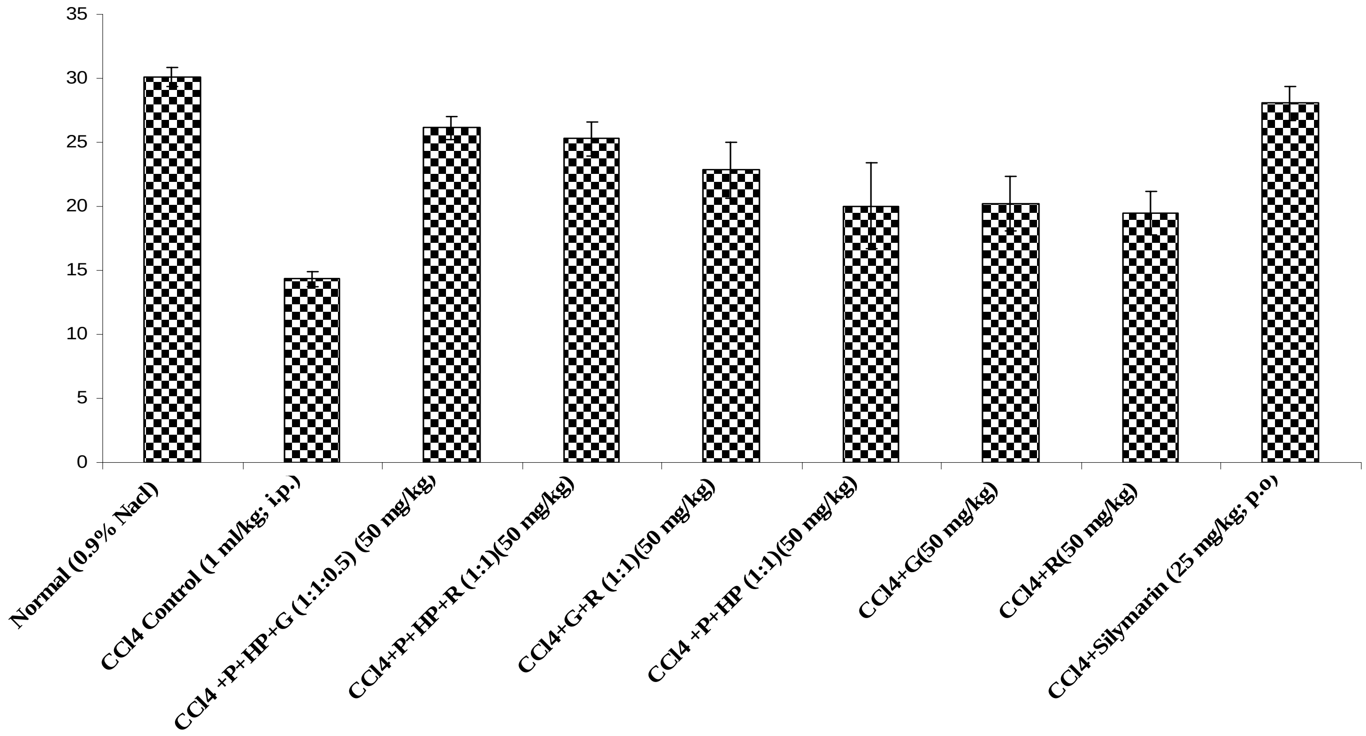
Phyllanthin-P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

Fig.2. Effect of *Phyllanthus amarus* and Silymarin on total bilirubin and total protein in CCl_4 intoxicated rats



Phyllanthin-P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

Fig.3. Effect of *Phyllanthus amarus* and Silymarin on catalase level in CCl4 intoxicated rats



Phyllanthin- P; Hypophyllanthin-HP; Geraniin-G; Rutin-R

Discussion

CCl_4 induced hepatic injuries are commonly used models for the screening of hepatoprotective damages[13-14] and the extent of hepatic damage is assessed by the levels of released cytoplasmic alkaline phosphatase and tranaminase (GOT, GPT) in circulation. The present investigation also revealed that the given dose of CCl_4 (1 ml /kg po) produced significant elevation in SGPT, SGOT and ALP levels indicating an impaired liver functions. The massive production of reactive species may lead to depletion of protective physiological moieties (glutathione and tocopherols etc) and ensuing widespread propagation of the alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes. The investigation further reveals that the all test molecules are effective against liver disease but lignan, gallotanoid and flavonoid (Phyllanthin; Hypophyllanthin; Geraniin) mixture offer maximum protection against impaired antioxidant status induced by CCl_4.

Liver injuries produced by a various hepatic toxic substances have been recognized as a major toxicological problem for years[15]. In the experimental study for the production of hepatotoxicity for both animals and humans CCl_4 an established hepatotoxicant.In liver cytochrome P-450 metabolized CCl_4 to trichloromethyl radical that reacts with GSH to form a GSH containg radical and causes various pathological and toxicological manifestations[16].

The primary stage of hepatic injury is concerned with the cytotoxicity of CCl_4 results activation of lipid peroxidation and covalently binding with lipid and proteins[17].The kupffer cells are activated in presence of CCl_4 formation of harmful cytokines that cause of the death of the hepatocytes and oxidative damage[18].

The activation of neutrophillic leukocytes occurred due to the cellular infiltration and it magnifies inflammatory response and cellular injury/death because of release of superoxide anions and other harmful mediators.

Conclusion

In conclusion, the present study demonstrated that *P.amarus has* hepatoprotective effect in CCl_4 induced liver damage. So the isolated and purified active principle or enriched fractions such as Phyllanthin, Hypophyllanthin, Geraniin and Rutin are responsible for the hepatoprotectvie activity of *P.amarus*. However, when the single molecules are subjected to animal experiment they were not able to show the action in a grater extant but when they were combined to each other they showed the perfect synergistic effects.

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