HEPATOPROTECTIVE ACTIVITY OF THE ISOLATES FROM THE LEAVES OF *PSIDIUM GUAJAVA* LINN.

Chanchal K Roy*, Amit Kumar Das

Krupanidhi College of Pharmacy,
Chikka Bellundur, Vartur Hobli Post, Bangalore-560 035, India.

Summary

The study was designed to evaluate the hepatoprotective activity of different isolates obtained from the methanolic extract of leaves of *P. guajava* Linn. in acute experimental liver injury induced by carbon tetrachloride and paracetamol. The isolates (I1, I2, I3, I5, and I6) were obtained by carrying out column chromatography using methanol:water:formic acid (7.5:2.0:0.5) as mobile phase and silica gel (SG 60 F_{254}) as stationary phase. The hepatoprotective effects observed by the isolates (I1, I2, I3, I5, and I6) were compared with a known hepatoprotective agent, silymarin (100 mg/kg p.o.). In the acute liver damage induced by different hepatotoxins, Isolate 1 (I1) (200mg/kg p.o.) significantly reduced the elevated serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin in carbon tetrachloride and paracetamol induced hepatotoxicity. The other isolates obtained isolate 2 (I2), isolate 3 (I3), isolate 5 (I5) and isolate 6 (I6) did not show significant hepatoprotective activity when compared to standard. Characterization of the active isolate (I1) was done by spectroscopic analysis using the IR, Mass, Proton NMR and 13 C NMR and compared. Structural analysis suggested that the isolated compound (I1) confirmed with Quercetin structure.

Keywords: Hepatoprotection, *Psidium guajava*, Quercetin, Carbontetrachloride, Paracetamol.

* ***Correspondent Author***
Phone: +91-80-65973260
Fax: +91-80-25526580
E-mail: chanchalr@gmail.com.

Introduction

Liver diseases, such as jaundice, cirrhosis and fatty liver are very common worldwide. In India, numerous medicinal plants are used for the treatment of liver disorders. One of the plants used traditionally is guava plant, *Psidium guajava* Linn (Myrtaceae). It is believed in folklore that the water decoction of the leaves of this plant can cure jaundice within three days. It is used widely in Mangalore district of Karnataka. *Psidium guajava* contains a number of chemical constituents, which are reported to posses antibacterial[1], antidiarrheoal[2], antimycobacterial[3], antihyperglycemic[4], antimalarial[5], cytotoxic[6] and antioxidant activities[7]. The anti-oxidant activity is due to presence of a number of constituents; the major ones are caryophyllene oxide, caryophyllene and a number of tannins[8]. Since, anti-oxidants are known to reduce the development of chemically induced liver damage; the effect of different extracts (petroleum ether, chloroform, ethyl acetate, methanol and water) of the leaves of the plant *P. guajava* were evaluated for hepatoprotective activity. Results of the above analysis showed that methanolic extract of the plant showed maximum hepatoprotection[9].So, the present work was designed to evaluate the constituent(s) responsible for hepatoprotection.

Materials and Methods

Plant materials: The fresh leaves of *P. guajava* were collected during November from Koramangala area in Bangalore. The Regional Research Institute, Bangalore identified and authenticated the plant. A specimen (RRCBI, Acc. No. 12473) has been preserved for future reference. The fresh leaves were collected and dried under shade. The leaves were powdered (coarse) and subjected to soxhlet using petroleum ether, chloroform, ethyl acetate, methanol and water respectively. The extracts obtained were evaporated in rotary evaporator to get a powdery mass. The yield was found to be 1.93%, 5.03%, 4.00%, 8.33% and 7.29% (w/w) of petroleum ether, chloroform, ethyl acetate, methanol and water extracts respectively.

Apparatus: Analytical balance: AB204-S, FT-IR- Perkin Elmer, Mass Spectroscopy-MICROMASS, H NMR- , 13C NMR- .

Chemicals: Petroleum ether, Chloroform, Ethyl acetate and Methanol were analytical grade purchased from E. Merck Chemicals. Powdered silica gel G and TLC Aluminium sheets pre-coated with silica gel 60 F_{254} were also purchased from E. Merck. Carbon tetrachloride was purchased from S D Fine Chemicals Ltd., Mumbai, India and paracetamol was obtained from Lupin Ltd. Silymarin was obatained as a gift sample from Micro Labs, Hosur, India.

Isolation and Characterization: Methanolic extracts was concentrated till it retained a clear consistency and was kept in refrigerator (4-8^0 C) for a week. The mobile phase was prepared by using methanol: water: formic acid in ratio of 7.5:2.5:0.5. Silica gel G was dissolved in the mobile phase. The slurry was poured into the column and allowed to settle down overnight. The methanolic extract was mixed with the mobile phase and poured in the column.

The fractions, 10ml elutes were collected and monitered simultaneously on TLC plates using methanol: water: formic acid (7.5:2.5:0.5) as mobile phase. The fractions eluted showing only one spot on TLC [R_f: 6.2] were pooled and evaporated to dryness at 25 ± 2^0C, yielded pale yellow coloured fine powers. The compound was re-crystallized in aqueous ethanol to produce 1 gm of pure quercetin. The purity was confirmed by TLC and identity was confirmed by comparing data of melting point, IR, Mass spectra, H NMR and 13C NMR with the procured pure sample.

Experimental animals: Wistar albino rats weighing 200-250 g of either sex were used. The experimental protocol was approved by the Institutional Animal Ethics Committee and animals were maintained under standard conditions in the animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Evaluation of hepatoprotective activity - Hepatoprotective activity was evaluated using acute hepatic injury models induced by carbon tetrachloride and paracetamol.

a. Acute hepatitis models

(i) Carbon tetrachloride (CCl_4) induced acute toxicity: The CCl_4 was diluted with liquid parafiin (1:1) before administration. The animals were divided into 8 groups consisting of 6 animals for each. The animals were then subjected to either one of the following treatments for 9 days.

Group 1: Control group: Animal of this group received distilled water, *p.o.* for nine days.
Group 2: CCl_4 group: Animals of this group received CCl_4 0.2 mg/Kg/day on ninth day.
Group 3: Standard group: Animal of this group received silymarin 100mg/Kg/day, *p.o* for nine days.
Group 4: Test group: Animal of this group received Isolate 1(I1), 200mg/Kg/day, *p.o*. for nine days.
Group 5: Test group: Animal of this group received Isolate 2(I2), 200mg/Kg/day, *p.o.* for nine days.
Group 6: Test group: Animals of this group received Isolate 3(I3), 200mg/Kg/day, *p.o.* for nine days.
Group 7: Test group: Animals of this group received Standard Quercetin (Q), 200mg/Kg/day, *p.o.* for nine days.
Group 8: Test group: Animal of this group received Isolate 5 (I5), 200mg/Kg/day, *p.o.* for nine days.
Group 9: Test group: Animals of this group received Isolate 6(I6), 200mg/Kg/day, *p.o.* for nine days.

Food was withdrawn 12 hr before carbon tetrachloride administration to enhance the acute liver damage in animals of groups 2, 3, 4, 5, 6, 7, 8 and 9. The animals were sacrificed 24 hr after the administration of CCl_4. Blood samples were collected and the serum was used for assay of marker enzymes such as aspartate aminotransferase (AST)[10], alanine aminotransferase (ALT)[10], alkaline phosphatase (ALP)[11] and serum bilirubin[12].

The liver was immediately isolated and washed with normal saline, blotted with filter paper and weighed. The liver was then subjected to histopathological examination[13].
(*ii) Paracetamol (PCM) induced liver toxicity:* The same procedure as mentioned above was followed except that the liver was damaged using PCM (1g/kg, p.o.) diluted with sucrose solution (40% *w/v*). PCM was administered in 3 divided doses on day 9 and animals were sacrificed 48 hr after administration of PCM[14].

Statistical analysis:
The statistical significance was assessed using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. The values are expressed as mean $\pm$ SEM and $P \leq 0.05$ were considered significant.

Results

TLC and R_F values of isolates:
Different solvent systems were used to isolate the active constituent responsible for hepatoprotectve activity. After various trial and error methods, the most suitable solvent system was found to be methanol, water and formic acid in ratio of 7.5:2.5:0.5 respectively. After finding the exact solvent system, it was run through a column packed with silica gel with the extract for isolation of the constituents. Five different isolates (I1, I2, I3, I5 and I6) were collected having different R_f values and it was compared with the standard quercetin (Q) procured commercially (Table 1).

Table 1: The R_f values obtained from the different isolates using the different solvent system are as follows:

SNo	Isolates	Solvents	Rf
1	I 1	Methanol:Water:Formic Acid.	6.2
2	I 2	Methanol:Water:Formic Acid.	7.5
3	I 3	Methanol:Water:Formic Acid.	8.6
4	Q	Methanol:Water:Formic Acid.	6.3
5	I 5	Methanol:Water:Formic Acid.	8.0
6	I 6	Methanol:Water:Formic Acid.	8.3

***Qualitative Chemical Analysis*:** The primary chemical analysis was performed and the investigated results are reported. (Table 2)

Table 2:- The qualitative chemical analysis of different isolates.

EXTRACTS/TEST	**I 1**	**I 2**	**I 3**	**Q**	**I 5**	**I 6**
Test for Flavanoids	+	+	+	+	+	+
Test for Saponins	+	+	+	+	+	+
Test for Cardiac glycosides	-	-	-	-	-	-
Test for Tannins	-	-	-	-	-	-
Test for Carbohydrates	-	+	+	+	-	+
Test for Reducing Sugars	-	-	-	-	-	-
Test for Steroids	-	-	-	-	-	-
Test for proteins and Amino Acids	-	-	-	-	-	-
Test for Anthraquinone glycosides	-	-	-	-	-	-

Analysis of Isolates

Isolate 1(I 1): (Figure 1)
Physical State:
Colour: Pale yellow.
Odour: Odourless.
State: Solid (Crystalline)
Melting Point: 316^{o}C.
Molecular weight: 302.
Rf: 6.2.
IR data (KBr cm^{-1}): 3406 (OH stretching), 3100 (C-H aromatic), 1665 (C=O), 1610 (OH bond), 1561 (C=C stretching), 1241 (C-O stretching), 1199 (C-C stretching).
NMR data (δ ppm; DMSO-d_6 solvent): 6.21 (s, 1H, Ar-H), 6.38 (s, 1H, Ar-H), 7.76 (s, 1H Ar-H), 7.60 (d, 1H, Ar-H), 7.6 (d, 1H, Ar-H), 7.71 (broad, 1H, OH), 8.48 (broad, 1H, OH), 8.78 (broad, 1H, OH).
Mass Spectroscopy: 302 (M^+, 100%), 285 (4%), 257 (6%), 245 (8%). (Figure 2)

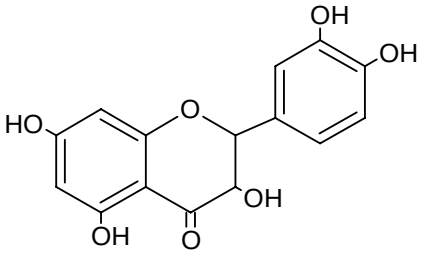
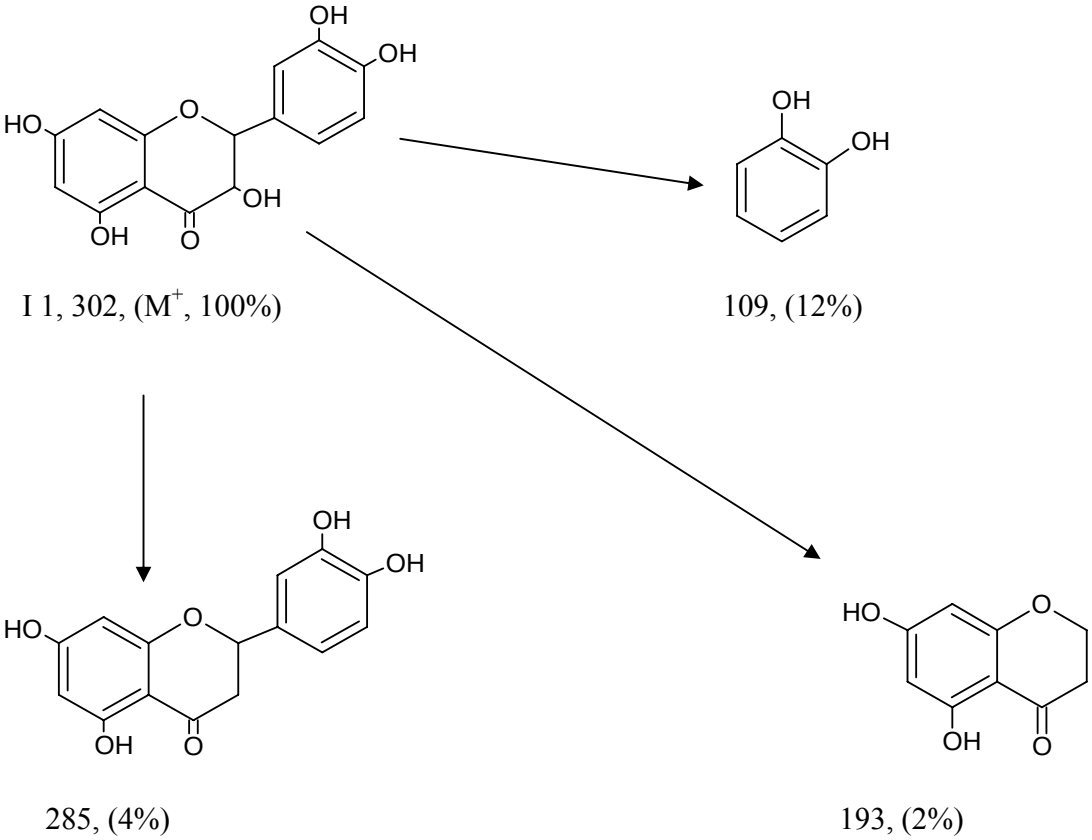


Figure 2: Probable fragmentation pattern of isolate I 1 (I 1):



Isolate 2 (I 2):

Physical State:
Colour: Dark brown.
Odour: Odourless.
State: Solid (Crystalline)
Melting Point: 195^{o}C.

Molecular weight: NA.
Rf: 7.5.
IR data (KBr cm^{-1}): 3402 (OH stretching), 2935 (C-H aromatic), 2077 (), 1629 (C=O), 1516 (), 1324 (C-O stretching), 1204 (C-C).
NMR data: NA

Isolate 3 (I 3):

Physical State:
Colour: yellow.
Odour: Odourless.
State: Solid (Crystalline)
Melting Point: 145^{o}C.
Molecular weight: NA.
Rf: 8.6.
IR data (KBr cm^{-1}): 3410 (OH stretching), 2929 (C-H aromatic), 1610 (C=O), 1384 (C-O stretching), 1196 (C-C).
NMR data: NA.

Standard Quercetin (Q):
Standard quercetin was obtained from Lab & General export Pvt Ltd., Bangalore 29.
Physical State:
Colour: Pale yellow.
Odour: Odourless.
State: Solid (Crystalline)
Melting Point: 324^{o}C.
Molecular weight: 358.
Rf: 6.2.
IR data (KBr cm^{-1}): 3408 (OH stretching), 3340 (C-H aromatic), 1664 (C=O), 1610 (OH bond), 1561 (C=C stretching), 1262 (C-O stretching), 1199 (C-C stretching).
NMR data (δ ppm; DMSO-d_6 solvent): 6.19 (s, 1H, Ar-H), 6.41 (s, 1H, Ar-H), 7.76 (s, 1H Ar-H), 7.57 (d, 1H, Ar-H), 7.68 (d, 1H, Ar-H), 7.76 (broad, 1H, OH), 9.39 (broad, 1H, OH), 10.39 (broad, 1H, OH).
^{13}C NMR :
Mass Spectroscopy: 358 (M^{+}, 100%), 302 (4%), 207 (1%).

Isolate 5 (I 5)
Physical State:
Colour: Orange yellowish powder
Odour: Odorless.
State: Solid (Powder)
Melting Point: 158^{o}C.
Molecular weight: 160
Rf: 8.0.

IR data (KBr cm^{-1}): 3433 (OH stretching), 2940 (C-H aromatic), 1638 (C=O), 1512 (C=C stretching), 1275 (C-O stretching), 1164 (C-C stretching)
NMR data (δ ppm; DMSO-d_6 solvent): 12.5 (COOH), 9.39 (3, OH), 10.39 (CH_2 OH).
^{13}C NMR:
Mass Spectroscopy: 160 (M^+, 100%), 149 (10%), 130 (1%), 121 (4%).

Isolate 6 (I 6)
Physical State:
Colour: Dark yellow.
Odour: Odourless
State: Solid (Crystalline)
Melting Point: 228^oC.
Molecular weight: 358
Rf: 8.3.
IR data (KBr cm^{-1}): 3410 (OH stretching), 2838 (C-H aromatic), 1638 (C=O), 1611 (OH bond), 1561 (C=C stretching), 1263 (C-O stretching), 1200 (C-C stretching).
NMR data (δ ppm; DMSO-d_6 solvent): 6.20 (s, 1H, Ar-H), 6.42 (s, 1H, Ar-H), 7.69 (s, 1H Ar-H), 9.35 (broad, 1H, OH), 10.83 (broad, 1H, OH).
^{13}C NMR:
Mass Spectroscopy: 358 (M^+, 100%), 302 (4%).

Pharmacological studies:

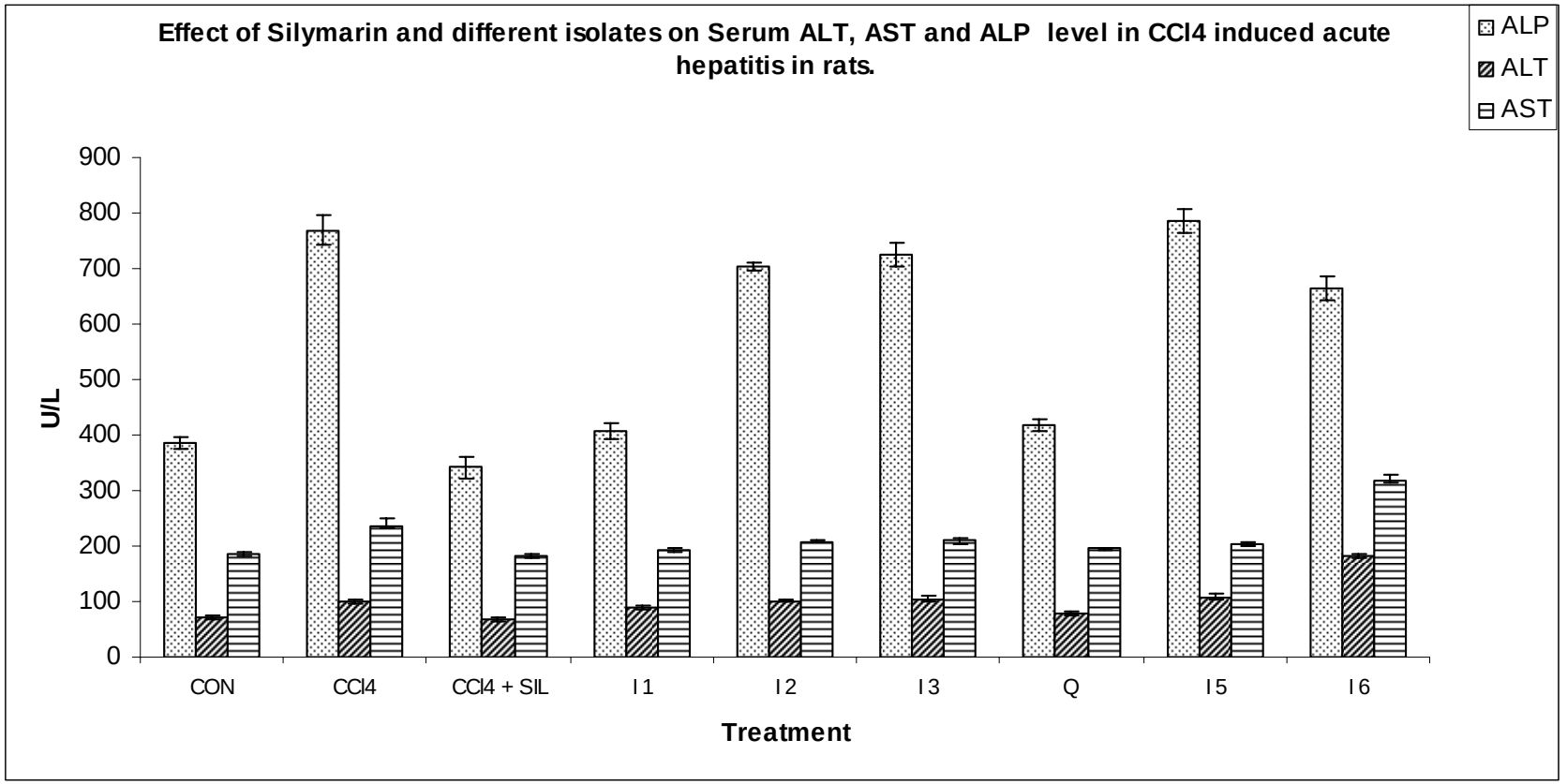
Carbon Tetrachloride (CCl_4) Induced Acute Hepatic Injury

Serum Biochemical markers and liver weight:

A significant difference in biochemical markers was observed between normal and CCl_4 control groups. Comparative analysis on the effect of ALT, AST and ALP and between the extracts revealed that standard quercetin and isolate I1 extract (200mg/Kg body weight) have almost similar activity compared to the standard silymarin (100mg/Kg body weight). The other isolates did not show significant activity when compared to standard silymarin, a known hepatoprotective agent. Significant reduction of liver weight was also seen in animals treated with standard quercetin and isolate I1, whereas other extracts did not show any reduction in the liver weight (Figure 1, 2 and 3).

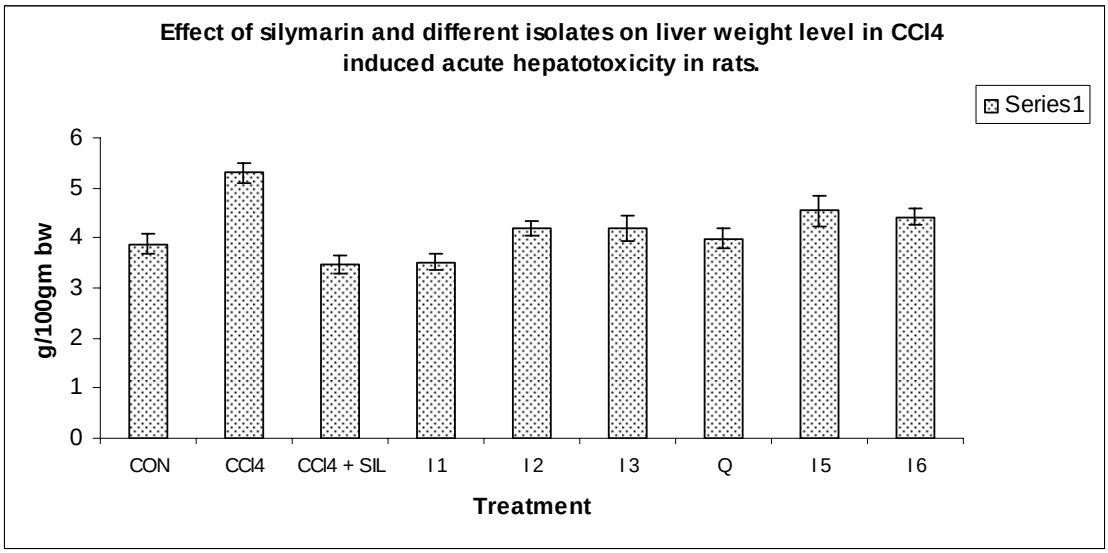
Histological examination of the liver tissue from CCl_4 treated animals revealed that CCl_4 had produced profound inflammation and congestion especially in the sinusoids. Hydropic degeneration and steatosis in the periportal region was also observed (fig1a). In animals pretreated with silymarin, and *Psidium guajava* methanolic extract reduced the inflammation, degenerative changes and steatosis (Figure 4).

Figure 1: Effect of Silymarin, quercetin (Q) and different isolates on Serum ALT, AST and ALP level in CCl_4 induced acute hepatitis in rats.



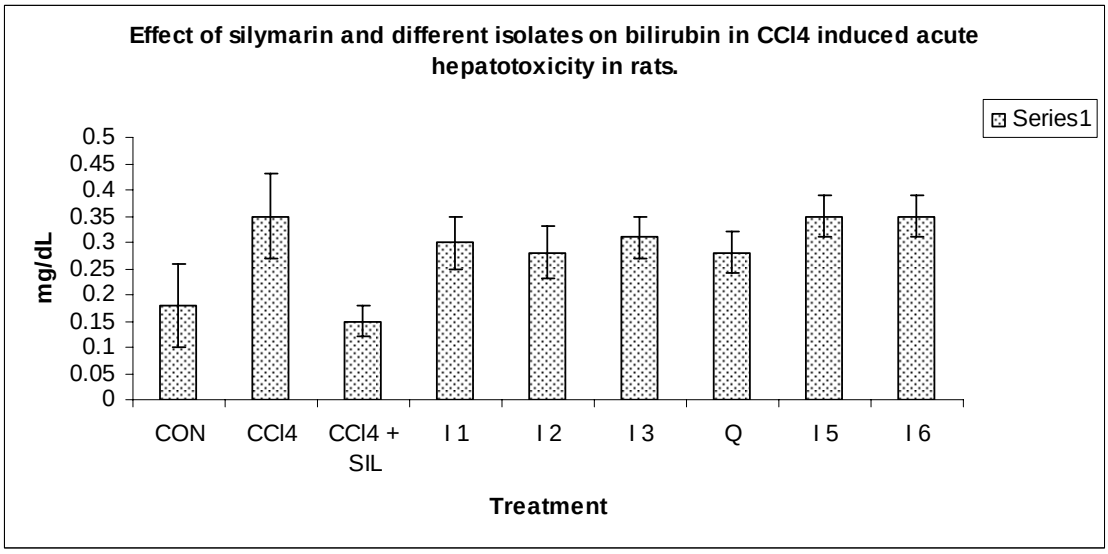
Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 2: Effect of silymarin, quercetin (Q) and various isolates on Liver weight in Carbon tetrachloride (CCl_4) induced acute hepatotoxicity in rats.



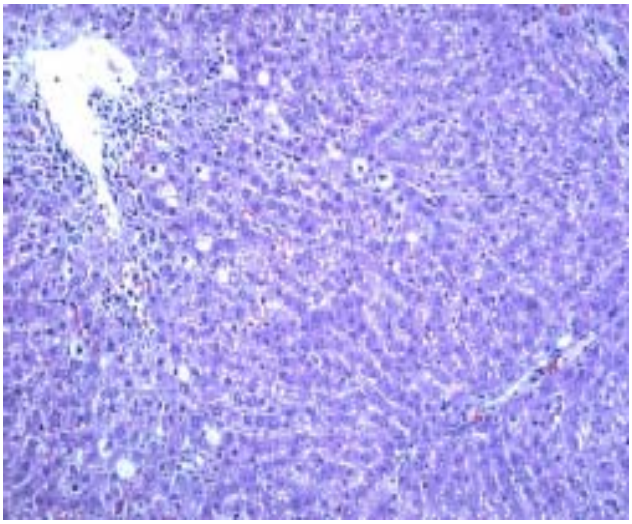
Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 3: Effect of silymarin, quercetin(Q) and different isolates on bilirubin in CCl_4 induced acute hepatotoxicity in rats.

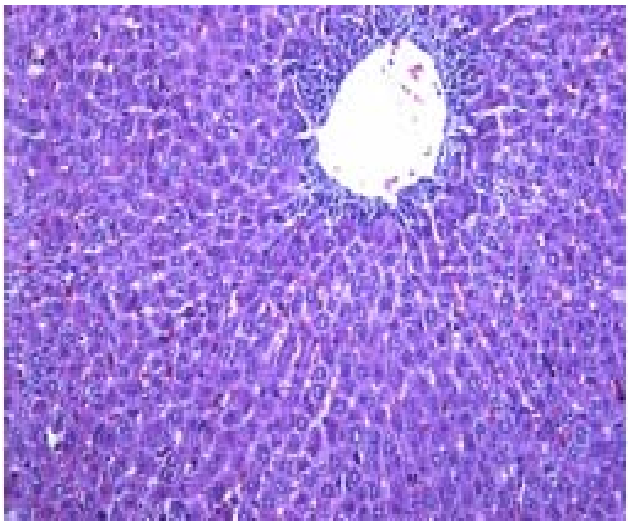


Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 4: Effect of *P. guajava* leaf extract on acute liver injury induced by CCl_4 (a: CCl_4 treated control, b: CCl_4 + Isolate 1 [H & E X200])



(a)



(b)

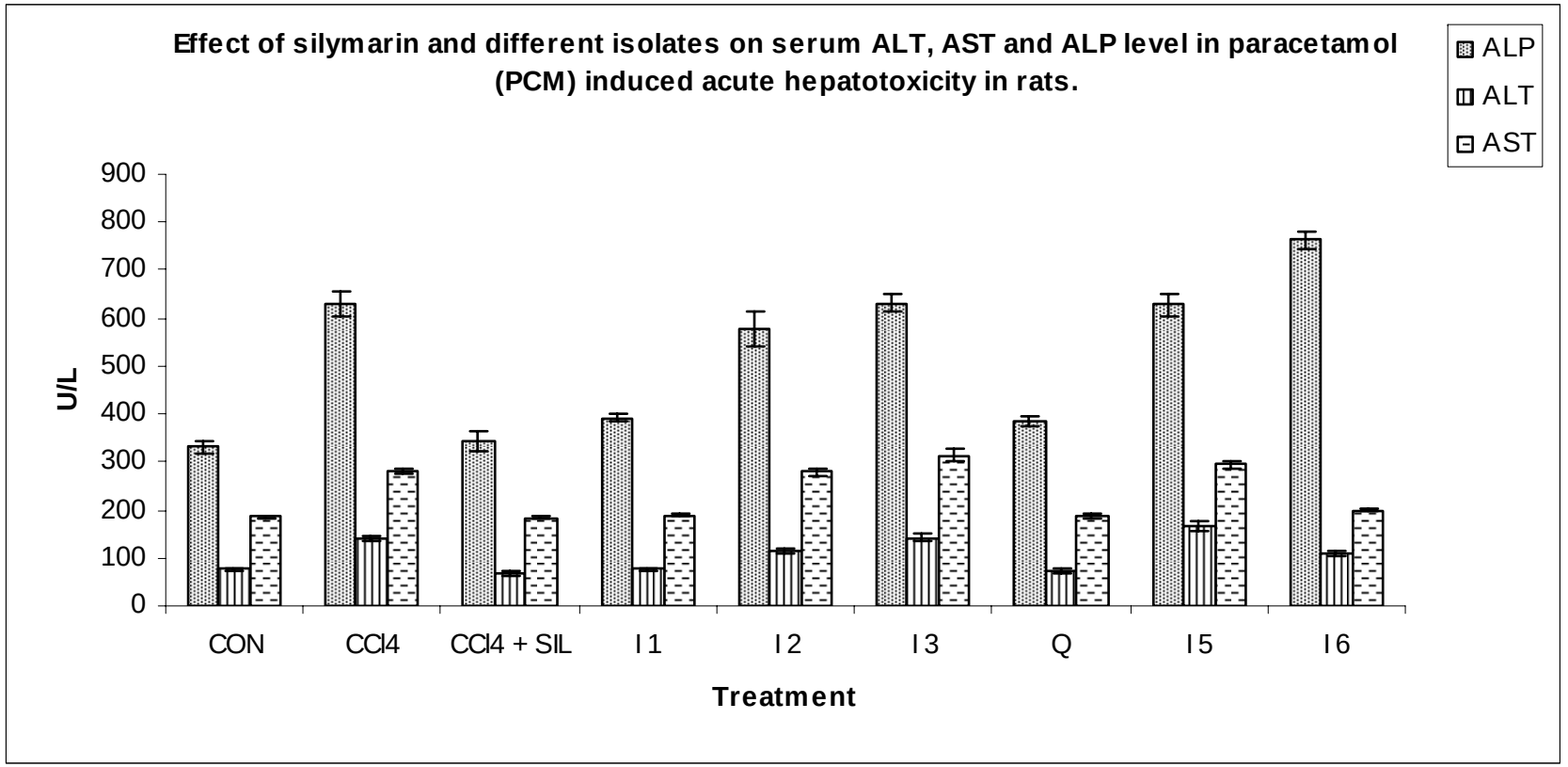
Paracetamol (PCM) Induced Acute Hepatic Injury

Serum Biochemical markers and liver weight:
Forty eight hours after treatment with paracetamol, the parameters ALT, AST, ALP and bilirubin levels in the serum increased remarkably. A significant difference in biochemical markers was observed between normal and PCM control groups.

Comparative analysis on the effect of ALT, AST and ALP and between the extracts revealed that standard quercetin and isolate I1 extract (200mg/Kg body weight) have almost similar activity compared to the standard silymarin (100mg/Kg body weight). The other isolates did not show significant activity when compared to standard silymarin except I6 which showed a mild reduction in the biochemical markers namely ALP, ALT and AST. Significant reduction of the liver weight was observed in groups administered with standard silymarin and Isolate 1 (I1) whereas the other extracts did not show any reduction of the liver weights (Figure 5, 6 and 7).

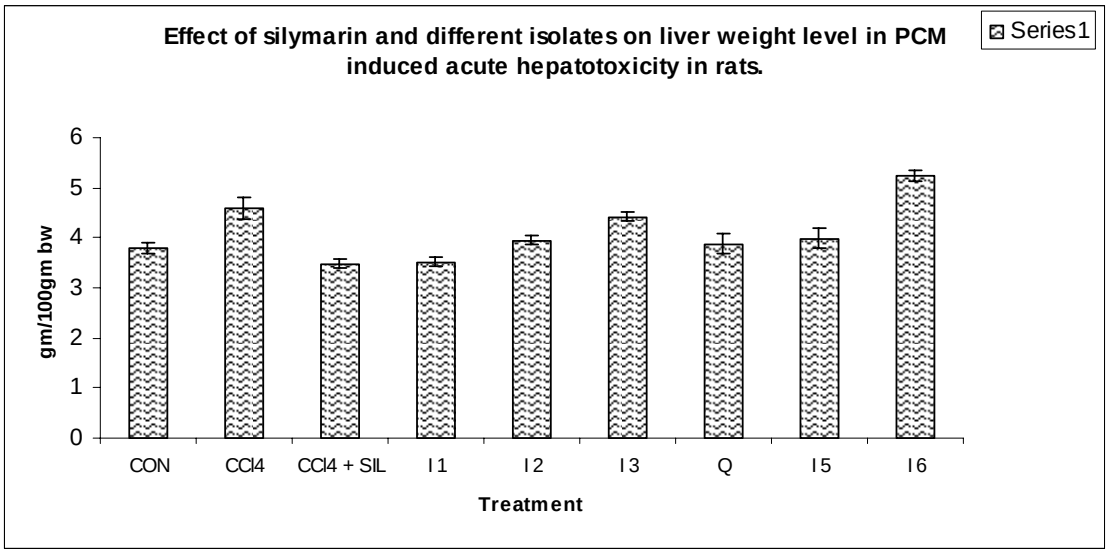
Histopathological examinations of the liver tissues showed severe congestion of blood vessels, mild hydropic degeneration, pyknosis of nucleus and occasional necrosis in PCM treated animals (Figure 8a). Silymarin reduced the pyknosis of hepatocytes when compared to PCM treated control. Animals treated with methanolic extract of *Psidium guajava* showed mild hydropic degeneration and there was no pyknosis or congestion (Figure8b).

Figure 5: Effect of silymarin, quercetin (Q) and different isolates on serum ALT, AST and ALP level in paracetamol (PCM) induced acute hepatotoxicity in rats.



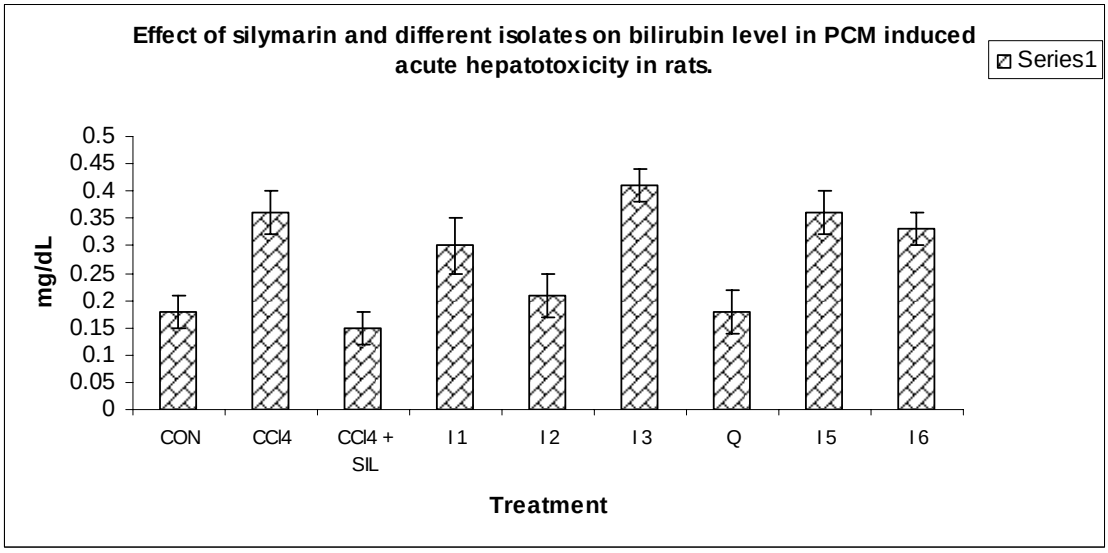
Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 6: Effect of silymarin, quercetin (Q) and different isolates on liver weight level in PCM induced acute hepatotoxicity in rats.



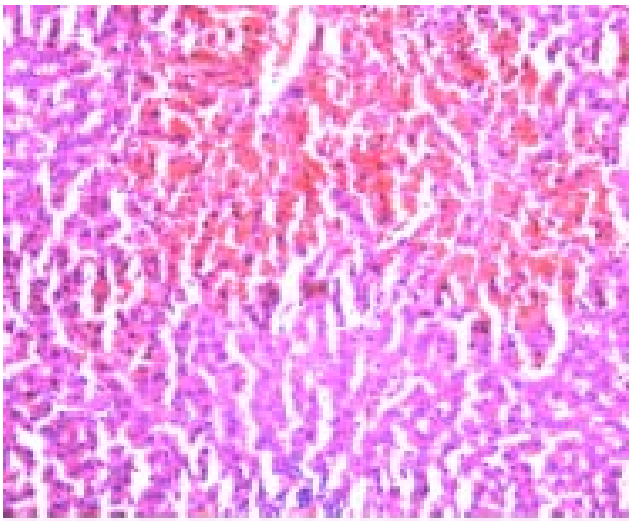
Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 7: Effect of silymarin, quercetin (Q) and different isolates on bilirubin level in PCM induced acute hepatotoxicity in rats.

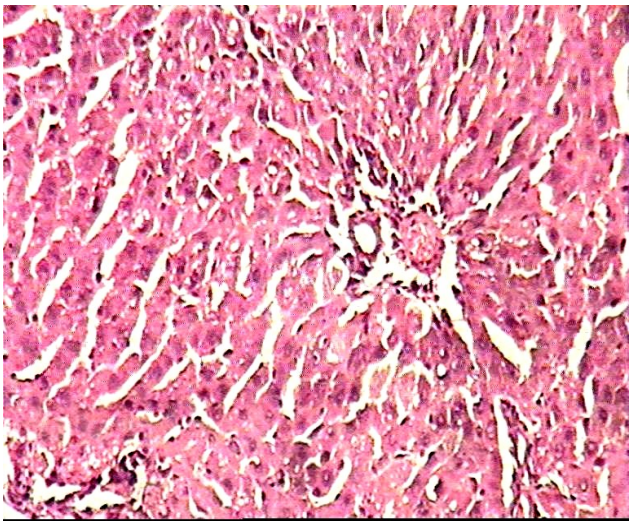


Values are mean ± S.E.M, n = 6, [a] $p<0.001$ vs. vehicle control, [b] $p<0.01$, c $p>0.05$ vs. vehicle control, [ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ vs. CCl_4 treated control.

Figure 8: Effect of *P. guajava* leaf extract on PCM induced acute liver injury (a: PCM treated control, b: PCM + Isolate 1 [H & E X200]).



(a)



(b)

Discussion

The methanolic extract of *Psidium guajava* leaves showed significant hepatoprotective activity when administered at doses of 200 mg/kg orally[9]. The effect produced by the dose of methanolic extract of *P. guajava* leaves was similar to that produced by silymarin (100mg/kg, p.o.), a well known hepatoprotective agent. Different solvent systems were

used to isolate the active constituent responsible for hepatoprotectve activity. After various trial and error methods, the most suitable solvent system was found to be methanol, water and formic acid in ratio of 7.5:2.5:0.5 respectively. Five different isolates (I1, I2, I3, I5 and I6) were collected having different R_f values and it was compared with the standard quercetin (Q) procured commercially.

The preliminary phytochemical investigation of the isolates of the methanolic extract of *P. guajava* showed that all the isolate contains flavanoids and saponins, whereas isolate I2, I3, Q and I5 showed the presence of carbohydrate.

CCl_4 is one of the most commonly used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effects of CCl_4 are largely due to generation of free radicals[15]. Drugs having antioxidant activity are effective in treating CCl_4 induced hepatotoxicity. Different extracts of this plant including the water extract are reported to increase the reduction of 2,2-diphenyl-1-picrylhydryzyl (DPPH) (Qian and Nihorimbere, 2004). The CCl_4 induced a significant increase in serum ALT, ALP, AST, bilirubin and liver weight, which is due to blocking of secretion of hepatic triglycerides into the plasma[16]. Silymarin, isolate 1 (I1) and Standard quercetin (Q) prevented the increase of serum ALT, ALP AST, bilirubin and liver weight in rats.

It is known that PCM induces liver injury through the action of its toxic metabolite, N-acetyl-p-benzoquinoneimine, produced by the action of Cytochrome P-450. This metabolite reacts with reduced glutathione (GSH) to yield non-toxic 3-GS-yl-paracetamol. Depletion of GSH causes the remaining quinone to bind to cellular macromolecules leading to cell death[17]. Damage induced in the liver is accompanied by the increase in the activity of some serum enzymes. The anti-hepatotoxic action of the isolate 1 (I1) and standard quercetin (Q) was substantiated by significant attenuation of the increased levels of serum enzymes in rats intoxicated with PCM. Drugs having antioxidant activity are also effective in treating paracetamol induced hepatotoxicity by scavenging the free radicals produced by PCM metabolism, thereby preventing the liver damage induced by both PCM metabolite and due to depletion of glutathione. As mentioned earlier that *Psidium guajava* is a known antioxidant (Qian and Nihorimbere, 2004) and this activity may be responsible for its effect in PCM induced hepatotoxic model. The PCM induced a significant increase in liver weight, which is due to the blocking of secretion of hepatic triglycerides into the plasma (Yoko *et al*., 2005). Isolate 1 and standard quercetin (Q) prevented the increase in liver weight of rats pretreated with PCM.

In conclusion characterization of the active isolate (I1) was done by spectroscopic analysis using the IR, Mass, Proton NMR and 13 C NMR and compared with standard.. Structural analysis suggested that the isolated compound (I1) confirmed with Quercetin structure.

Acknowledgement

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